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Inactivated HSV-2 in MPL/Alum Adjuvant Provides Nearly Complete Protection Against Genital Infection and Shedding Following Long Term Challenge and Rechallenge

Christopher S. Morello[1], **Kimberly A. Kraynyak**[1], **Michael S. Levinson**[2], **Zhijiang Chen**[3], **Kuo-Fen Lee**[3], and **Deborah H. Spector**[1,2,*]

[1]Department of Cellular and Molecular Medicine University of California, San Diego, La Jolla, CA 92093-0712

[2]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093-0712

[3]Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, La Jolla, CA 92037

Abstract

Herpes Simplex Virus Type 2 (HSV-2) infection can result in life-long recurrent genital disease, asymptomatic virus shedding, and transmission. No vaccine to date has shown significant protection clinically. Here, we used a mouse model of genital HSV-2 infection to test the efficacy of a vaccine consisting of whole, formalin-inactivated HSV-2 (FI-HSV2) formulated with monophosphoryl lipid A (MPL) and alum adjuvants. Vaccine components were administered alone or as a prime-boost immunization together with DNA vaccines encoding a truncated glycoprotein D2 (gD2t) and two conserved HSV-2 genes necessary for virus replication, UL5 (DNA helicase) and UL30 (DNA polymerase). Our results show: 1) Compared with mock immunized controls, mice immunized with FI-HSV2 plus MPL/Alum consistently showed protection against disease burden and total viral shedding while the mice immunized with gD2t protein with MPL/alum did not; 2) Protection against genital disease and viral replication correlated with the type of boost in a prime-boost immunization with little advantage afforded by a DNA prime; 3) Intramuscular (i.m.) immunization with FI-HSV2 in MPL/Alhydrogel adjuvant provided nearly complete protection against vaginal HSV-2 shedding after a lethal intravaginal (i.vag.) short-term challenge and long-term rechallenge; 4) Single formulation immunization with DNA vaccines, FI-HSV2, and MPL in an aluminum phosphate (Adju-Phos) adjuvant did not increase protection relative to FI-HSV2/MPL/Adju-Phos alone; and 5) addition of MPL/Alum to the FI-HSV2 was required for optimal protection against disease, viral replication, and latent virus load in the dorsal root ganglia (DRG). Most notably, an optimized vaccine formulation of FI-HSV2 MPL/Alhydrogel given i.m. completely protected against detectable vaginal HSV-2 shedding in the majority of animals and HSV-2 latent DNA in the DRG of all animals.

*Corresponding Author Cellular and Molecular Medicine 9500 Gilman Dr. 0712 La Jolla, CA 92093-0712 Phone: (858) 534-9737 Fax: (858) 534-6083. dspector@ucsd.edu.

Keywords

Herpes Simplex Virus Type 2 (HSV-2); inactivated vaccine; MPL/alum; prime-boost

1. Introduction

Herpes Simplex Virus type 2 (HSV-2) is a common sexually transmitted infection [1]. It can cause serious disease in neonates [2] and immune compromised hosts [3] and is associated with increased risk of human immunodeficiency virus (HIV) acquisition [4, 5]. HSV-2 replicates in the genital epithelium and is transported to the dorsal root ganglia (DRG) where it establishes a lifelong infection with reactivation and both symptomatic and asymptomatic shedding [6].

The correlates of protective immunity against HSV-2 are unknown. Studies in HSV-2/HIV-1 co-infected individuals have shown that CD4+ T cell loss correlates with increased HSV-2 shedding [7]. Neutralizing antibodies are important, but not sufficient in protecting against infection, as evidenced by failed vaccine trials [8]. During primary infection, CD8+ cytotoxic T cells may prevent acute ganglion infection [9], and reduce HSV-2 replication and shedding during recurrence [10, 11]. Despite many strategies, no successful HSV-2 vaccine has yet been licensed [9]. A promising candidate, the GlaxoSmithKline (GSK) vaccine consisting of secreted gD2 protein formulated with monophosphoryl lipid A (MPL) and alum, did not show significant protection against HSV-2 infection or genital disease in the latest Phase 3 study, but did show efficacy against HSV-1 infection and genital disease [12]. Replication-defective virus vaccines for HSV-2 are also in the pipeline as they have been successful in animal models [13-17]. Chemically inactivated HSV-2 vaccines have been tested extensively in humans, but subsequently dismissed due to a lack of controls required for accurate data interpretation (reviewed in [18]). The data did show, however, the need for a durable immune response not elicited by inactivated virus alone [18]. Inactivated virus formulated with MPL/alum or other adjuvants, or given together with other vaccine platforms in a prime-boost combination, may provide the necessary enhanced immune responses.

We recently evaluated a DNA prime-inactivated virus boost strategy for its efficacy against intravaginal (i.vag.) HSV-2 challenge in guinea pigs, a model for studying both acute and recurrent disease [19]. Guinea pigs were primed with plasmids encoding gD2t, UL5, and UL30, and then boosted with formalin-inactivated HSV-2 (FI-HSV2) in MPL/Alhydrogel. After i.vag. challenge, this group showed a 97% reduction in recurrent lesion days compared with the mock controls, had the highest reduction in days with recurrent disease, and contained the lowest mean HSV-2 DNA load in the dorsal root ganglia. However, the FI-HSV2/MPL/Alhydrogel component alone (the pVAX empty vector DNA – FI-HSV2 group), with the exception of one “nonresponder” animal, elicited complete protection against both acute and recurrent lesions and detectable HSV-2 DNA in the DRG. Cumulative acute disease scores and numbers of recurrent disease days were also comparable to the UL5, UL30, gD2t DNA-FI-HSV2 group.

In this report, we used the mouse model to further characterize the immunity and protection afforded by the DNA, FI-HSV2, and adjuvant. We also tested single formulation vaccines in order to simplify and expedite administration and to increase protective responses. Although HSV-2 does not spontaneously reactivate in mice, this i.vag. challenge model offers advantages in that it provides a lower cost system for rapidly evaluating vaccine candidates, has better characterized immunity to the virus with a wide variety of available reagents for the evaluation of specific T cell responses, has been optimized for progesterone treatment

allowing for uniform susceptibility to HSV-2 infection, and allows the use of inbred, transgenic, and congenic strains. Together, experiments in the mouse model have demonstrated that protection against HSV-2 infection can be mediated by both cell mediated and humoral immunity.

2. Materials and Methods

2.1. Vaccines

FI-HSV2 and a formalin inactivated mock preparation (FI-Mock) were prepared from HSV-2 infected or uninfected cells, respectively. Extracellular virus was purified and inactivated as previously described [19], and dextran sulfate wash-derived virus was purified as previously described for ELISA antigen [19] and then formalin inactivated. Cell associated virus was obtained from the clarified supernatant of HSV-2 infected Vero cells that were sonicated in virus containing extracellular media. The virus was subsequently pelleted and inactivated as described [19]. No infectious virus was detected after inactivation of any preparation. The preparation used for each experiment is denoted in each figure legend. All FI-HSV2 immunizations contained 10^7 PFU equivalents and 12.5 μg of MPL (Sigma L6895). Alum was purchased from Thermo Pierce (Imject alum) or Accurate Chemical & Scientific (Adju-Phos and Alhydrogel), with Al doses described in figure legends.

Plasmids expressing HSV-2 strain G UL5, UL30, and gD2t (aa 1 – 327) and the gD2t protein subunit vaccine were constructed and prepared as previously described [19].

2.2. Mice and Ethics Statement

Female BALB/c mice were purchased from Charles River Laboratories, housed in microisolator cages, and acclimated for at least 2 weeks prior to use. Studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All use of vertebrate animals was approved by the Institutional Animal Care and Use Committee, University of California, San Diego.

2.3. Intravaginal HSV-2 Challenge Model

Mice were treated with medroxyprogesterone acetate (Depo-Provera, Sigma M1629) [20] and i.vag. swabbed with a DPBS-moistened polyester tipped swab (MicroPur 1001D, PurFybr Solon, Rhinelander, WI) immediately prior to instillation of 5×10^4 PFU of HSV-2 strain G (ca. 10 LD_{50} doses) by micropipette. The HSV-2 preparation used for challenge was described previously [19].

Anogenital disease was scored as described in the Fig. 1 legend. Vaginal virus shedding of infectious virus was measured by plaque assay [19], and the limit of sensitivity of each assay is denoted in each figure.

2.4. Antibody Quantification

2.4.1. HSV-2 Virion Specific IgG—ELISAs were as previously described [19] except that alkaline phosphatase goat anti-mouse conjugates specific for IgG (Sigma), IgG1 or IgG2a (SouthernBiotech) were used, and endpoint titers were Fit Spline interpolated (GraphPad Prism 5.0d). Specifically, plates were coated overnight with 50 μl per well of either live, dextran sulfate-released HSV-2 (2.35×10^6 PFU) or an equal protein mass of uninfected Vero cell sonicate (0.1 μg), with subsequent UV-treatment of coated HSV-2 plates. Pre-challenge serum samples from individual mice were intially diluted 1:40 and then serially diluted 4-fold to 655,360 for analysis.

2.4.2. Neutralizing Antibody Assay—Complement-dependent HSV-2 neutralizing antibodies were measured by plaque reduction assay as described previously [21].

2.5. Intracellular Cytokine Staining

Eleven days following the second injection, mice were *in vivo* restimulated by HSV-2 injection in the footpad. Four days later, splenocytes were stimulated *in vitro* with 10 PFU per cell of HSV-2 (or an equivalent volume of a mock preparation) for 2 hours. Brefeldin A was added for an additional 8 hours.

For staining, a viability dye (LIVE/DEAD fixable violet; Molecular Probes, Invitrogen) and Fc block (CD16/32; BD) was added for 30 minutes at 4°C. Antibodies to surface markers CD8 (CD8-Ax488; Clone 53-6.7; BD) and CD4 (CD4-Ax647; clone RM4-5; BD) were added for 30 minutes at 4°C and then cells permeabilized and fixed using the BD Cytofix/Cytoperm kit. Antibodies to CD3 (CD3-PE-Cy5; Clone 145-2C11; BD) and IFN-γ (IFN-γ-PE; Clone XMG1.2; BD) were included in the intracellular stain for 45 minutes at 4°C. Data from 50,000-100,000 live CD3+ T cells were collected on a BD FACSCanto flow cytometer and analyzed with BD FACSDiva software at the Research Flow Cytometry Core Facility of the San Diego Center for AIDS Research and the Veterans Medical Research Foundation and VA San Diego Healthcare System, La Jolla, CA.

2.7 Quantification of HSV-2 DNA in DRG

Four week postchallenge lumbosacral DRG from each surviving mouse and 4 naïve mice were removed, pooled, and frozen. The DRG DNA from each pool was extracted and quantified by spectrophotometry. Cross-contamination safeguards, sample storage, DRG DNA extraction, and HSV-2 copy number determined by TaqMan quantitative PCR (Applied Biosystems, Inc.) using primers and a probe specific for gG2 were as previously described [19]. Each reaction contained 300 ng of DRG DNA and the DNA load for each mouse is expressed as HSV-2 DNA copy number per 300 ng of DRG DNA. Uniformity of each template was ensured by TaqMan quantification of the mouse adipsin gene (GenBank accession no. X04673.1): sense primer (TGT GGC AAT GGC AAA AAG C), antisense primer (TGT TAC CAT TTG TGA TGT TTT CGA T), and probe (6-FAM-CGT CTA TAC-ZEN-CCG AGT GTC ATC CTA CCG GA-Iowa Black F Quencher).

2.6. Statistical Analysis

Kruskal-Wallis analysis determined statistical significance for all data groups and Dunn's multiple comparison tests (GraphPad Prism 5.0d) compared all pairs of vaccine groups. Significance scores, $^{(*)}P < 0.05$; $^{(**)}P < 0.01$; $^{(***)}P < 0.001$; and (ns), not significant.

3. Results

3.1 FI-HSV2 provides more consistent protection against HSV-2 genital disease and shedding than gD2 subunit

To examine the protective efficacy of the protein-based "boost" vaccine components, mice were immunized twice with FI-HSV2, FI-Mock, or gD2t protein (each plus MPL/Imject Alum), and i.vag. challenged. FI-HSV2 was completely protective against death (Fig. 1A), with both anogenital disease (Fig. 1B) and vaginal virus shedding (Fig. 1C) significantly reduced below FI-Mock controls ($P < 0.001$). On day 2, vaginal virus titer reductions in the FI-HSV2 mice were reduced 3.6 Logs compared with FI-Mock ($P < 0.001$), although FI-HSV2 and gD2t-mediated protection were variable (Fig. 1D).

Next, we examined the protective efficacy of DNA priming (gD2t DNA alone or with UL5 and UL30 DNAs) followed by the above boost types in MPL/Alum (see Fig. 2A for

timeline). Boosting with gD2t or FI-HSV2 gave similar protection against death (Fig. 2B), and compared with pVAX – FI-Mock, all 3 of the FI-HSV2 boosted groups had significant reductions in total disease burden ($P < 0.01$ for the pVAX primed group and $P < 0.001$ for the others) (Fig. 2C) and total virus shedding ($P < 0.001$ for pVAX, $P < 0.01$ for gD2t DNA, and $P < 0.05$ for UL5, UL30, and gD2t DNAs) (Fig. 2D). The gD2t DNA – gD2t protein group also showed significantly reduced disease burden and total virus shedding ($P < 0.001$). Total shedding was lower in FI-HSV2 boosted mice compared to gD2t protein, but the differences were not statistically significant. While immune correlates were not examined in this experiment, independent intracellular cytokine staining (ICS) experiments using transfected stimulator cells showed that UL5 and UL30, but not gD2t) specific IFN-γ+ CD8+ T cells were detectable at levels of 0.5 – 1.0% of CD8+ T cells following DNA immunization (data not shown).

3.2 Co-immunization with plasmid DNA and FI-HSV2 in MPL/Adju-Phos as a single formulation does not enhance FI-HSV2 mediated protection

We next tested whether the vaccine components could be combined into a single formulation, as immune responses were shown to be elicited against both plasmid DNA and protein following codelivery in aluminum phosphate (Adju-Phos) [22]. Mice were injected with FI-HSV2 and MPL/Adju-Phos with or without the UL5, UL30, and gD2t DNAs. Two days post short-term challenge, both vaccinated groups had significantly reduced titers compared to the negative control group ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 3A, left). In the FI-HSV2 alone group, no virus was detected in 4 of the animals on day 2 and in 6 animals on day 4 (Fig. 3A, right).

We next tested the durability of responses. At 2 days post long-term challenge, the FI-HSV2 alone group had a significant 4 Log reduction in titers relative to Mock ($P < 0.001$), with rates of undetectable virus similar to those following short-term challenge (Fig. 3B). On day 4, all of the pVAX FI-HSV2 mice had detectable virus.

Levels of HSV-2 specific IgG prior to short-term challenge were similarly high in both vaccinated groups, and decreased by 1.5 - 2.5-fold in the long-term challenge animals (Fig. 3C left versus right). Surprisingly, neutralizing antibody titers prior to long-term challenge were only significantly above Mock in animals that received antigenic DNA ($P < 0.001$ compared to pVAX FI-Mock) (Fig. 3D).

3.3 Immunization with FI-HSV2 in Alhydrogel or Adju-Phos adjuvants is highly protective against HSV-2 replication following challenge and rechallenge

The best protection against virus shedding was observed in FI-HSV2 MPL/Adju-Phos immunized mice. We next compared two immunization routes, intramuscular (i.m.) and subcutaneous (s.c.), in addition to the adjuvants Adju-Phos and Alhydrogel (a well-defined aluminum hydroxide compared to Imject), and assessed the subsequent protection against a second challenge.

On day 2 postchallenge, virus shedding in all the FI-HSV2 groups was significantly reduced by 3 Logs compared to mock controls ($P < 0.01$ or $P < 0.05$) (Fig. 4A, left). Half or more of the mice in each FI-HSV2 group had shedding levels at or below the detection limit. By day 4 postchallenge, no or very low level virus was detectable in any of the FI-HSV2 immunized mice (Fig. 4A, right).

Mice immunized i.m. with FI-HSV2 in Alhydrogel or Adju-Phos had similar high levels of virus-specific IgG that were statistically greater than Mock ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 4B, left). FI-HSV2 in Alhydrogel i.m. elicited significantly higher IgG compared to s.c. ($P < 0.05$). Although all groups were significantly protected against

shedding, FI-HSV2 given i.m., but not s.c., elicited significant neutralizing antibody titers ($P < 0.01$) (Fig. 4B, right).

Nearly half of the animals receiving FI-HSV2 had no detectable shedding, so we next tested whether this high level protection would also be effective against re-challenge. After long-term rechallenge, only 2 of 23 mice immunized with FI-HSV2 had detectable vaginal virus (Fig. 4C y-axis) with levels that were low compared with naïve controls (Fig. 4D). Strikingly, 5 of 8 FI-HSV2/Alhydrogel/i.m. mice had no detectable virus on day 2 postchallenge or post-rechallenge (Fig. 4C, middle).

3.4 The addition of MPL/Alhydrogel to the FI-HSV2 vaccine is required for protection against HSV-2 replication, disease, and latent viral load in the DRG

We next determined whether formulation of FI-HSV2 in MPL/Alhydrogel was necessary for optimal protection and confirmed the above effect of immunization route on protection. On day 2 postchallenge, mice immunized with FI-HSV2 in MPL/Alhydrogel had statistically significant ca. 4 Log reductions in mean virus titer relative to Mock ($P < 0.001$), with 5 or 2 of 8 animals immunized i.m. or s.c. having no detectable virus, respectively (Fig. 5A, left). By contrast, shedding levels following immunization with FI-HSV2 in DPBS were not significantly different than Mock. In the i.m. injection groups, MPL/Alhydrogel provided significantly lower virus levels than did DPBS ($P < 0.05$). On day 4, the FI-HSV2 MPL/Alhydrogel groups had very similar mean titers, with no detectable virus in 6 of 8 animals in each group (Fig. 5A, right).

Mice immunized i.m. with FI-HSV2 MPL/Alhydrogel were completely protected against any disease beyond very slight erythema ($P < 0.001$) (Fig. 5B). While the FI-HSV2 MPL/Alhydrogel s.c. and DPBS i.m. groups were also significantly protected from disease relative to controls ($P < 0.01$), the DPBS s.c. group was not. Two mice died in each of the DPBS groups.

Four weeks postchallenge, DRG from the surviving mice and 4 naïve mice were harvested to quantify latent HSV-2 DNA levels by real-time qPCR (Fig. 5C). Strikingly, animals immunized i.m. with FI-HSV2 in MPL/Alhydrogel had no detectable HSV-2 DNA. Only two mice from the FI-HSV2 MPL/Alhydrogel s.c. group had detectable HSV-2 DNA; the mouse with 14 genome copies had the highest titers on day 2 and the mouse with 18 copies had undetectable virus.

I.m. immunization with FI-HSV2 MPL/Alhydrogel elicited the highest titers of virus specific total IgG (Fig. 6A, left) and neutralizing antibodies (Fig. 6B), with IgG titers approximately 10-fold higher than in the FI-HSV2/DPBS i.m. group (Fig. 6A, left). FI-HSV2 DPBS given i.m., but not s.c., elicited significant IgG levels relative to controls ($P < 0.05$). Addition of MPL/Alhydrogel to the FI-HSV2 s.c. immunization increased IgG levels by 7.6-fold and to a significant level over Mock ($P < 0.01$). We tested whether the injection route or addition of MPL/Alhydrogel biased the HSV-2 specific antibody response toward a Th1 (IgG2a) or Th2 (IgG1) response. Addition of MPL/Alhydrogel to the FI-HSV2 groups gave significantly increased IgG1 responses compared to their respective DPBS groups (i.m.: $P < 0.01$ and s.c.: $P < 0.05$), suggesting a Th2 bias (Fig. 6A, right).

Figure 6B shows that the highest levels of neutralizing antibody titers resulted from i.m. vaccination with FI-HSV2 ($P < 0.001$ compared to FI-Mock). Addition of MPL/Alhydrogel i.m. gave a 2.4-fold increase (not significant) in neutralization titers compared to the DPBS i.m. group. Interestingly, neither s.c. group was statistically different than FI-Mock.

Finally, an ICS assay was performed to analyze IFN-γ+ T cell responses to the inactivated virus (Fig. 6C). Low levels of HSV-2 specific CD4+ IFN-γ+ T cells were detected in the splenocytes of vaccinated and *in vivo* restimulated mice (net mean range from 0.15 to 0.43%), compared to the control group (net mean= 0.05%). However, CD8+T cell responses were undetectable above background. Stimulation of splenocytes with a pool of overlapping peptides spanning gD2 did not result in any CD4+ or CD8+ T cell responses above background (data not shown).

4. Discussion

In this study, we determined that the best protection against HSV-2 disease and shedding was obtained by i.m. injection of FI-HSV2/MPL/Alhydrogel. This vaccine prevented HSV-2 disease other than mild inflammation, reduced virus shedding by 3 to 4 Logs, protected the majority of animals from detectable vaginal virus even after two challenges, and provided complete protection against HSV-2 DNA in DRG. The high level efficacy was dependent upon formulation with MPL/Alhydrogel or MPL/Adju-Phos. The long-term challenge experiment with FI-HSV2/MPL/Adju-Phos demonstrated that responses were durable, as similar high level protection was observed at 3 and 9 weeks post-boost.

We found that the route of administration of the same tube of formulated FI-HSV2/MPL/Alhydrogel could affect the immune response, but not the resulting high level protection (Figs. 4-6). In 2 independent experiments, i.m. immunization elicited significantly higher levels of virus neutralizing antibodies compared to the s.c. and mock groups (Figs. 4B and 6B). Clinical studies of vaccines for influenza [23], diptheria and tetanus (DT) [24], and others [25, 26] have compared i.m. versus s.c. administration. In general, similar levels of antibody titers were attained with either route, but overall fewer adverse events (local and/or systemic) were observed following i.m. injection. The immunological mechanism for the better protection against disease found by i.m. delivery of the same FI-HSV2 vaccine is unknown, but is likely related to differential effects of administration route on the levels, functions, or localization of vaccine induced immunity.

Similar to a recent study comparing HSV-2 subunit vaccines [27], we found that no single measure of immunity correlated with protection, including neutralizing antibody, total IgG, IgG subtypes, or T cell responses. FI-HSV2/MPL/Alhydrogel i.m. immunization trended towards eliciting the highest levels of total IgG, IgG1, IgG2a, and neutralizing antibodies (Fig. 6). A previous study showed that purified IgG2a from the sera of HSV-2 immune BALB/c mice had 3- to 4-fold higher neutralizing activity than the IgG1 fraction [28], and in immunization studies using gD, bias towards an IgG2a (Th1) antibody response was associated with higher levels of protection [29, 30]. We found that FI-HSV2/MPL/Alhydrogel i.m. elicited levels of virus specific CD4+ IFN-γ+ T cells in the spleen similar to those observed in BALB/c mice immunized with replication-defective HSV-2 vaccines as measured with a similar assay [21, 31]. Our assay detected virus specific T cells in the spleen to document systemic responses to a parenterally administered vaccine. However, the T cell types and levels at the sites important for protection against mucosal HSV-2 challenge, such as the vaginal mucosa or DRG, are not known. Further experiments using adoptive transfer of immunoglobulin or T cell subsets from FI-HSV2 immunized mice to naïve mice or immune depletion of T cell subsets in immunized mice would be required to rigorously measure the relative contributions of these immune mechanisms for protection.

In general, FI-HSV2/MPL/Alhydrogel immunization consistently resulted in ca. 4 Log reductions in peak virus replication, which is similar to what was observed in mice immunized with replication-defective virus vaccines [31], subunit vaccines with experimental adjuvants [32], and live attenuated vaccines [33, 34]. Most notably, the

majority of FI-HSV2/MPL/Alhydrogel i.m. immunized mice had no detectable virus shedding, suggesting they may have had sterilizing immunity.

Compared with a single glycoprotein subunit, a potential advantage of a whole virus vaccine is that it may deliver a broader diversity of CD4+ and antibody targets in the context of the virus particle. While we did not measure the specificities of the antibodies elicited by FI-HSV2, we considered the possibility that protection was largely due to responses against the FI-HSV2 derived gD2. However, the cell associated FI-HSV2 used in Fig. 1 contained 2.9 μg of gD2 compared to 5 μg in the gD2t protein group, indicating that gD2 was not the key to protection. Subsequent FI-HSV2 preparations derived from dextran sulfate washes contained only 20 ng gD2 per dose and elicited the highest protection. Thus, the optimal protection from FI-HSV2 was not likely due to the nanogram quantities of gD2 protein but rather the total response to the virion-associated antigens.

We have now shown that FI-HSV2/MPL/Alhydrogel protects mice against virus shedding after challenge and long-term rechallenge, and guinea pigs against acute and recurrent disease [19]. The protection in animal models appears to be at least as high as that engendered by attenuated or replication-defective HSV-2 vaccines, strategies that have the potential for the establishment of latency or recombination with a wild-type virus. While vaccination with inactivated virus eliminates many safety concerns, a major criticism of the first-generation inactivated HSV-2 vaccines has been the poor durability of elicited responses. However, we show that formulation with adjuvants such as MPL/Alhydrogel challenges this dogma. These exciting results demand further consideration of inactivated HSV-2 vaccines formulated with the modern, clinically approved adjuvants.

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- ▶ Significant protection was achieved more frequently than with gD2t subunit.
- ▶ Formulation of DNA with inactivated HSV-2 did not enhance the protective efficacy.
- ▶ MPL/Alum was essential for optimal protection against HSV-2 disease and shedding.
- ▶ Injection route affected disease and types of immunity but not virus replication.
- ▶ Optimal vaccine delivery completely protected against latent HSV-2 in ganglia.

A

Vaccine	Survivors (n=8)
FI-Mock	0
gD2t protein	5
FI-HSV2	8

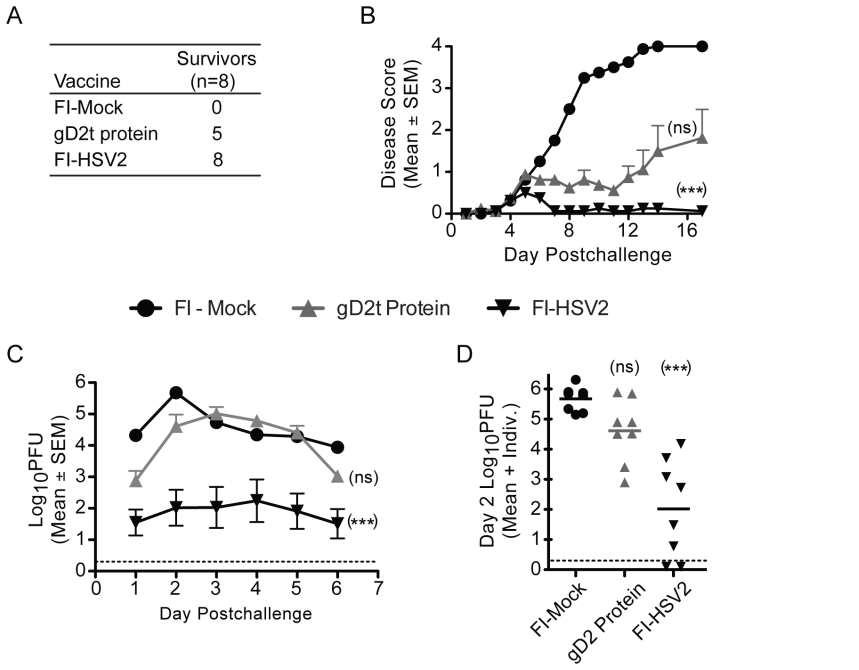

Fig. 1.

Protection against HSV-2 after immunization with FI-HSV2 or gD2t protein. Mice were s.c. immunized twice (mid-back), three weeks apart with either 1) cell-associated FI-HSV2 (approximately 250 μg of total protein), 2) an equal volume of a FI-Mock virus preparation, or 3) 5 μg purified gD2t protein. Each dose contained MPL and Imject alum equivalent to 725 μg Al. Mice were challenged 4 weeks following the last protein-based vaccination. (A) Twenty-one day postchallenge survival of each vaccine group. (B) Disease severity scores from 0 (no vaginitis), 1 (mild swelling or redness only), 2 (moderate swelling or erosions), 3 (severe genital maceration), or 4 (central nervous system involvement or death), with half scores given to intermediate disease levels. Mice discovered with hind limb paralysis were given a score of 4 and immediately sacrificed. Total disease burden (summed disease scores) of the FI-HSV2, but not gD2t protein group, was significantly reduced compared to FI-Mock. (C) Vaginal virus shedding, The dotted line shows the limit of sensitivity of the assay (2 PFU per swab), and assays yielding no plaques were assigned a Log_{10} titer of 0.1 for graphing and statistical analysis. Total shedding (summed Log_{10} PFU) in the FI-HSV2 group was significantly reduced compared to FI-Mock. (D) Vaginal virus titers of each mouse on day 2 postchallenge. Each symbol represents the Log_{10}PFU titer of each mouse, horizontal lines represent the group means, and the dotted line shows the assay detection limit (2 PFU per swab).

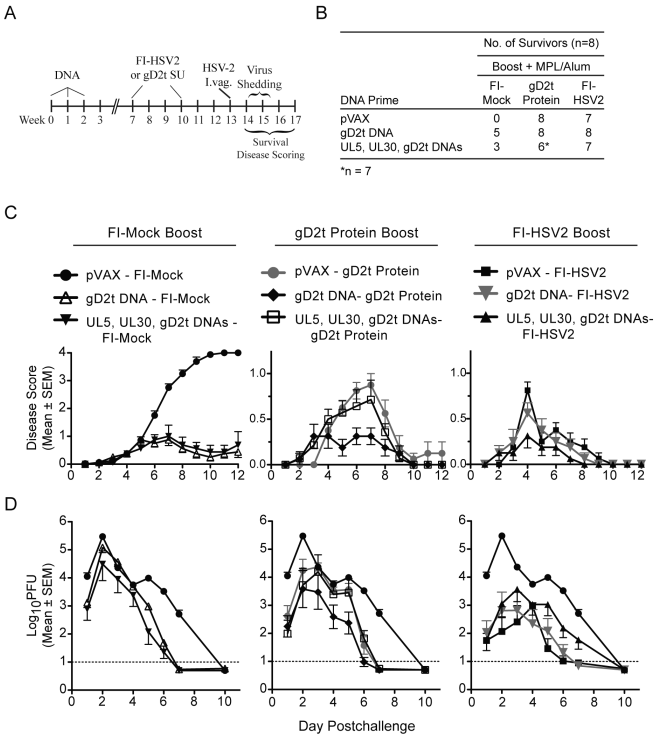

Fig. 2.

Protection against HSV-2 after prime-boost immunization. (A) Timeline of prime-boost immunization, challenge, and postchallenge outcome measurements. Mice were primed by 3 intradermal (i.d.) injections in the tail, 2 weeks apart, with either 1) 50 μg pVAX, 2) 50 μg gD2t DNA, or 3) a cocktail of 20 μg each of the UL5, UL30, and gD2t DNAs on the weeks shown. Mice from each DNA prime group were s.c. boosted with MPL and Imject Alum (equivalent to 43.3 μg of Al) together with either extracellular FI-HSV2 (containing 8.3 – 9.5 μg of protein), an equal volume of FI-Mock virus preparation, or 5 μg gD2t protein subunit (gD2t SU). (B) Postchallenge survival. One mouse in the UL5, UL30, gD2t DNAs – gD2t protein group developed a severe ear infection and was sacrificed prior to the challenge day. (C) Disease severity using the scale in Fig. 1, with each boost type shown in a separate panel. (D) Vaginal virus shedding as shown in Fig. 1C. Each panel shows the same boost type together with the pVAX – FI-Mock group.

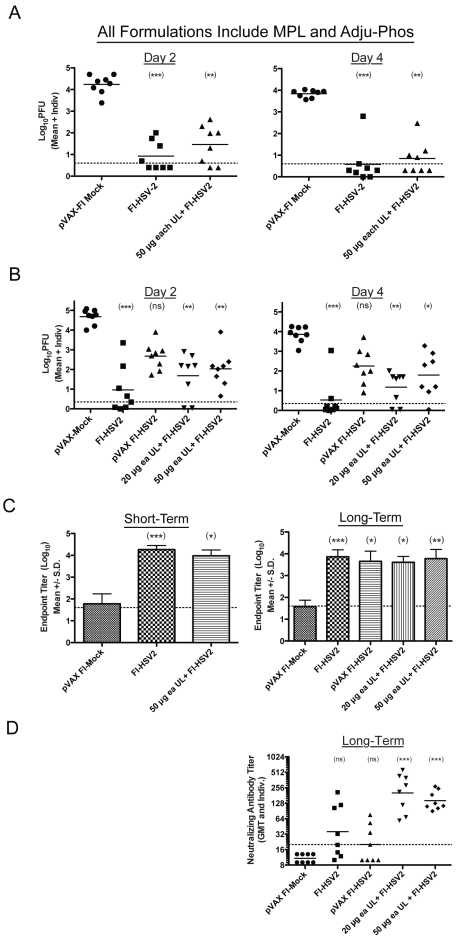

Fig. 3.

Protection against HSV-2 shedding with a single formulation vaccine. (A-D) Mice (n=8) were given two i.m. injections (into each tibialis anterior muscle) four weeks apart with dextran sulfate-derived FI-HSV2 (0.34 μg of protein), plasmid DNAs, MPL (12.5 μg), and Adju-Phos (90 μg Al). Groups received either 1) FI-HSV2, 2) 60 μg of pVAX DNA plus FI-Mock, 3) 60 μg of pVAX DNA plus FI-HSV2, 4) 20 μg each of UL5, UL30, and gD2t DNAs plus FI-HSV2, or 5) 50 μg each of UL5, UL30, and gD2t DNAs plus FI-HSV2. Following the second immunization, mice were rested for either 3 weeks (short-term) (A) or 9 weeks (long-term) (B), and then i.vag. challenged with HSV-2 as above. Vaginal HSV-2 shedding on day 2 (left panel) and day 4 (right panel) postchallenge as shown in Fig. 1D except assay detection limits were 4 PFU (A) and 2.22 PFU (B) per swab. (C,D) Virus specific antibody levels in immunized mice pre-challenge. Dashed lines represent the assay limits of sensitivity, and individual titers below the assay limit were assigned a value of one-half the assay limit for calculation and graphing purposes. (C) IgG titers measured by an ELISA against dextran sulfate-derived HSV-2 (2.35×10^6 PFU per well) before short-term (left panel) or long-term (right panel) challenge. The endpoint titer was defined as the highest, Fit Spline interpolated reciprocal dilution of serum at which the A_{405} of the virion-coated well was equal to twice the A_{405} of the same serum dilution in a well coated with an equal mass of Vero cell lysate. (D) Virus neutralizing antibody titers prior to long-term challenge. Endpoint neutralization titers were calculated as the highest reciprocal serum

dilution that resulted in a 50% reduction in the number of input PFU (50 PFU of dextran sulfate-derived HSV-2) using Fit Spline interpolation as above.

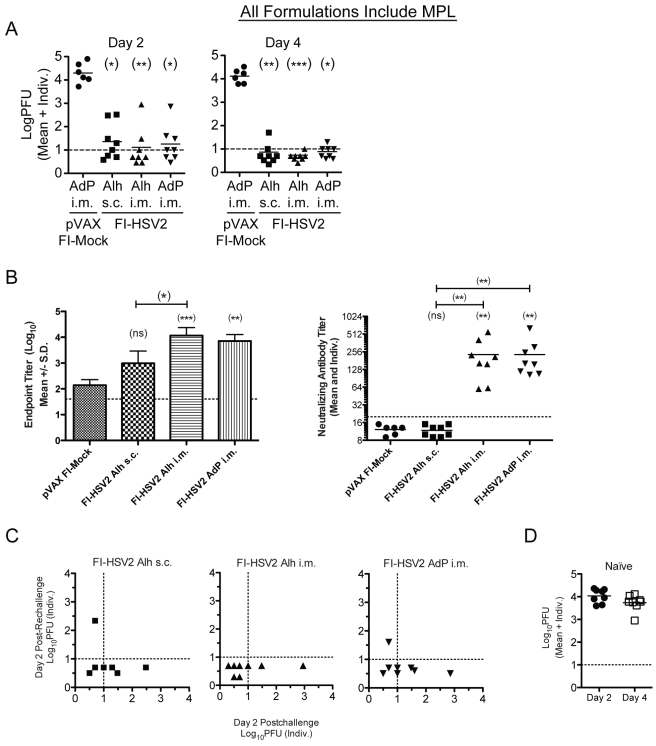

Fig. 4.

Protection against HSV-2 challenge and rechallenge after immunization with FI-HSV2 in MPL plus Alhydrogel or Adju-Phos. Groups of mice (n=8) were i.m. or s.c. immunized as indicated with extracellular FI-HSV2 (9.5 or 13.5 μg of protein for the first or second immunizations, respectively), MPL (12.5 μg), and either Alhydrogel (Alh, equivalent to 54 μg of Al) or Adju-Phos (AdP, equivalent to 90 μg of Al). A negative control group received 60 μg pVAX DNA mixed with MPL, Adju-Phos, and a volume of FI-Mock equal to the FI-HSV2 used in the other groups (2 mice in this group died prior to challenge). Mice received an identical immunization after 4 weeks and then challenged 3 weeks later. (A) Vaginal virus shedding on day 2 (Left panel) or day 4 (Right panel) postchallenge as in Fig. 1D. There were no significant differences between protected groups. (B) Virus specific antibody levels in immunized mice pre-challenge. Pre-challenge IgG titers (left panel) as in Fig. 3C and virus neutralizing antibody titers (right panel) as in Fig. 3D. (C), Surviving mice were medroxyprogesterone re-treated and rechallenged at 17 weeks after the initial challenge. One of the FI-HSV2/Alhydrogel/s.c. mice died after the first medroxyprogesterone re-treatment, and post-rechallenge, none of the other mice had disease scores above 0.5. Vaginal HSV-2 shedding is plotted as the $\log_{10}$ PFU on day 2 following HSV-2 challenge on the x-axis and on day 2 following rechallenge on the y-axis. Each FI-HSV2 immunization group is shown in a separate panel for clarity, and the limit of detection for both assays was 10 PFU per swab. No virus was detectable in any of the FI-HSV2 groups on day 4 post-rechallenge. (D) A naïve group of mice was i.vag. challenged with the same HSV-2 inoculum used for re-challenge of the groups in (C) and vaginal shedding on day 2 and day 4 are shown as in Fig. 1D. All naïve mice died following challenge.

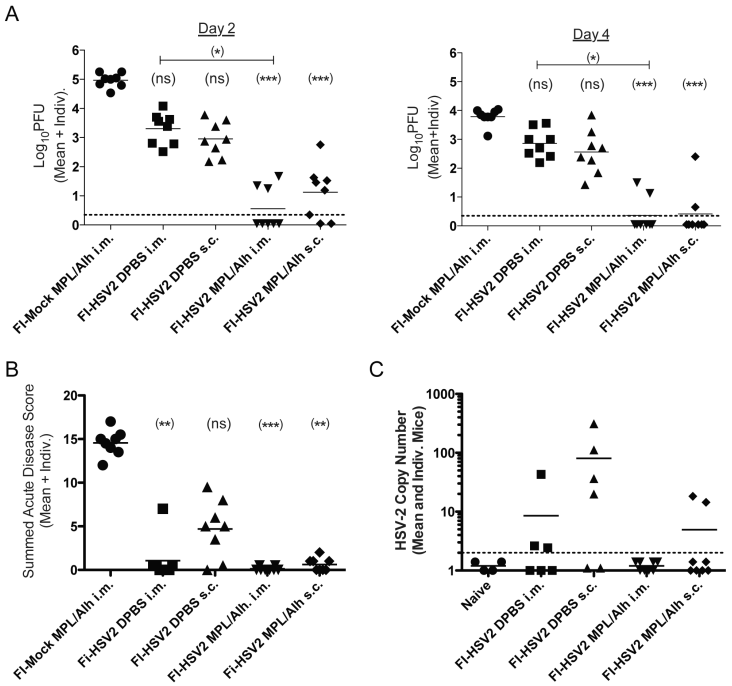

Fig. 5.

Immunization with FI-HSV2 formulated in MPL/Alhydrogel is required for protection against HSV-2 replication, disease, and latent viral load in the DRG. Groups of mice (n=8) were immunized s.c. or i.m. with dextran sulfate-derived FI-HSV2 (1 μg of protein) in either MPL and Alhydrogel (equivalent to 54 μg of Al) or DPBS. In addition, a negative control group received a volume of FI-Mock equal to the FI-HSV2 used in the other groups in MPL and Alhydrogel. Two injections were given 4 weeks apart, and mice were challenged 3 weeks later. (A) Vaginal virus shedding on day 2 (left panel) or day 4 (right panel) postchallenge as in Fig. 3A,B except that the limit of assay sensitivity was 2 PFU per swab. (B) Disease scores (using the scale described in Fig. 1) from days 1-14 postchallenge were summed for individual mice (each symbol shows the value for one mouse). (C) Protection against HSV-2 latent DNA load in the DRG. HSV-2 DNA copy number from four week postchallenge lumbosacral DRG from each surviving mouse and 4 naïve mice was determined by TaqMan quantitative PCR. Each reaction contained 300 ng of DRG DNA and the DNA load for each mouse is expressed as HSV-2 DNA copy number per 300 ng of DRG DNA. The line represents the limit of detection for the assay (2 copies of HSV-2 DNA per 300 ng of DRG DNA).

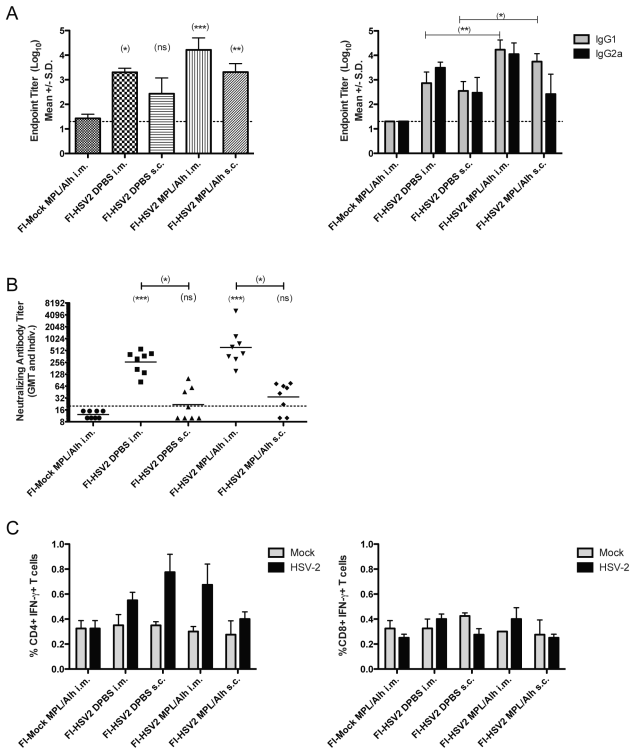

Fig. 6.

Virus specific antibody and T cell levels in mice immunized s.c. or i.m. with FI-HSV2 in MPL/Alhydrogel or DPBS. (A,B) Virus specific antibody levels in immunized mice. (A) ELISA against HSV-2 virion was used to measure prechallenge, total IgG (left panel) or IgG1 and IgG2a isotypes (right panel) in the sera of immunized mice. The mean endpoint reciprocal titers and SD are shown for each immunization group as in Fig. 3C. (B) Virus neutralizing antibody titers were measured against HSV-2 virion as in Fig. 3D. (C) The means and standard errors of virus specific CD4+ T cells (left panel) and CD8+ T cells (right panel) producing IFN-γ were measured by intracellular cytokine staining. Splenocytes from immunized and then *in vivo* restimulated mice (n=4) were collected and assayed 3 weeks following the second injection as described in Materials and Methods.