Immunization with a highly attenuated replication-competent herpes simplex virus type 1 mutant, HF10, protects mice from genital disease caused by herpes simplex virus type 2

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Genital herpes is an intractable disease caused mainly by herpes simplex virus (HSV) type 2 (HSV-2), and is a major concern in public health. A previous infection with HSV type 1 (HSV-1) enhances protection against primary HSV-2 infection to some extent. In this study, we evaluated the ability of HF10, a naturally occurring replication-competent HSV-1 mutant, to protect against genital infection in mice caused by HSV-2. Subcutaneous inoculation of HF10-immunized mice against lethal infection by HSV-2, and attenuated the development of genital ulcer diseases. Immunization with HF10 inhibited HSV-2 replication in the mouse vagina, reduced local inflammation, controlled emergence of neurological dysfunctions of HSV-2 infection, and increased survival. In HF10-immunized mice, we observed rapid and increased production of interferon-γ in the vagina in response to HSV-2 infection, and numerous CD4+ and a few CD8+ T cells localized to the infective focus. CD4+ T cells invaded the mucosal subepithelial lamina propria. Thus, the protective effect of HF10 was related to induction of cellular immunity, mediated primarily by Th1 CD4+ cells. These data indicate that the live attenuated HSV-1 mutant strain HF10 is a promising candidate antigen for a vaccine against genital herpes caused by HSV-2.

Keywords: genital herpes, live attenuated vaccine, HSV-1, HSV-2

INTRODUCTION

Herpes simplex virus (HSV): type 1 (HSV-1) and type 2 (HSV-2) belong to the alphaherpesvirus family. HSV-1 and HSV-2 have 50% DNA sequence homology (Kieff et al., 1972). Generally, HSV-1 infects via the oral route, whereas HSV-2 infects via the genital tract. Both exert neurotropic effects and spread to the nervous system (Corey and Spear, 1986; Whitley and Roizman, 2001). HSV-2 is the main causative agent of genital herpes worldwide (Tao et al., 2000). Epidemiological investigations have indicated that the prevalence of HSV-2 in the general population of the USA ranges from 10 to 60%, and genital herpes is one of the most common sexually transmitted diseases (Malvy et al., 2005; Xu et al., 2006). After primary infection via the genital tract, the virus establishes latency within the lumbosacral ganglia, and establishes a state of lifelong infection. Subsequently, the latent virus reactivates intermittently resulting in recurrent disease (Miller et al., 1998; Stanberry et al., 2000). In addition, genital herpes is linked to an increased susceptibility to sexually acquired and transmitting human immunodeficiency virus (HIV; Freeman et al., 2006; Kapiga et al., 2007), which is not markedly reduced by HSV antiviral therapy (Celum et al., 2008; Watson-Jones et al., 2008). A vaccine would provide a more effective means of preventing or limiting infection, and would greatly reduce the social and economic burden of HSV-2 infection. In developed countries, while childhood acquisition of HSV-1 has decreased, HSV-2 seroprevalence has increased, suggesting the possible protective effect of HSV-1 against HSV-2 infection (Xu et al., 2006). HSV-1 has also become a major causative agent of primary genital herpes in developed countries (Lafferty et al., 2000; Nieuwenhuis et al., 2006).

In the past, efforts to develop an HSV vaccine have included development of inactivated whole-virus vaccines, subunit glycoprotein preparations, DNA plasmids, and attenuated replication-competent viruses. These candidate vaccines were unsuccessful in clinical trials (Stanberry, 2004). The most successful vaccine in human trials was a subunit glycoprotein vaccine that included HSV-2 gD, a major viral envelope antigen, as an immunogen with alum and 2-o-deacylated monophosphoryl lipid A as adjuvants (Bernstein et al., 2003). Although the vaccine appeared safe and effective against genital herpes in guinea pigs, it failed to provide sufficient protection against primary infection in a clinical trial (Stanberry et al., 2002). Furthermore, immunization with HSV-2 gD subunit did not reduce the rate at which women acquired HSV-2 genital herpes (Rebbe et al., 2012). Therefore, new strategies for developing HSV vaccines are required. Meanwhile, there are established live vaccines for other alphaherpesviruses, e.g., a modified live virus vaccine that prevents pseudorabies virus infection (PRV/Marker Gold®) in pigs is commercially available (Swayne et al., 1993a,b; Van de Walle et al., 2003), and an attenuated live varicella-zoster virus vaccine that prevents chicken pox.
Vero cells (African green monkey kidney epithelial cells) were
sizes inducing latency or reactivation (Cappel, 1976).
HF10 is a spontaneously occurring HSV-1 mutant that lacks
functionality of UL43, UL49.5, UL55, UL56, and latency-
associated transcripts (Ushijima et al., 2007). We have demon-
strated that HF10 can be attenuated, and that it does not cause
any neurotropic effects in mice. Intranasal vaccination of mice
with HF10 conferred significant protection against lethal chal-
lenge with HSV-1 and HSV-2 (Mori et al., 2005). Thus, HF10 is
a promising live attenuated HSV vaccine candidate. It is also a
well-known oncolytic virus for cancer therapy (Fujimoto et al.,
2006; Kimata et al., 2006; Nakao et al., 2007). In this study, we
used HF10 as a live attenuated vaccine, and evaluated the immune
response generated and protective effect against HSV-2 genital
infection in mice. Subcutaneous inoculation of HF10-immunized
mice from lethal infection by HSV-2, and attenuated the devel-
opment of genital ulcer diseases. Furthermore, we observed
inhibition of virus replication and production of interferon-γ
(IFN-γ) by splenocytes in response to HSV-2 antigens in the serum
of immunized mice. HF10 also induced rapid accumulation of
CD4+ and CD8+ cells in the infective focus, and protected mice
against HSV-2 genital disease via induction of a cellular immune
response.

MATERIALS AND METHODS

VIRUSES, CELLS, AND ANTIBODIES
Vero cells (African green monkey kidney epithelial cells) were
grown in Eagle’s minimal essential medium (MEM) supplemented
with 10% calf serum. The HSV-1 mutant HF10, the wild-type
HSV-1 strains KH7 and KOS, and wild-type HSV-2 strain 186
were titrated in Vero cells. HF10 virus was inactivated by expo-
sure to ultraviolet (UV) light for 30 min using a GL15 UV
(Mitsubishi/Osram, Kakegawa, Japan). UV-inactivated virus was
not infectious when inoculated into Vero cells. NIH3T3 cells (mouse embryonic fibroblast cell line derived from BALB/c)
were grown in Dulbecco’s modified Eagle’s medium containing 10%
calf serum. NIH3T3 cells were infected with HSV-2 strain 186 at
a multiplicity of infection (MOI) of 3 in the presence of ganc-
ciclovir (GCV; 10 μg/mL) or cycloheximide (CHX; 20 μg/mL)
for 8 h, and then harvested for stimulating splenocytes. Anti-
HSV-1 polyclonal rabbit antibody was purchased from Dako
(Glostrup, Denmark). Anti-HSV-2 antibody was acquired from a
mixture of anti-HSV-2 UL17, UL42, UL46, UL48, and US11
antibodies generated in our laboratory by immunizing rabbits
(Conshima et al., 2000; Kato et al., 2000; Koshizuka et al., 2001). Anti-mouse CD4 antibody and fluorescein isothiocyanate (FITC)-
labeled anti-mouse CD8 antibody were purchased from Chemicon International (Temecula, CA, USA) and Thermo Scientific
(Rock-
ford, IL, USA), respectively. DRAQ5® (Biostatus Limited, Shipshed,
UK) was used to stain cell nuclei. Anti-mouse IgG-conjugated
FITC and anti-rabbit IgG-conjugated tetramethylrhodamine-5-
(6)-isothiocyanate (TRITC) were obtained from Sigma-Aldrich
(St. Louis, MO, USA).

MOUSE STRAINS, IMMUNIZATION, AND CHALLENGE
BALB/c and BALB/c nude mice were obtained from SLC (Hamam-
atsu, Japan). Six-week-old BALB/c mice were immunized
subcutaneously in the rear flank once with 100 μl phosphate-
buffered saline (PBS) containing 1 × 10^8 plaque-forming units
(PFU) of HF10, 1 × 10^6 PFUs of UV-treated HF10, or PBS only.
Mice were challenged 4 weeks or 4 months after immunization,
and 7 days prior to challenge they were subcutaneously injected
in the neck ruff with 3 mg Depo-Provera (Sigma-Aldrich). For
intravaginal challenge, mice were inoculated with 5 × 10^6 PFUs of
HSV-2 strain 186 (approximately 15 × 10^3) using a pipette.
For the safety study, 5 × 10^6 PFUs of HF10 or KH7 were sub-
cutaneously inoculated in the flank of 6-week-old BALB/c nude
mice. On days 1 and 5 after infection, mice were sacrificed, and skin
samples were harvested for histological and immunohistochemical
studies to detect HSV-1 antigens.

All experiments were approved by the University Committee
and conducted in accordance with the Guidelines for Animal
Experimentation of Nagoya University.

CLINICAL OBSERVATIONS
Mice were observed daily for signs of genital lesions. The severity
of disease was scored as follows: 0, no sign; 1, slight genital erythema
and edema; 2, moderate genital inflammation, 3, purulent genital
lesions and paralyzis; and 4, death.

EVALUATION OF ACUTE INFECTION
Vaginal tracts of mice were washed with 200 μL MEM contain-
ing 5% newborn calf serum for 1–5 days after challenge. These
were stored at –80°C for virus titration and IFN-γ assays. Viral
titers were determined using a standard plaque assay. IFN-γ con-
centration was determined using a Quantikine Immunoassay kit
(R&D Systems, Minneapolis, MN, USA) in an enzyme-linked
immunosorbent assay (ELISA).

NEUTRALIZING ANTIBODY ASSAY
Four weeks after immunization, blood samples were collected via
laparotomy from the abdominal aortic arch. After incubation at
37°C for 30 min, blood samples were centrifuged at 3000 rpm
for 10 min and serum was collected. To estimate neutralization
titers, diluted sera were added to 100 PFUs of HSV-1 strains HF10
and KOS or HSV-2 strain 186, incubated for 30 min at 37°C, and
the remaining infectious virus was detected on duplicate Vero cell
monolayers.

IMMUNOFLOUORESCENT STAINING OF VAGINAL TISSUES
Mice were deeply anesthetized with ketamine and xylazine and
their vaginas were excised. To examine the distribution of CD4+
and CD8+ cells, frozen sections were stained with a variety of anti-
bodies. In brief, 8 μm frozen sections were blocked with PBS/2%
fetal calf serum (FCS), reacted with mouse CD4 monoclonal anti-
body for 30 min, and stained with anti-mouse IgG-conjugated
FITC antibody for 30 min or FITC-labeled anti-mouse CD8 mono-
oclonal antibody for 30 min at 37°C. Slides were then washed
with PBS/2% FCS three times, fixed with 4% paraformaldehyde
for 15 min, and treated with 0.1% Triton X-100 for 10 min at
room temperature. Next, these slides were stained with polyclonal

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rabbit HSV-2 antibody (described in Section "Viruses, Cells, and Antibodies") for 30 min at 37°C, washed with PBS, and then treated with a secondary antibody (anti-rabbit IgG-conjugated TRITC) for 30 min at 37°C. Stained slides were washed, incubated with DRAQ5, and mounted with Fluoromount Plus (Diagnostic Biosystems, Pleasanton, CA, USA). Finally, slides were visualized using an LSM 510 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany).

EVALUATION OF CELLULAR IMMUNITY
Mice were deeply anesthetized with ketamine and xylazine, and their spleens were excised. Tissues were crushed through a 100-μm nylon cell strainer (BD Biosciences, Franklin Lake, NJ, USA). Erythrocytes were depleted using lysis buffer (BD Biosciences), and spleen cells were suspended in RPMI-1640 medium containing 10% FCS. Spleen cells were plated at 1 × 10⁷ cells/well (2 ml) for stimulation by HSV-2-infected NIH3T3 cells. NIH3T3 cells (2 × 10⁶ cells/35 mm dish) were infected with HSV-2 strain 186 at an MOI of 3 for 3 h. Spleen cells were also plated at 1 × 10⁶ cells/well (500 μl) for stimulation by NIH3T3 cells expressing HSV-2 viral antigens. To produce viral antigens, UL46 and US6 genes and ICP0 cDNA from HSV-2 strain 186 were amplified by polymerase chain reaction and cloned into pcDNA 3.1(+) expression vectors (Invitrogen, Carlsbad, CA, USA). Each plasmid (1.5 μg) was transfected into 1 × 10⁶ NIH3T3 cells/35 mm dish with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and incubated for 16 h. Plasmid-transfected or HSV-2-infected cells were frozen, thawed, and added to dishes containing splenocytes acquired as described above. Splenocytes were stimulated with these viral antigens at 37°C for 24 h and the medium was collected at 3 and 20 h to quantify IFN-γ concentrations.

STATISTICS
The statistical significance of differences in disease scores and viral titers on individual days was determined using a Student's t-test. Survival rates were estimated by the Kaplan–Meier method, and statistical significances were determined by the log-rank test. Differences in disease scores and viral titers on individual days was determined using a Student's t-test. The statistical significance of differences in disease scores and viral titers on individual days was determined using a Student's t-test. The statistical significance of differences in disease scores and viral titers on individual days was determined using a Student's t-test. Viral titers on day 5 were significantly lower in HF10-immunized mice than in unimmunized mice and UV-inactivated HF10-immunized mice. Immunization with HF10 protected mice from development of local genital symptoms and these mice exhibited no signs of systemic disease (Figure 2B); the survival rate was 83.3% (Figure 2C). In contrast, all mice in the control group developed severe genital symptoms and hind paralysis (Figure 2B). Eventually, all succumbed and died within 10 days of challenge (Figure 2C). Genital symptoms and paralysis in mice immunized with UV-inactivated HF10 were slightly weaker than those in unimmunized mice, albeit without statistical significance (Figure 2B). The survival rate of UV-inactivated HF10-immunized mice was 40% (Figure 2C). Thus, immunization with UV-inactivated HF10 partially protected mice from HSV-2 genital disease. We then

RESULTS
CLEARANCE OF HF10 AFTER SUBCUTANEOUS INOCULATION INTO BALB/c NUDE MICE
To confirm the low virulence of HF10, we subcutaneously inoculated HF10 or wild-type KH7 into eight BALB/c nude mice and compared their virulence. No HF10-inoculated nude mice developed zoster or died. In contrast, KH7 infection caused severe zoster formation and death in all mice. HF10 was detected 1 day after inoculation but cleared by day 5 (Figure 1). Conversely, KH7-infected cells were still detectable on day 5. These results confirm the low virulence of HF10 (Mori et al., 2005).

IMMUNIZATION WITH HF10 PROTECTS MICE AGAINST HSV-2 GENITAL DISEASE
To determine the efficacy of HF10 as a vaccine, we subcutaneously immunized either BALB/c mice with HF10 or UV-inactivated HF10, or performed mock immunization. After 1 month, each group was challenged by intravaginal inoculation of wild-type HSV-2 stain 186. Mice immunized with HF10 had significantly lower titers of virus shedding in the vaginal mucosa 1 day after challenge (Figure 1A). The shedding of strain 186 in HF10-immunized mice was diminished by day 5 after challenge. In control mice, the viral titer decreased by day 3 and then increased (Figure 1A). Viral titers on day 5 were significantly lower in HF10-immunized mice than in unimmunized mice and UV-inactivated HF10-immunized mice. Immunization with HF10 protected mice from development of local genital symptoms and these mice exhibited no signs of systemic disease (Figure 2B); the survival rate was 83.3% (Figure 2C). In contrast, all mice in the control group developed severe genital symptoms and hind paralysis (Figure 2B). Eventually, all succumbed and died within 10 days of challenge (Figure 2C). Genital symptoms and paralysis in mice immunized with UV-inactivated HF10 were slightly weaker than those in unimmunized mice, albeit without statistical significance (Figure 2B). The survival rate of UV-inactivated HF10-immunized mice was 40% (Figure 2C). Thus, immunization with UV-inactivated HF10 partially protected mice from HSV-2 genital disease. We then
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FIGURE 2 | Immunization of mice with HF10 followed by lethal HSV-2 challenge via vaginal inoculation. One month after immunization with HF10, 5 x 10^5 PFUs of wild-type HSV-2 strain 186 were inoculated into mice vaginas. Unimmunized mice and UV-inactivated HF10-immunized mice were used as controls. (A) Replication of strain 186 in the genital mucosa was determined by viral titrations of vaginal washes (200 μl). Viral titers were significantly lower in HF10-immunized mice on day 5 than in both unimmunized mice (P < 0.05) and UV-inactivated HF10-immunized mice (P < 0.05). (B) Clinical symptoms were monitored and scored for 8 days. HF10-immunized mice had lower disease scores than unimmunized mice (on day 8, P < 0.0001). Disease scores were not significantly different between UV-inactivated HF10-immunized mice and unimmunized mice (on day 8, P = 0.001). (C) Survival curve derived by the Kaplan–Meier method. HF10-immunized mice survived longer than unimmunized mice and UV-inactivated HF10 mice (on day 28, P < 0.0001 and P = 0.001, respectively).

confirmed the persistent efficacy of HF10 immunization against vaginal infection by HSV-2. Four months after immunization, mice immunized with HF10 were also protected against genital disease to the same level as mice challenged after 1 month (data not shown).

Histological examinations of vaginal tissues were performed (Figure 3). In unimmunized mice, HSV-2 antigens were present in mucosal epithelial cells and subepithelial lamina propria of vaginal tissue at days 1 and 4 after challenge, and mucosa in the infective focus dropped out from the epithelium 6 days after challenge. In contrast, in HF10-immunized mice, HSV-2 antigen staining was restricted to the mucosal surface at days 1 and 4 and was undetectable 6 days after challenge. These results indicate that mice immunized with HF10 were protected against severe genital disease caused by HSV-2.

IMMUNE RESPONSES AGAINST HSV-2 STRAIN 186 IN HF10-IMMUNIZED MICE

To determine neutralizing antibody titers against HSV-2 strain 186 in HF10-immunized mice, we collected serum 1 month after HF10 immunization and investigated its ability to neutralize HSV-1 strains HF10 and KOS and HSV-2 strain 186 based as determined by reductions in plaque formation (Figure 4A). Serum inhibited HF10 plaque formation at a dilution of 1:128 and KOS plaque formation at a dilution of 1:64. The titers producing a 50% reduction in plaque formation by HF10 and KOS were between 16 and 32. Serum had little effect on plaque formation by HSV-2 strain 186 at lower concentrations, but at a dilution of 1:2 caused a 40% reduction. Plaque formation by UV-inactivated HF10-immunized mouse serum was lower than that of HF10-immunized mice (Figure 4B). To evaluate cellular immune responses, we stimulated spleen cells from each immunized or unimmunized mouse with 186-infected NIH3T3 cells and examined IFN-γ production kinetics. IFN-γ accumulated in the medium of splenocytes from HF10-immunized mice at both 5 and 20 h after stimulation, although IFN-γ levels were similar to those produced by UV-inactivated HF10-immunized mice (Figure 4C). We then investigated IFN-γ concentrations in vaginal washes after challenge (Figure 4D). Although IFN-γ production was recognized in mouse vaginas immunized with UV-inactivated HF10, the quantity was not statistically significantly different from that produced by unimmunized mice. In contrast, IFN-γ concentration in HF10-immunized mice was significantly higher than that in unimmunized mice.

To confirm that memory cells of HF10-immunized mice cross-reacted with HSV-2 proteins, we expressed HSV-2 ICP0, UL46, and gD in NIH3T3 cells (Muller et al., 2009), used them to stimulate splenocytes, and quantified IFN-γ concentration in the medium. Splenocytes from HF10-immunized mice produced considerable amounts of IFN-γ in response to gD-, ICP0-, and UL46-expressing NIH3T3 cells (Figure 5). To confirm that protein synthesis was necessary, we stimulated splenocytes with 186-infected NIH3T3 cells treated with ganciclovir or cycloheximide. Splenocytes stimulated with ganciclovir-treated cells produced high levels of IFN-γ (474 pg/ml) at 20 h after stimulation compared to the production by cycloheximide-treated cells (82 pg/ml), indicating the requirement for protein synthesis (Figure 5). There was little or
FIGURE 3 Immunohistochemical evaluation after HSV-2 challenge. After inoculation of wild-type HSV-2 strain 186 into HF10-immunized mice or unimmunized mice vaginas, vaginal mucosal lesions were excised at days 1, 4, and 6 after challenge, and HSV-2 antigens were stained. Arrows indicate HSV-2-infected cells.

After inoculation of wild-type HSV-2 strain 186 into HF10-immunized mice or unimmunized mice vaginas on days 1 and 3 after challenge (Figure 6). In HF10-immunized mice, CD4+ cells localized to the infective focus and invaded the mucosal subepithelial lamina propria on both 1 and 3 days after challenge (Figure 6A). CD8+ cells were only detected 1 day after challenge (Figure 6B). In unimmunized mice, there were no detectable CD4+ and CD8+ cells 1 day after challenge (Figures 6A,B), but there were a few CD4+ cells in the infective focus 3 days after challenge.

DISCUSSION

Genital herpes is an intractable disease of major public health importance. It causes significant morbidity and psychosocial distress and increases the risk of HIV transmission (Freeman et al., 2006; Kapiga et al., 2007). Previous HSV-1 infection provides protection against primary genital HSV-2 infection and its severity, to some extent (Mertz et al., 1992; Bryson et al., 1993). Therefore, immunization with HSV-1 may be useful for preventing infection with or disease caused by HSV-2.

In this study, we evaluated the ability of the spontaneously occurring HSV type 1 mutant HF10 to serve as a vaccine against HSV-2-mediated genital disease. The safety of the vaccine must be considered because HF10 is a replication-competent virus. We determined the complete DNA sequence of HF10 and found that the virus lacks functional expression of UL43, UL49.5, UL55, UL56, and latency-associated transcripts (Ushijima et al., 2007). In addition, HF10 exhibits a relatively high divergence in proteins compared to HSV-1 strain 17. All of these changes occurred spontaneously. UL56 associates with the kinesin motor protein KIF1A, and its absence reduces the neuroinvasiveness of HSV (Rosen-Wolff et al., 1991; Berkowitz et al., 1994; Koshizuka et al., 2002). The LAT promoter region is also reported to be associated with neurovirulence (Jones et al., 2005; Peng et al., 2005). We previously found that HF10 lacks neuroinvasiveness and is at least 10,000-fold less virulent than wild-type HSV-1 in mouse models (Nishiyama et al., 1991; Jiang et al., 1995; Mori et al., 2005). To confirm the safety of HF10, we subcutaneously inoculated HF10 into BALB/c nude mice. HF10 was cleared from the skin by day 5, and no nude mouse developed zoster or died. Using clinical trials of HF10 as cancer virotherapy, we have been evaluating the safety of HF10 through various approaches in preclinical tests (Fujimoto et al., 2006; Kimata et al., 2006; Nakao et al., 2007). Recently, the US Food and Drug Administration approved a phase I clinical trial of HF10 against refractory head and neck cancer (Clinical Trials Gov, Identifier: NC7010717185). Considering the results collectively, we believe that HF10 is a safe vaccine candidate. One striking advantage of HF10 is that it is a naturally occurring HSV-1 mutant and is not genetically engineered. Therefore, in terms of ethical aspects, it is not necessary to consider the safety of foreign gene expression.

Our results indicate that immunization with HF10 protected mice from HSV-2 primary genital infection. Immunization inhibited viral replication in the vagina, reduced local inflammation, controlled emergence of neurological manifestations of HSV-2 infection, and increased survival. We speculate that HF10 can induce adaptive immunity against HSV-2 strain 186. However, serum from the HF10-immunized mice elicited few neutralizing effects against HSV-2. Therefore, humoral immunity does not play a key role in the protection against HSV-2 genital disease. In previous studies, the transfer of serum from HSV-immunized mice to unimmunized mice did not reduce HSV replication in...
FIGURE 4 | Immune responses of HF10-immunized mice. (A) Serum was obtained from HF10-immunized mice (n = 3), and its neutralizing ability against HSV-1 strains (HF10 and KOS) and an HSV-2 strain (186) was investigated by the reduction in plaque formation. (B) Mice were immunized with UV-inactivated HF10 or HF10. After 4 weeks, serum (n = 3) collected and neutralizing ability against HF10 was assayed by the reduction in plaque formation. (C) IFN-γ produced by splenocytes stimulated with HSV-2 strain 186-infected NIH3T3 cells. Splenocytes (1 × 10^7 cells/dish) from unimmunized (n = 3), UV-inactivated HF10-immunized (n = 3), or HF10-immunized (n = 3) mice were incubated with 186-infected NIH3T3 cells (1 × 10^7 cells/dish) for 5 and 20 h, and the supernatants were collected. Supernatants from three mice were combined and assayed for IFN-γ concentrations using an ELISA. (D) IFN-γ levels in mice vagina after HSV-2 challenge. Genital tracts of unimmunized, UV-inactivated HF10-immunized, and HF10-immunized mice were washed 0, 1, and 3 days after challenge with strain 186, and the washes were assayed for IFN-γ concentrations by ELISA. *p < 0.05 between unimmunized mice and HF10-immunized mice. ND; not detected.

the vaginal mucosa, but rather protected the nervous system and prevented lethality (McDermott et al., 1998; Eis-Hubinger et al., 1993). Mucosal immunity against vaginal HSV-2 infection does not depend on IgA. Regarding cellular immunity, Th1 CD4+ cell help is required for the entry of CD8+ T cells into the genital mucosa during the effector phase of the immune response (Nakanishi et al., 2009). Furthermore, CD4+ effector T cells themselves play a direct antiviral role. In the mouse vaginal infection model, Th1 CD4+ cells enter the infected vagina and produce high levels of IFN-γ, which blocks viral replication (Haima et al., 2008). Conversely, CD8+ T cells are important for preventing reactivation of latent HSV in neurons (Zhu et al., 2007). Thus, T cell-mediated immunity plays an important role in the prevention of HSV genital disease. Our results indicate that HF10-immunized mice rapidly produced high levels of IFN-γ in their vagina in response to vaginal infection by HSV-2. Furthermore, the vaginal sections of HF10-immunized mice revealed many CD4+ cells concentrated in the infective focus and invading the mucosal subepithelial lamina propria 1 and 3 days after challenge. CD8+ cells were detected only 1 day after challenge. Taking our data and those of previous reports into consideration, protection of HF10-immunized mice against HSV-2 genital disease appears to be associated with cellular immunity mediated mainly by Th1 CD4+ T cells.

In this study, the serum of UV-inactivated HF10-immunized mice had a lower neutralizing titer against HF10 than did that of live HF10-immunized mice. In mice immunized with UV-irradiated HF10, IFN-γ concentration in vaginal washes after HSV-2 challenge were not significantly increased compared to those of unimmunized mice. As a result, UV-inactivated HF10 immunization could not completely protect mice against HSV-2 challenge. These results indicate that UV-inactivated HF10...
FIGURE 5 | Cellular immune responses of HF10-immunized mice to HSV-2 proteins. Splenocytes (1 × 10^6 cells) from HF10-immunized or unimmunized mice were collected and incubated at 37°C for 3 h. NIH3T3 cells (1 × 10^6 cells) transfected with expression vectors for HSV-2 gD, ICP0, and UL46 were added into dishes to stimulate splenocytes. To confirm protein synthesis, 186-infected NIH3T3 cells were treated with either 10 μg/ml ganciclovir (186/GCV) or 20 μg/ml cycloheximide (186/CHX) for 8 h and used for stimulation. The media were collected at 5 and 20 h after addition and assayed for IFN-γ concentrations by ELISA. “Cells” denote uninfected NIH3T3 cells used as a negative control.

FIGURE 6 | Accumulation of CD4^+ and CD8^+ T cells in the infection focus of vagina after HSV-2 challenge. Unimmunized and HF10-immunized mice were challenged with HSV-2 by intravaginal infection, and vagina was excised 1 or 3 days later. Frozen sections were stained with anti-CD4 (green), anti-CD8 (green), and anti-HSV-2 antibodies (red). Cell nuclei were counterstained with DRAQ5 (blue). Images were captured with confocal microscopy at ×200 magnification. (A) Anti-CD4. (B) Anti-CD8.
immunization induced weaker acquired immunity, in accordance with the belief that killed viral vaccines are inferior to live vaccines.

Recently, it has been reported that replication-defective HSV-1 that include the CMV promoter driving the HSV-1 gD expression cassette (CJ9-gD) effectively protects guinea pigs against HSV-2 genital disease by inducing a Th1 type cellular immunity (Brans et al., 2008). In mice, CJ9-gD induces strong and persistent humoral and Th1-associated cellular immunity against both HSV-1 and HSV-2 (Brans et al., 2009). In this study, splenocytes from CJ9-immunized mice produced IFN-γ in response to gD-, ICP0-, and UL49-expressing NIH3T3 cells, indicating that memory T cells responding to epitopes of HSV-2 gD, ICP0, and a tegument protein encoded by UL46 can be induced in CJ9-immunized mice. Taken together with the IFN-γ production in the vagina, CJ9 immunization could induce Th1 type cellular immunity and protect against severe HSV-2 infection. Due to the high amino acid sequence homology between gD-1 and gD-2 (Lasky and Dowdenbo, 1984), the memory T cell response to gD may play an important role in the mechanism underlying protection against HSV-2 infection by CJ9 inoculation.

In summary, we demonstrated that immunization with HF10, a non-engineered, naturally occurring HSV-1 mutant, protects mice against severe genital disease caused by HSV-2. Immunization with HF10 inhibited HSV-2 replication in the vagina, reduced local inflammation, blocked neuro-invasiveness, and increased survival. The protective effect was related to the induction of cellular immunity mediated mainly by Th1 CD4+ T cells. These results indicate that HF10 is a promising candidate antigen in a vaccine against both HSV-1 and HSV-2 infection. Moreover, our data support the hypothesis that previous infection with HSV-1 provides a degree of protection against primary genital HSV-2 infection and attenuates its severity.

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REFERENCES

Arens, A. M., and Gordon, A. A. (1996). Live attenuated varicella vaccine. Annu. Rev. Microbiol. 50, 59–126.

Belkin, R. B., Levine, P. A., Bernstein, D. L., Wald, A., Levine, M. J., Stapleton, J. T., Gottschalk, J., Morrow, P. A., Devlin, M. G., Stosor, R., A. D., Dubois, G., Heineman, V. C., Schabes, J. M., Dau, D. C., and Herpes Trial for Women. (2012). Efficacy results of a trial of a herpes simplex virus type 1 and HSV-2 disease in mice immunized with a vaccine against both HSV-1 and HSV-2 infection. Moreover, our data support the hypothesis that previous infection with HSV-1 provides a degree of protection against primary genital HSV-2 infection and attenuates its severity.

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REFERENCES

Arens, A. M., and Gordon, A. A. (1996). Live attenuated varicella vaccine. Annu. Rev. Microbiol. 50, 59–126.

Belkin, R. B., Levine, P. A., Bernstein, D. L., Wald, A., Levine, M. J., Stapleton, J. T., Gottschalk, J., Morrow, P. A., Devlin, M. G., Stosor, R., A. D., Dubois, G., Heineman, V. C., Schabes, J. M., Dau, D. C., and Herpes Trial for Women. (2012). Efficacy results of a trial of a herpes simplex virus type 1 and HSV-2 disease in mice immunized with a vaccine against both HSV-1 and HSV-2 infection. Moreover, our data support the hypothesis that previous infection with HSV-1 provides a degree of protection against primary genital HSV-2 infection and attenuates its severity.

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McDermott, M. R., Brais, L. J., and Luo et al. HF10 protects HSV-2 genital disease

Miller, C. S., Danaher, R. J., and Jacob, R.

Mertz, G. J., Benedetti, J., Ashley, R., M., (2009). Herpes simplex virus type 1 clone, 2 tegument proteins contain sub-
dominant T-cell epitopes detectable in BALB/c mice after DNA immu-
ination and infection. J Gen Virol 90(Pt 5), 1155–1163.

Nakimshi, Y., Lu, B., Gerard, C., and Inoue, A. (2009). CD8+ T lymphocyte mobilization to varicella-
zenic virus-infected tissue requires CD8+ T- cell help. Nature 462, 516–520.

Nakos, A., Takada, S., Shimoyama, S., Kanaya, H., Kimata, H., Takahara, J., Suzuki, Y., Masuko, T., Koderu, Y., Nakada, T., Goshima, R., Nozawa, Y., and Imaz, E. (2007). Clinical experience of mutant hor-
pes simplex virus HSV therapy for cancer. Curr Cancer Drug Targets 7, 169–174.

Nasu, K., On Doork, J. E. M., Moller, J. P., Fuss, N. A., Muzumdar, H. A., and van der Meijden, W. L. (2006). Importance of herpes simplex virus type 1 (HSV-1) in primary genital herpes. Acta Derm. Venereol. 86, 129–134.

Nishiya, Y., Kineru, H., and Daikoku, T. (1991). Complementary lethal mutation of the central nervous system by nonrearranging herpes simplex virus types 1 and 2. J Virol 65, 4520–4525.

Ottman, N. M., Levin, M. J., Johnson, G. R., Schmidt, K. E., Strauss, S. E., Gell, L. D., Artho, R. D., Sum-
borkill, M. S., Gershon, A. A., Davis, L. E., Winstead, A., Roland, K. D., Williams, H. M., Zhang, J. H., Podar, P. N., Brand, C. E., Mersi-
son, V. A., Geitzl, J. C., Brooks, P. A., Kaufman, C. A., Pachucki, C. T., Nazar, K. M., Bertsch, W. P., Griffin, M. R., Brandt, F., Roots, N. E., Marques, R. A., Kran, S. K., Goodman, B. P., Goff, D. J., Gannam, J. W., Lott, J., Klotz, J., Malisoni, R., Rei-
ch, W. A., Crawford, G. E., Yolk, S. S., Lob, Z., Tonee, J. F., Greenen, B. R., Kato, P. M., Harbaum, B., Hayward, A. R., Ibnerr, M. R., Kiy-
ishkis, T. C., Chen, C. Y., Chan, S., Low, W. Y., Wamats, P. W., Silber, J. F., and Sanger Prevention Study Group. (1995). An in vivo method to prevent herpes yester and pothe-
retic neuritis in older adults. N Engl J Med 332, 2271–2280.

Peng, W., Henderson, G., Jinman, M., Bar-Mohammed, L. A., Wein, C., Chochili, S. L., and Jones, C. (2005). The focus encompassing the latency-associated transcript of hor-
pes simplex virus type 1 infectious virus and delayed viremia expression in productively infected neurobla-
toma cells and trigeminal Ganglia of acutely infected mice. J Virol 79, 6162–6171.

Rosson-Wolff, A., Lamal, W., Berkowitz, C., Becker, Y., and Darai, G. (1991). Elimination of UL96 gene by insertion of Iac conjugate between molecular position 110031 to 121753 of the herpes simplex virus type 1 genome abrogates interproetopathogenic pathway in true shoves and mice. Virovirology 20, 203–221.

Stanberry, L. R. (2004). Clinical trials of prophylactic and therapeutic hor-
pes simplex virus vaccines. Herpes 11(Suppl. 1), 181A–180A.

Stanbury, L. R., Cunningham, A. L., Minik, A., Sertli, I. L., Spruance, S. L., Aoki, F. Y., and Lacey, C. J. (2000). Prospects for control of herpes simplex virus disease through immunization. Clin Infect Dis. 30, 549–566.

Spruance, S. L., Cunningham, A. L., Berens, D. J., Mindlik, A., Sacks, S., Tying, A., Hula, F. Y., Hasen, M., Dren, M., Vande-
polpe, P., Dubi, O., and Glen-
mauSkllembuske herpes Vaccin Efficacy Study Group. (2002). Glycoprotein-B-adjuvant vaccine to prevent geni-
tal herpes. N Engl J Med. 347, 1652–1661.

Swanson, S. L., McMillen, J., and Hill, H. H. T. (1995). Diagnostic compati-
bility of a thymidine kinase, inverted repeat, pl and gp,g-modified live gene-deleted PRV vaccine with three dirifental ELISA. J Vet. Diag. Invest. 7, 347–350.

Swanson, S. L., McMillen, J., and Hill, H. H. T. (1995b). Evaluation of the safety and efficacy of a thymidine kinase, inverted repeat, pl and gp,g gene-deleted pseudorabies vaccine. J Vet. Diag. Invest. 7, 541–548.

Tao, G., Kassler, W. J., and Rein, D. B. (2000). Medical care expenditures for genital herpes in the United States. Am. J. Public Health 90(7), 12–20.

Ushijima, Y., Luo, C., Goshima, F., Uchihida, Y., Luo, C., Goshima, F., Yumode, A., Kimura, H., and Nishiyama, Y. (2007). Determination and analysis of the DNA sequence of highly attenuated herpes simplex virus type 1 mutant, HF10, a poten-
tial oncolytic virus. Microbes Infect. 9, 142–149.

Van de Walle, G. R., Fearson, H. W., Nazarysz, J. H., and Parent, M. B. (2003). Antibody-induced interme-
ialization of viral phosphoprotein and gI-
gl Fc receptor activity protect pseudor-
bacteria virus-infected monocytes from efficient complement-mediated lysis. J. Gen. Virol 84(Pt 4), 993–998.

Watan-Jones, D., Wein, H. A., Rau-
nicka, M., Changalzhha, J., Banley, R., Murguez, R., Tannin, C., Ross, D., Dervet, D., Clayton, T., Balba, R., Knight, L., Hambliton, J., Le Goff, J., Balse, L., Himys, R., HSV Test Team, and Steering and Data Monitoring Committees. (2008). Effect of herpes simplex suppression on incidence of DHS among women in Tanzania. N. Engl. J. Med. 358, 1580–1571.

Whitley, R. J., and Roizman, B. (2001). Herpes simplex virus infec-
tions. Lancet 357, 1513–1528.

Xu, F., Storngab, M. R., Kottiri, B. J., McQuillan, G. M., Lao, F. K., Nihlman, A. J., Berman, S. M., and Markowitz, E. E. (2006). Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States. JAMA 296, 966–973.

Xu, J., Kolda, D. M., Cao, J., Vasquez, J., Huang, M. L., Hahlib, F., Veld, A., and Corey, L. (2007). Virus-specific CD8+ T cells accumulate near sen-
sory nerve endings in genital skin during subclinical HSV-2 neuroinva-
sion. J Exp. Med. 204, 405–403.

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