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A Mucosal Vaccination Approach for Herpes Simplex Virus Type-2

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Abstract

An estimated 1 out of every 5 Americans is infected with herpes simplex virus type 2 (HSV-2). Efforts in developing a potent vaccine for HSV-2 have shown limited success. Here we describe a heterologous vaccination strategy for HSV-2 based on an intramuscular DNA prime followed by a liposome-encapsulated antigen boost delivered intranasally. Both portions of the vaccine express the immunogenic HSV-2 glycoprotein D. In female Balb/c mice, this heterologous immunisation regimen stimulated high titers of serum neutralising antibodies, a DNA priming dose dependent T helper type response, enhanced mucosal immune responses and potent protective immunity at the portal of entry for the virus: the vaginal cavity. A clear synergistic effect on immune responses and protection from infection was seen using this heterologous immunisation approach. Suboptimal DNA prime (0.5 μg) followed by the liposome boost resulted in an 80% survival rate when mice were infected 2 weeks after immunisation. A higher dose of DNA priming (5 μg) followed by the liposome boost resulted in sterilising immunity in 80% of mice. The vaccine induced durable protection in mice, demonstrated by a 60% survival rate when lethal infections were performed 20 weeks after the immunization primed with 0.5μg of DNA vaccine.

Keywords

HSV-2; mucosal vaccine; heterologous prime and boost; DNA vaccine; liposome; mucosal delivery

1. Introduction

Herpes simplex virus type 2 (HSV-2) is endemic in the human population. According to the Centers for Disease Control and Prevention (CDC) approximately 20% of the US adult population is infected with HSV-2 [1], which can result in significant morbidity and psychological suffering. After initial replication in epithelial cells, virus enters neurons

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innervating the site of infection and enters latency. Periodically, HSV-2 will reactivate, replicate, and form new viral particles and travel down the axon to the original infected site, where it will undergo another round of lytic replication in the mucosal epithelium. Recurrences of genital ulcers typically occur 4 times per year [2]. Asymptomatic shedding of virus in the absence of vesicle formation is also a common occurrence. As many as 70% of new cases of HSV-2 are reported to be acquired from partners with asymptomatic shedding [3] and it is estimated that HSV-2 infected women shed virus from the genital tract a total of 15–20% of days [4]. Although HSV-2 generally results in mucosal lesions, HSV-2 infections involving other organs and surfaces are not uncommon [5]. For example, HSV-2 infection can involve the central nervous system where it induces the abrupt onset of fever and focal neurological symptoms. In addition, vertical transmission of virus from mother to infant and infections in immune compromised individuals can lead to viral encephalitis and/or dissemination of virus throughout the body [6]. In the absence of treatment with nucleoside analogs, the mortality rate for these infants is 50% [6]. In addition to causing primary disease on its own, HSV-2 is also a positive cofactor for HIV-1 transmission and has been associated with a 2–4 fold risk of acquiring HIV-1 [7].

No successful vaccine for HSV-2 has been marketed. To date, the only vaccine candidate with proven efficacy provides only limited protection against HSV-2, and solely in female patients that are seronegative for herpes simplex virus type 1 (HSV-1) [8]. The partial success of this vaccine is believed to be due to the costimulation of antibody and T cell responses.

Clinical trials and animal studies have indicated that any successful HSV-2 vaccine candidate must initiate protection in multiple forms. Humoral immunity is important for protection from extracellular virion particles during initial exposure, during vertical transmission of virus from mother to child and during reactivation of virus when extracellular particles are transmitted from neuron to epithelial cell [9,10]. Infections in B cell-deficient mice indicate that while HSV-specific antibody limits infection, other arms of the immune system are required to prevent infection [11]. Cellular immunity is necessary for clearance of virus-infected epithelial cells during primary and recurrent infections, resolution of lytic infections in sensory ganglia and possibly in the prevention of reactivation [12–18]. Depletion studies have demonstrated that protection against HSV-2 re-infection is primarily controlled by $CD4^+$ T cells rather than $CD8^+$ T cells or antibodies [19–21]. Further, long term immunity appears to be dependent upon mucosal rather than systemic immunisation, highlighting the importance of local mucosal responses [22].

Few vaccination strategies are safe and effective in raising protective immunity at mucosal surfaces [23]. Vaccines are also restricted in their ability to raise cell-mediated immune responses necessary to eliminate intracellular infections [24,25]. To address these issues, we have developed a heterologous immunisation platform that entails a DNA prime followed by a liposome-encapsulated protein boost [26]. These components are safe, easily prepared, and have a demonstrated capability in the study of vaccine development [27,28]. Using the Hepatitis B surface antigen (HBsAg) as a model antigen, we previously showed that this regimen induces: strong, high avidity serum and mucosal antigen-specific IgG and IgA antibodies; a T helper type 1 (Th1)-biased immune response; and antigen-specific cytotoxic T lymphocytes (CTLs; [26]). Here we demonstrate the successful application of this vaccination regimen for the development of an HSV-2 vaccine.

2. Material and Methods

2.1 vaccine constituents

To produce the gD vaccine, the DNA sequence encoding gD was synthesised *in vitro* to generate a gene sequence fully optimised for expression in mammalian cells (GeneArt; North Carolina). The synthesised gene was cloned into the DNA vaccine-specific vector, pDNAVACC (Nature Technology; Nebraska) and sequenced to confirm identity.

The gD antigen was purchased from Vybion (Ithaca, New York). The antigen was produced in *Pichia pastoris* and comprised the extracellular domain of gD.

gD encapsulated liposomes were prepared as previously described [26]. For optimisation of the vaccine, both negatively and positively charged liposomes containing 3 or 15 μg of gD protein per 50 μl volume were produced. Liposomes were lyophilised and stored at −20° C until the day of use.

2.2 Mice and immunisations

Pathogen free, barrier maintained female Balb/c mice (H-2^d) 6–7 weeks of age were obtained from Harlan (Indianapolis, IN). All mice were maintained under specific-pathogen-free conditions. Mice were anesthetised prior to vaccination using a ketamine/xylazine mixture. gD DNA vaccine or empty plasmid was administered intramuscularly on Days 0 and 2. gD-liposomes or empty liposomes were administered 3 weeks after the DNA prime (50 μl total dose per mouse per time point in both nostrils). All animals were housed in sterile microisolator cages and had no evidence of spontaneous infection. Animals were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of BRM, and in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

2.3 Sample collection

Blood samples were collected by orbital bleed and collecting blood by capillary action into clot activating Microvette ®microtubes (Sarstedt, Newton, NC). Vaginal washes were performed by instilling 40 μl of sterile saline intravaginally to anesthetised mice, gently flushing the cavity, and collecting the wash with a pipet tip. This procedure was repeated, and then the first and the second washes were combined and diluted in 300 μL of serum-free Dulbecco's Modified Eagle Medium (DMEM) and stored at −70°C. Vaginal washes were taken every other day for 7 days post infection (p.i.).

2.4 Measurement of antibody responses

gD-specific antibody responses (IgG, IgG1 and IgG2a and mucosal IgG and IgA) were measured using ELISA assays. Antibodies and mouse Ig isotype standards were purchased from Southern Biotech (Birmingham, AL). Assays were developed and optimised using 40μg/ml of recombinant gD in binding buffer to directly coat the plates. ELISAs were developed using Sure-Blue™TMB microwell peroxidase substrate (KPL, Gaithersburg, MD) and 2 N sulphuric acid to stop the reaction. Plates were read using a Multiskan Ascent plate reader (Thermo Electron Corp., Mountain View, CA). Results are expressed as μg/ml or ng/ml of IgG and IgA.

2.5 In vitro IFN-γ stimulation and ELISA Assay

1×10^6 of splenocytes were collected from immunised and HSV-2 infected mice. The cells were stimulated in vitro with the HSV-gD protein used for immunisation (10 μg/ml) in 6

well plates for 48 hours. Cell culture supernatants and vaginal wash samples were analysed for IFN-γ using the mouse "Femto-HS" high sensitivity ELISA Ready-SET-Go kit (eBioscience, San Diego, CA). Briefly, 3-fold dilutions of sample or standard were incubated with IFN-γ capture antibody coated plates. IFN-γ was detected with the provided detection antibody and Avidin-HRP / TMB substrate solution and the reaction was stopped with 2N sulphuric acid. Plates were read on a 96-well ELISA plate reader at 450nm wavelength and the data was converted to concentration (pg/ml) using the IFN-γ standard curve.

2.6 Virus and viral challenge

The clinical isolate, HSV-2 strain MS purchased from the ATCC was grown and titered in Vero cells. LD50 was titrated in Balb/c mice prior to the challenge experiments. Five days prior to infection, mice were injected subcutaneously with 2mg of medroxyprogesterone (Depo-Provera, Pfizer, St. Louis, MI). On the day of infection, animals were anesthetised intraperitoneally with a ketamine/xylazine mixture and instilled intravaginally with a 20μl suspension containing the indicated virus dose. Animals were monitored for body weight and clinical signs of disease for at least 21 days after infection. Lesions were scored according to the following scale: 0 = no visible redness or lesions, 1 = redness or mild swelling, 2 = erosions, vesicles, or moderate swelling, 3 = several large vesicles, 4 = large ulcers with severe maceration and/or urinary retention and/or hind limb paralysis. Animals that reached a clinical score of 4 were immediately euthanized.

2.7 Virus titration

Samples from the vagina were thawed, diluted in ten-fold serial dilutions and titrated for plaque forming units (PFU) on Vero cells. Plates were incubated for two days, the culture medium was discarded and cells were fixed and stained with 0.5% methylene blue (Thermo Scientific, Waltham, MA) in 70% methanol.

2.8 Neutralisation assay

Neutralising HSV-2 antibody titers were measured with a complement-dependent Neutralisation assay. Guinea pig complement (Biomeda; Foster City, CA) was added to the medium at a final dilution of 1:125. The complement-medium mixture was used as diluent in the preparation of virus dilutions and further serum dilutions. A 0.15-ml amount of three-fold dilutions of serum was combined with an equal volume of virus dilution (calculated to produce approximately 100 plaque-forming units) and incubated for 2 hours at 37°C. After removal of growth medium, 0.3ml of each serum-virus mixture was added to duplicate Vero cell cultures grown to near confluence in 35-mm tissue culture plates. These plates were incubated and shaken at 37°C for 1h. The inoculum was removed and medium containing 2% FBS and 7.5μg/ml pooled human immunoglobulin (Sigma; St Louis, MO) was added to each plate. Plates were incubated at 37°C in a $C0_2$ incubator for two days, the culture medium discarded and cells were fixed and stained with 0.5% methylene blue in 70% methanol. Plaques were counted and the percentage of plaque reduction was measured compared to the control (virus incubated without serum).

2.9 Statistics

Statistical analysis was performed using the GraphPad Prism software. The equality of surviving animals was tested by log rank analysis. Means between two groups were compared using a paired T test. Groups of three or more means were compared using one way analysis of variance and the Tukey multiple comparison test. P values <0.05 were considered statistically significant.

3. Results

3.1 Optimisation of vaccine components

We previously demonstrated that liposome charge and size are critical for induction of optimal responses to HBsAg when delivered intranasally [26]. To confirm this observation for the gD antigen, we immunised mice homologously (3 weeks apart) with either negatively or positively charged liposomes with average sizes of either 0.2 or 1–4 microns. Each liposome condition was tested with either 3 or 15μg of incorporated gD. As observed for HBsAg, only negatively charged liposomes induced measurable gD-specific IgG responses (data not shown). We also observed that 15μg of protein encapsulated in liposomes sized at 1–4 microns induced the highest responses (data not shown). From these experiments, the optimal liposome composition was chosen as 15μg of gD encapsulated in negatively-charged liposomes sized at 1–4 microns (hereinafter called gD LIP.)

Prior research has demonstrated that vaccination with multiple, high doses of gD DNA alone is only sufficient to protect mice and guinea pigs from developing disease following a lethal challenge with HSV-2, while virus replication can be only partially contained [29,30]. However, multiple, high doses (up to 3mg) of DNA vaccination alone is not similarly immunogenic in humans [31]. A recent paper from the same group showed that this DNA vaccine raised HSV-specific T-cell responses, in what the authors termed “immune seronegatives” [32]. As these were human studies, it is not known how effective the seronegative, cellular-positive responses might be in preventing or modifying subsequent challenge. To develop a vaccine that will be effective in the human population, we hypothesised that a prime and boost vaccination regimen that relies on synergistic responses (including mucosal immune responses) after heterologous immunisation with both the DNA and antigen-liposome components of the vaccine would have a higher chance of success. To test our hypothesis, we first titrated the gD DNA vaccine in mice to determine the optimal dose of vaccination. Efficient expression of HSV-2 gD protein by our gD DNA vaccine construct was confirmed by both Western blot and ELISA (data not shown).

Female Balb/c mice were immunised with various doses of the gD DNA vaccine (0.05 to50 μg of gD DNA). Three weeks after immunisation serum was collected and pooled from immunised mice and total IgG responses to gD were measured using an ELISA assay as described in the *materials and methods*. At the second lowest vaccination dose (0.25μg of DNA), 4/6 mice developed antibodies to gD. Higher doses of DNA resulted in a dose dependent response from all animals (Figure 1, Panel A). Mice immunised with 0.5, 5 or 50 μg of gD DNA had gD-specific antibody titers of 28, 94 and 203 μg/ml IgG, respectively. This was greater than the IgG response seen in mice that had been infected with HSV-2 viruses (1000 pfu) for 5 weeks (~ 20μg/mL total IgG; data not shown). These results indicate that the DNA vaccine expresses gD protein in sufficient quantities to elicit antigen-specific antibody responses.

To determine whether the DNA portion of the vaccine was protective in mice against disease and mortality, we challenged gD DNA vaccine alone vaccinated mice with either a low (5LD50) or high (100LD50) dose of HSV-2. As shown in Figure 1, Panel B, all but the lowest dose of DNA vaccine provided protection from challenge with 5LD50 of HSV-2. Greater than 50% of animals immunised with all but the two highest doses of DNA vaccine died or were moribund within 2 weeks after infection with 100LD50 of HSV-2 (Panel C). The higher dose of viral challenge helped reveal that protection against HSV-2 infection was dependent upon the dose of DNA vaccination: the greater the concentration of DNA vaccine, the greater the number of animals that survived. Based upon these results, we chose 0.5μg and 5.0μg as low (suboptimal) and high DNA vaccine doses to be used in future studies.

3.2 Heterologous immunisation induces synergistic HSV gD-specific IgG responses

To test the ability of the full vaccine (gD DNA + gD LIP) to induce immune responses, female Balb/c mice were immunised heterologously by priming with an *i.m.* injection of 0.5μg of gD DNA (given on days 0 and 2) followed by an *i.n.* boost of gD LIP given 3 weeks later. To evaluate the contribution from each of the vaccine components, separate groups of mice were immunised with either gD DNA followed by empty liposomes (gD DNA) or empty vector followed by gD LIP (gD LIP). In addition, we tested the effect of increasing the priming dose of DNA vaccine by immunising an additional group of mice with 5μg gD DNA+gD LIP. Naïve mice served as a negative control. The results are shown in Figure 2.

Serum IgG was barely detectable after a single vaccination with either of the components alone (Panel A). In contrast, IgG levels after immunisation with 0.5μg gD DNA + gD LIP reached greater than 1100μg/mL, demonstrating a clear synergistic response. Animals immunised with 5μg of gD DNA+gD LIP had serum antibody responses two times that of animals immunised with 0.5μg of gD DNA+gD LIP (2393 μg/mL and 1151 μg/mL, respectively). The difference in IgG levels after immunizing with either component alone versus both components in combination was highly significant ($p<0.0001$). Vaginal antibody responses (IgG and IgA) followed a similar pattern (Panel B), however the differences did not reach statistical significance. When the gD liposome boost was delivered *i.m.* after DNA priming (0.5μg dose) only a marginal increase in serum IgG was observed in the absence of any mucosal responses (data not shown).

A plaque-based Neutralisation assay was used to compare the ability of serum antibodies to neutralize whole HSV-2 virus. Neither serum collected from naïve mice, mice immunised with 0.5μg gD DNA alone or gD LIP alone induced measureable neutralising antibody (data not shown). In contrast, serum samples collected from mice immunised with 0.5μg gD DNA + gD LIP strongly neutralized HSV-2, resulting in a 70% reduction in the number of plaques seen when the serum was diluted 1:150. Neutralising activity could be enhanced by using 10x more priming dose of gD DNA (5μg), followed by the same gD LIP boost procedure. Pooled serum samples from mice vaccinated with 5μg gD DNA+gD LIP showed a 94% reduction in plaque number after a 1:150 dilution of the sample.

3.3 Heterologous immunisation induces a Th-1 to balanced Th1/Th2 helper response

The ratio of antigen-specific IgG1 and IgG2a antibodies was used to characterize the T helper type bias of an immune response [26,33,34]. IgG1:IgG2a ratios≤0.5 indicate a Th1-biased immune response, while a ratio of ≤ 2.0 indicates a Th2-biased immune response. Ratios between 0.5 and 2.0 indicate a mixed response. To evaluate the contribution from each vaccine component, we used quantitative ELISA assays to determine the IgG1:IgG2a ratio obtained after administration of each individual component and the two components combined. We also compared results after immunisation with either 0.5μg gD DNA or 5μg gD DNA (Figure 3). Immunisation with 0.5μg gD DNA or gD LIP alone resulted in IgG1:IgG2a ratios of 0.84 and 27.9, respectively, indicating mixed and Th2-biased responses. The ratio after immunisation with 5μg of gD DNA alone was 0.39 indicating a Th1 biased response. The ratios after immunisation with both components were 0.47 and 0.33, respectively after priming with 0.5μg or 5μg of gD DNA. These results demonstrate that: 1) gD DNA alone immunisation induces a mixed or Th1 biased response depending upon the amount of DNA used; 2) gD LIP alone induces a Th2 biased response; 3) Th1 biased responses established by DNA priming are maintained after boosting with the liposome encapsulated protein component of the vaccine.

3.4 Induction of HSV-2 gD specific IFN-γ

In addition to serum and vaginal IgG, and CD4 T cells, IFN-γ is also a major protective mechanism in HSV-2 infections [35]. While IFN-γ is not essential for virus clearance, it plays an important role in enhancing T cell-mediated clearance mechanisms [36]. IFN-γ produced locally in the genital tract enhances virus clearance and may ultimately be important for reducing the amount of virus available to infect sensory ganglia.

Examination of specific T cell responses to gD is limited in Balb/c mice as gD-specific epitopes have yet to be defined in this strain. To determine whether whole gD protein could be used to stimulate gD specific T cells, we vaccinated mice with either 0.5μg gD DNA, gD LIP or 0.5μg gD DNA+gD LIP and then infected the animals with HSV-2 two weeks after the liposome immunisation. We reasoned that infection would act as an additional boost thereby enhancing immune responses and increasing the chance of detecting gD-specific T cell responses. Ten days after infection, splenocytes were isolated and incubated with 10μg/mL of gD protein. The supernatants were collected 48 hours post stimulation and examined in an ELISA assay to detect IFN-γ. As shown in Figure 4 Panel A, IFN-γ was readily detected from infected animals vaccinated with either of the components alone or by heterologous immunisation, while IFN-γ release was minimal in the one surviving naïve animal (152 pg/ml). Vaccination with gD DNA alone induced the highest IFN-γ response that was significantly greater than the level observed after vaccination with gD LIP or gD DNA+gD LIP ($p<0.05$). The less IFN-γ production found in gD DNA+gD LIP vaccinated animals may due to the protective immune response established by the vaccination, which led to decreased virus infection and therefore limited its boosting effect on IFN-γ production. More importantly, IFN-γ secretion was enhanced in vaccinated groups of mice suggesting gD specific T cell response was established by the immunisation and vigorously recalled upon virus challenge.

In a separate experiment, we directly measured IFN-γ levels in the vaginal wash samples of naïve or vaccinated mice infected with HSV-2 for varying amounts of time. Groups of mice were vaccinated with 0.5μg DNA, 5μg DNA, gD LIP, 0.5μg gD DNA + gD LIP or 5μg gD DNA + gD LIP and infected 2 weeks after the last immunisation. Vaginal washes were collected on Days 0 (before infection), 1, 3, 5 and 7 post infection and used in an ELISA assay to directly measure IFN-γ levels. IFN-γ was undetectable on Days 0, 1 but increased on Day 3 and peaked on Day 5 post infection (as shown in Figure 4, Panels B and C), a point in time when memory T cells are responding to challenge virus and naïve immune cells undergo an innate immune response [34,35]. On Day 3 post infection, IFN-γ levels were lowest in naïve animals. Higher levels of IFN-γ were seen in animals vaccinated with either component alone, with the highest responses seen in animals immunised with gD DNA as responses were recalled in previously primed cells. Interestingly, on day 5 IFN-γ levels in mice vaccinated with 0.5μg DNA + gD LIP were lower than in animals vaccinated with gD LIP alone, and levels in animals vaccinated with 5μg DNA + gD DNA were almost undetectable. On Day 5 post infection, naïve animals showed a robust innate response to virus, which were higher than that of animals vaccinated with gD DNA alone, gD LIP alone or 0.5μg gD DNA + gD LIP while levels in those animals vaccinated with 5μg DNA + gD LIP remained almost undetectable. On day7 post infection, IFN-γ level in naïve animals became lower (64 pg/ml) than that of animals vaccinated with 0.5μg gD DNA alone (255 pg/ml), gD LIP alone (128 pg/ml) or 0.5μg gD DNA + gD LIP (108 pg/ml) while the IFN-γ levels in those animals vaccinated with 5μg DNA + gD LIP remained undetectable. The lack of IFN-γ response in animals vaccinated with 5μg DNA+ gD LIP may suggest that viral replication was effectively inhibited probably by neutralising antibodies upon virus challenge, based on the correlation between virus replication and IFN-γ secretion.

3.5 Heterolgous immunisation protects animals from a lethal dose of HSV-2

We infected animals immunised with 0.5μg gD DNA or gD LIP alone and animals immunised with 0.5μg gD DNA+gD LIP to test the ability of the heterologous immunisation to provide protection from live virus. Two weeks after the last immunisation, naïve and immunised animals were inoculated intravaginally with 100X LD50 of HSV-2. A summary of the clinical scores is shown in Table 1 and survival curves are shown in Figure 5. All infected naïve animals progressed to severe signs of disease; 4/5 of the animals were moribund or died as a result of the infection. The fifth animal developed a score of 3.5. The average maximum clinical scores among infected animals immunised with 0.5μg gD DNA or gD LIP alone were 2.7 and 3.0, respectively. No significant differences in survival were observed after vaccination with either 0.5μg gD DNA or gD LIP alone compared to unvaccinated animals ($p=0.09$ and 0.55, respectively). In contrast, mortality was significantly reduced in animals vaccinated with the heterologous regimen as compared to unvaccinated animals ($p=0.01$). Only 2/10 animals (20%) that underwent heterologous immunisation were moribund/died. A majority of the animals immunised with 0.5μg gD DNA+gD LIP developed only minor signs of disease. Three infected animals did not develop any signs of clinical disease, while 4 did not progress beyond a clinical score of 2.0.

In a separate experiment, animals were vaccinated with 5μg gD DNA alone or 5μg gD DNA +gD LIP prior to infection. In this experiment, animals were only held until 10 days post infection so that spleens could be harvested. At the time of sacrifice of the animals (Day 10), 5/6 animals vaccinated with 5μg gD DNA alone had a clinical score of 3 while the sixth animal had a clinical score of 2. Importantly, only 1/5 animals immunised with 5μg gD DNA+gD LIP showed clinical score of 4, while the remaining 4 animals showed no clinical signs of disease.

Vaginal samples were collected from unvaccinated naïve animals, animals immunised with 0.5μg gD DNA or gD LIP alone, or animals immunised with both 0.5μg gD DNA+gD LIP on days 1, 3, 5 and 7 post infection. In addition, samples were also collected from the animals immunised with 5μg gD DNA alone or those animals immunised with 5μg gD DNA +gD LIP that showed no clinical signs of disease by Day 10 post infection. Samples were titrated in Vero cells for detection of virus. In unvaccinated animals, virus could be detected in the vagina 1 day post infection (Figure 6). Viral titers peaked at 3 days post infection and declined thereafter, paralleling IFN-γ levels (Figure 4). On days 1, 3 and 5 post infection viral replication was detected in all samples isolated from animals immunised with any of the vaccine components alone or 0.5μg gD DNA+gD LIP. On these days viral titers were decreased to varying degrees depending upon the vaccine used, however no statistical differences were observed between these groups. In contrast, no virus could be recovered from animals immunised with 5μg gD DNA+gD LIP that showed no clinical signs of disease (4/5 mice) at any point post infection.

3.6 Heterologous immunisation induces long lasting immunity

To test the ability of heterologous immunisation to induce long-lasting protective immunity, animals immunised with 0.5μg dose gD DNA+gD LIP were infected at 10 or 20 weeks after the booster immunisation. Results were compared to naïve animals, and animals immunised with gD DNA alone. Figure 7 shows the results from the 10 and 20 week infections compared to those obtained in Figure 5, in which animals were infected 2 weeks after boosting. Only one naïve animal (1/15; 7%) survived infection among the 3 time points that were investigated. Two weeks after the booster, 50% of the animals immunised with gD DNA alone survived infection. Immunity induced by gD DNA alone appeared to decrease over time as only 20% of animals survived infection at 10 and 20 weeks after boosting. The mortality rate at 20 weeks after vaccination was significantly different from the mortality

rate seen at 2 weeks after vaccination (p=0.048). In contrast, heterologous immunisation with gD DNA+gD LIP induced long-lasting protective immunity. At 10 and 20 weeks after boosting, 89 and 60% of animals survived infection, respectively. Comparison of mortality rates after infection at 2, 10 and 20 weeks post vaccination revealed no significant differences between all groups (p>0.373).

4. Discussion

Many different HSV-2 immunisation strategies have been developed and evaluated, including the use of whole inactivated virus, live attenuated virus, live replication defective virus, subunit vaccines and DNA vaccines. However, a safe and effective vaccine for HSV-2 is still not available. As demonstrated in this work, we have developed a new HSV-2 vaccine comprised of a DNA prime given intramuscularly and a liposome-encapsulated boost delivered intranasally. The vaccine induces clear synergistic responses comprised of humoral, T cell and mucosal immunity. The vaccine is flexible and potency can be enhanced by increasing the dose of DNA vaccine. In fact, sterilising immunity was observed in 80% of mice receiving the higher dose of DNA vaccine in combination with the liposome component. Protection is durable as >50% of animals are still protected from lethal disease 20 weeks after final immunisation (primed with suboptimal DNA dose)

We sought to build upon the existing strategies for development of an HSV-2 vaccine by using a heterologous immunisation approach that targeted the mucosa, a major portal of entry for viruses. There are several advantages to using DNA vaccines over purified protein. Antigen is presented in a native state, and epitopes can be presented by the MHC class I pathway to activate CTL. DNA vaccines are generally designed to contain multiple bacterial unmethylated CpG motifs which can activate host defense mechanisms leading to innate and acquired immune responses [37,38]. Human trials indicate that DNA vaccination is well-tolerated and induces priming of immune responses, but overall will require enhancement of immune responses to generate protective immunity [39].

The current focus in DNA vaccine technology is on the generation of better vectors for higher expression of the gene of interest and the use of heterologous immunisation regimens to boost immune responses [39,40]. In our approach, high expression of HSV-2 gD was achieved through the use of optimised humanized codons and the use of a strong mammalian promoter. This resulted in an increase in immunogenicity while lowering the dose of DNA required for priming. Previous work demonstrated that 3 doses of 100-200μg of gD DNA were required to provide protection from live HSV-2 challenge in contrast to the 0.5–5μg of DNA used in our studies [29,41].

We employed liposomes as the second component of the heterologous immunisation strategy. The success of liposomes in vaccines is attributed to their capacity to sequester antigens and release them slowly, in addition to an adjuvant role. Liposomes are non toxic yet retain intrinsic adjuvant properties that can enhance both cellular and humoral immune responses due to their ability to deliver antigen that can be processed for presentation by both major histocompatibility complex (MHC) class I and II molecules. Aerosolisation of liposomes has also been successfully demonstrated, suggesting future use of liposomes for intranasal delivery [42,43]. Experimental liposomal vaccines against viral and parasitic diseases such as HIV and malaria have been developed that are safe and highly immunogenic in human trials [44]. We and others have also demonstrated that encapsulation of hepatitis B protein enhances antibody responses [26,45]. In addition, it has been shown that administration of liposome encapsulated antigen to mucosal sites generates substantial mucosal secretory IgA and CTL responses [26].

Critical to the success of the prime and boost protocol is the combination of vaccine components and delivery of the liposome boost intranasally, leading to Th-1 biased synergistic systemic and mucosal responses. Previous work has shown that a Th1 response and serum neutralising antibodies are correlated with protection from lethal HSV-2 challenge in mice and guinea pigs [46]. The T helper type established by DNA priming (*i.m.*) determines the overall T helper type after heterologous immunisation. While intranasal administration of liposome vaccine alone induces extremely biased Th2 responses, a preferred Th-1 biased response was observed after heterologous immunisation. Delivery of the boost intranasally is critical for eliciting synergistic systemic and mucosal immune responses. The vaginal cavity is believed to be a tolerance induction site for immune responses. Accordingly, using HBsAg, we failed to induce significant immune responses after direct inoculation of liposome encapsulated antigen into the vagina, even after DNA priming (data not shown). However, induction of mucosal responses was observed in the lung and induction of pulmonary T cell responses provided protection from intranasal challenge after intranasal delivery of antigen [26]. We now extend these findings to show that the immunisation regimen induces protective immune responses in the vaginal cavity, a remote site from the location of immunisation.

Although the protective mechanism remains to be further characterised, we believe immune memory is very important for the local protection in the vagina. Immune memory either established at or recruited to the mucosal surfaces would be especially important. We hypothesize that recall responses involving recruitment of functional effecter T cells and antibody secretion cells to the vaginal cavity should be correlated with the observed mucosal protection upon vaginal challenge with HSV-2. The vaginal IFN-γ levels on 3 and 5 days post HSV-2 challenge provide the direct evidence of antigen specific immune memory and recruitment (Figure 4).

Altogether we have demonstrated the success of a new candidate vaccine for HSV-2. This heterologous immunisation regimen meets many criteria for a potent HSV-2 vaccine. The vaccine stimulates high titers of serum antibodies, a Th-1 biased immune response and potent mucosal immunity. It also induces a strongly protective and durable immune response in mice. The use of suboptimal and high DNA doses demonstrated a titration of response that can be used for development of a human vaccine. The DNA prime and liposome boost strategy is effective as well as safe. No viral vectors or bacterial toxin-based adjuvants are used in the protocol. Success of this new mucosal HSV-2 vaccine in mice warrants further testing and optimisation in larger animal models prior to human trials.

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Abbreviations

i.m.	intramuscular
i.n.	intranasal

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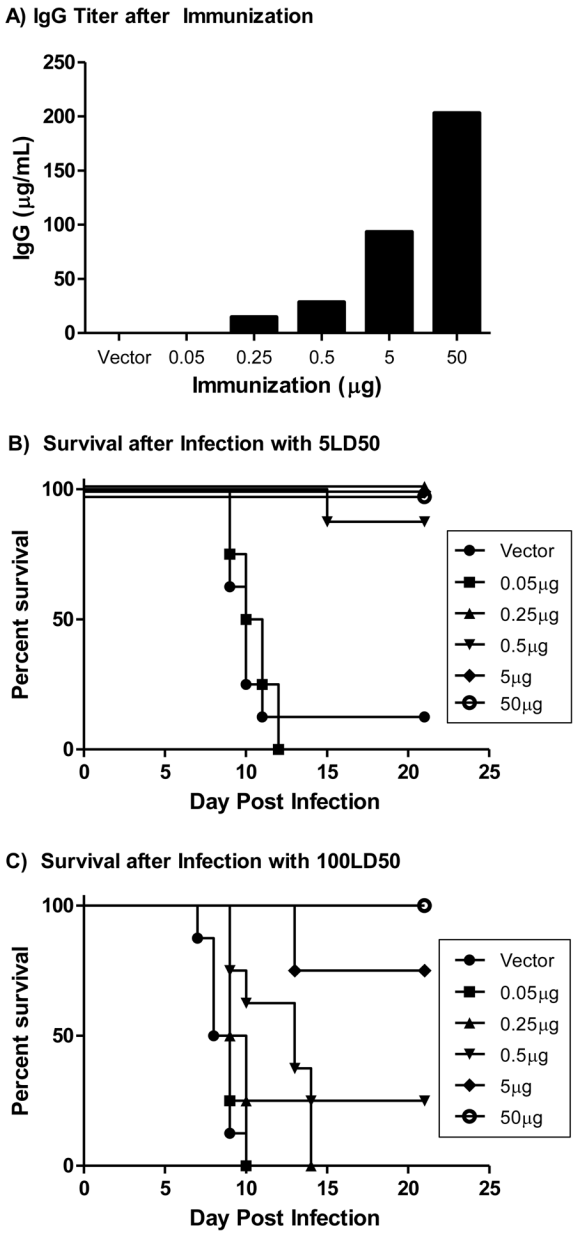

Figure 1.

Antibody responses and survival curves after DNA immunisation. Adult female Balb/c mice were immunised *i.m.* with either vector control or with 0.05, 0.25, 0.5, 5 or 50μg gD DNA vaccine. Three weeks after immunisation, serum IgG levels were measured from pooled samples (**A**). 6 weeks after immunisation mice were injected with medroxyprogesterone and infected with either 5LD50 (**B**) or 100LD50 of HSV-2 (**C**). Mice were observed daily for clinical scores and mortality. (n=8 for naïve and 0.5μg DNA; n=4 for 0.05, 0.25, 5 or 50μg DNA).

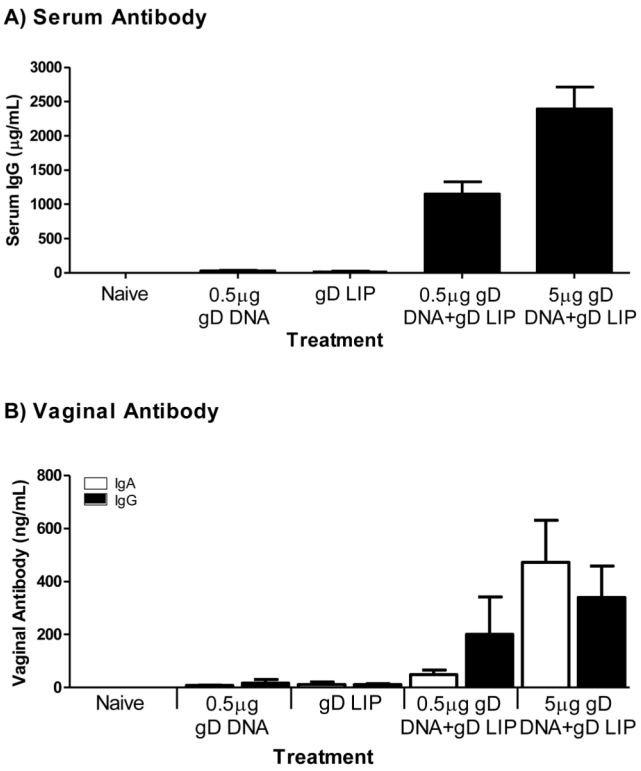

Figure 2.

Serum and vaginal antibody responses to heterologous immunisation. Female Balb/c mice were either left untreated (naïve), immunised with 0.5μg of gD DNA vaccine *i.m.* on days 0 and 2 (gD DNA), 15μg of gD protein encapsulated in liposomes (gD LIP) or 0.5μg or 5μg of gD DNA *i.m.* on days 0 and 2 followed by gD LIP *i.n.* 2 weeks after the last DNA immunisation (gD DNA+gD LIP). Serum and vaginal antibody responses were measured 2 weeks after the liposome immunisation. Average gD-specific serum IgG (**A**) and vaginal antibody (**B**) responses +/− SEM are shown. n= 2, 5, 5 and 8 for naïve, gD DNA, gD LIP and gD DNA+gD LIP groups, respectively.

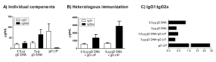

Figure 3.

IgG1:IgG2a ratios in immunised mice. Female Balb/c mice were immunised with either 0.5μg or 5μg of gD DNA vaccine alone *i.m.* on days 0 and 2 (gD DNA), 15μg of gD protein encapsulated in liposomes alone (gD LIP) or 0.5μg or 5μg of gD DNA *i.m.* on days 0 and 2 followed by gD LIP *i.n.* 2 weeks after the last DNA immunisation (gD DNA+gD LIP). Serum gD-specific IgG1 and IgG2a responses were measured 2 weeks after the liposome immunisation. Average gD-specific IgG responses +/- SEM are shown. **A**. Average responses after vaccination with individual components. **B**. Average responses after heterologous immunisation. n= 8 for 0.5μg gD DNA alone, 0.5μg gD DNA+gD LIP and 5μg gD DNA+gD LIP; n=7 for gD LIP alone; n=6 for 5μg gD DNA alone. **C**. IgG1:IgG2a ratio.

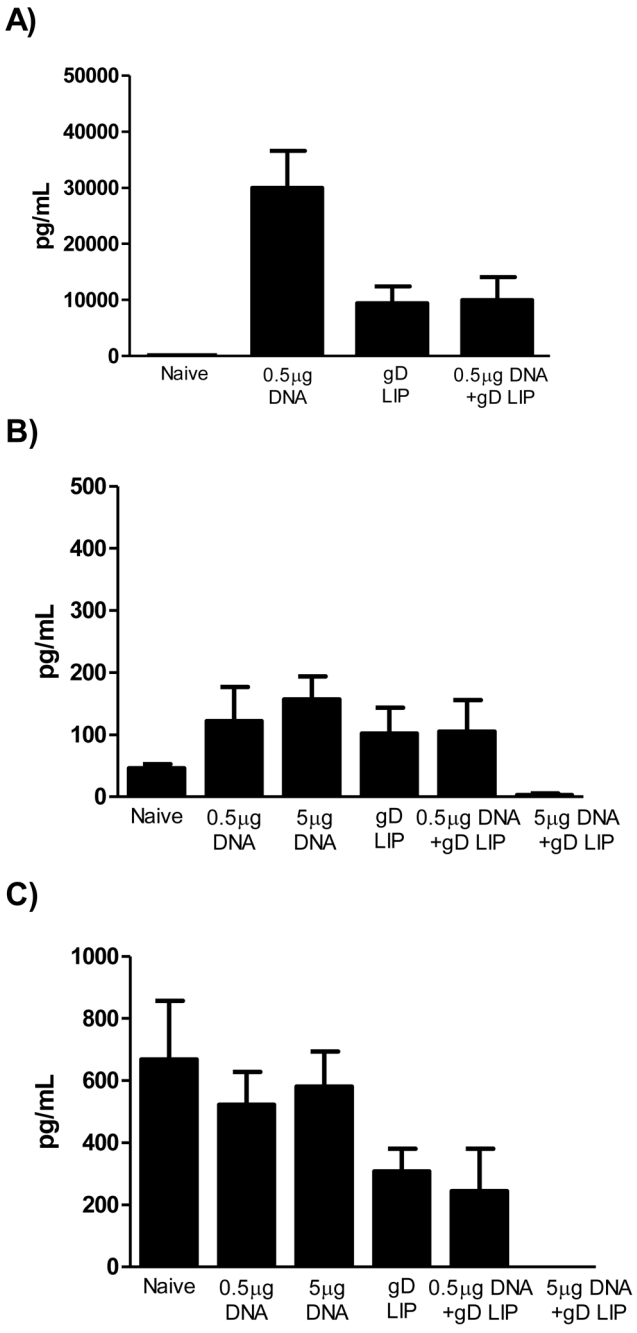

Figure 4.

IFN-γ production in vaccinated and infected mice. Female Balb/c mice were either left untreated, immunised with 0.5μg or 5μg of gD DNA vaccine *i.m.*, 15μg of gD protein encapsulated in liposomes or 0.5μg or 5μg of gD DNA *i.m.* followed by gD LIP *i.n.* 2 weeks after the last DNA immunisation. Two weeks after the last immunisation, animals were injected with progesterone and inoculated with 100LD50 of HSV-2. **A.** On Day 10 post infection, spleens were isolated from one naïve animal, 3 animals immunised with 0.5μg of DNA, 5 animals immunised with gD LIP or 4 animals immunised with 0.5μg DNA+gD LIP and used to make whole cell suspensions. Cells were incubated with 10μg/ml gD protein for 48 hours prior to collecting cell supernatants. IFN-γ levels in the supernatants were

measured using the “Ready-Set-Go” cytokine ELISA kit from eBioscience. **B and C.** Vaginal samples were collected on Days 3 (B) and 5 (C) post infection from naïve mice (n=6) and mice immunised with 0.5μg or 5μg gD DNA (n=6), gD LIP (n=6) or 0.5μg or 5μg gD DNA+gD LIP (n=5). IFN-γ levels in the samples were measured using the “Ready-Set-Go” cytokine ELISA kit from eBioscience.

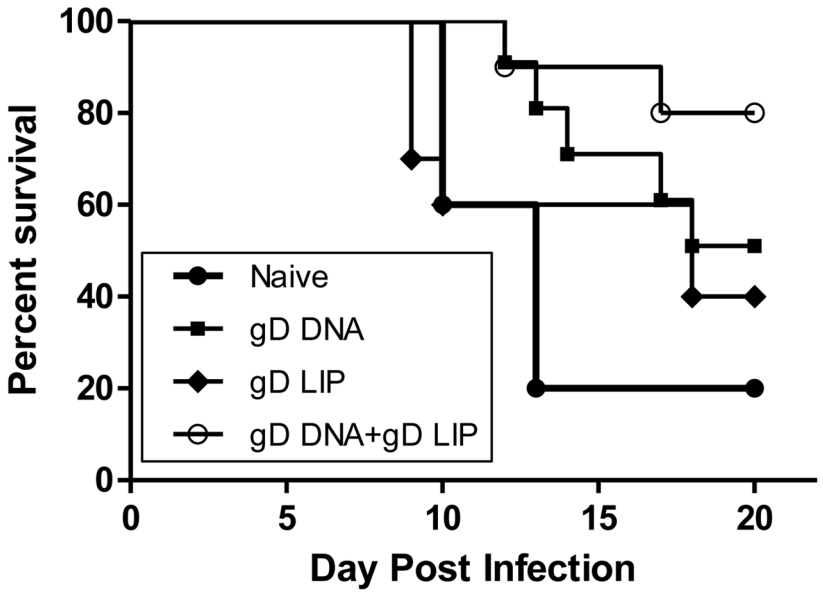

Figure 5.

Survival curves of immunised animals. Female Balb/c mice were either left untreated (naïve), immunised with 0.5μg of gD DNA vaccine *i.m.* on days 0 and 2 (gD DNA), 15μg of gD protein encapsulated in liposomes (gD LIP) or 0.5μg of gD DNA *i.m.* on days 0 and 2 followed by gD LIP *i.n.* 2 weeks after the last DNA immunisation (gD DNA+gD LIP). Two weeks after the last immunisation, animals were injected with progesterone and inoculated with 100LD50 of HSV-2. Animals were examined daily for 3 weeks for clinical signs of disease. Animals with a clinical score of 4 were sacrificed. n=5 for naïve mice; n=10 for gD DNA, gD LIP and gD DNA+gD LIP.

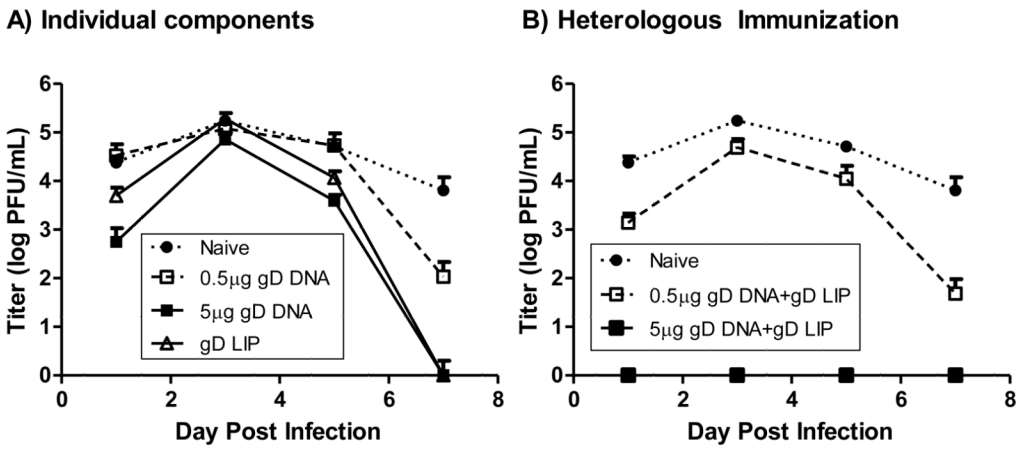

Figure 6.

Titration of virus isolated from vagina of vaccinated animals infected 2 weeks after immunisation. Female Balb/c mice were either left untreated (naïve), immunised with 0.5μg or 5μg of gD DNA *i.m.* on days 0 and 2, 15μg of gD protein encapsulated in liposomes (gD LIP) or 0.5μg or 5μg of gD DNA *i.m.* on days 0 and 2 followed by gD LIP *i.n.* 2 weeks after the last DNA immunisation (gD DNA+gD LIP). Two weeks after the last immunisation, animals were injected with progesterone and inoculated with 100LD50 of HSV-2. Vaginal washes were collected on days 1, 3, 5 and 7 post infection and titrated in Vero cells. **A**. Viral titers after vaccination with individual components. **B**. Viral titers after heterologous immunisation. Titers obtained from unvaccinated mice are shown on both panels for comparison. n=5 for naïve mice, 0.5μg gD DNA, 5μg gD DNA, gD LIP and 0.5μg gD DNA +gD LIP; n=6 for 5μg gD DNA and n=4 for 5μg gD DNA+gD LIP.

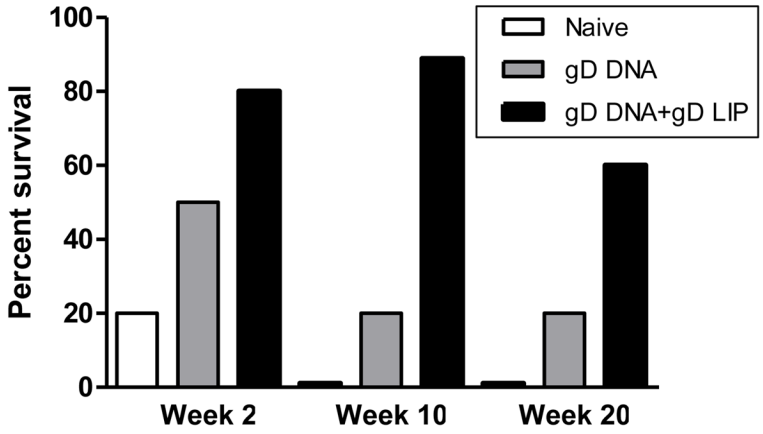

Figure 7.

Long-lasting protective immunity at 10 and 20 weeks post immunisation. Female Balb/c mice were immunised as described in the legend to Figure 5. At 10 and 20 weeks after the last immunisation, animals were injected with progesterone and infected intravaginally with 100LD50 of HSV-2. n=5 for naïve; n=10 for gD DNA at week 2 and 20; n=9 for gD DNA at week 10; n=10 for all gD DNA+gD LIP groups.

Table 1

Clinical Scores Are Decreased in Animals Immunised by Heterologous Immunisation

Immunisation	# of Animals with Clinical Score (%)			
	0	1–2	>2	Death
Naïve	0	0	5 (100%)	4 (80%)
0.5μg gD DNA	0	1 (10%)	9 (90%)	5 (50%)
gD LIP	0	0	10 (100%)	6 (60%)
0.5μg gD DNA+gD LIP	3 (30%)	4 (40%)	3 (30%)	2 (20%)

0–normal; 1- redness or mild swelling; 2–raw patch, blister, moderate swelling; 3–multiple blisters or raw patches; 4 - large blisters with severe and/or deep sores; and/or urinary retention; and/or hind limb paralysis. Animals demonstrating a clinical score of 4 were considered moribund and were sacrificed. n=10 for each group.