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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 immunisation primed with 0.5 μg of DNA vaccine.



## 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 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- to 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]. Cellu-

*Abbreviations:* *i.m.*, intramuscular; *i.n.*, intranasal.

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lar 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. Materials 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, NY). 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 ($H\text{-}2^d$) 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 40 μg/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

$1 \times 10^6$ 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 h. 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 450 nm 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 2 mg 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 20 μl 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 10-fold serial dilutions and titrated for plaque forming units (PFU) on Vero cells. Plates were incubated for 2 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 comple-