

Nucleic Acid Vaccine Encoding gD2 Protects Mice from Herpes Simplex Virus Type 2 Disease

John D. Kriesel, S. L. Spruance, Raymond A. Daynes, and Barbara A. Araneo

Departments of Medicine and Pathology, University of Utah School of Medicine; Paradigm Biosciences, Salt Lake City, Utah

Nucleic acid vaccinations with plasmids pWW65, containing the sequence for herpes simplex type 2 (HSV-2) gD2, and pRSVnt, lacking the gD sequence, were studied. Groups of mice were immunized with pWW65 alone, pWW65 plus 1,25-dihydroxyvitamin-D3 (D3), or pRSVnt. Clinical disease (vaginitis), serum and vaginal washing antibody levels, and vaginal washing virus titers were measured after intravaginal HSV-2 challenge. No animals (0/10) in the pWW65 + D3 group, 6/10 animals in the pWW65 group, and 10/10 animals in the pRSVnt group developed severe disease by postchallenge day 13 ($P < .001$, $P = .04$ vs. pRSVnt). Virus titers in vaginal washings were significantly reduced in the pWW65 and pWW65 + D3 groups versus the pRSVnt group ($P < .001$). Increasing levels of serum anti-gD2 antibodies were measured 2 and 6 days after challenge among animals in the pWW65 and pWW65 + D3 groups but not among animals in the pRSVnt group. Vaccinations with a plasmid containing the gD2 gene are immunogenic and provide some protection from HSV-2-induced disease.

Wolff and colleagues [1, 2] were the first to show that direct transfection of murine muscle is a feasible way to transfer exogenous genes *in vivo*. This technique has been used to show immunogenicity of a human immunodeficiency virus (HIV) plasmid construct inoculated repeatedly into mice and monkeys [3, 4]. Inoculation of "naked DNA" into living tissues and the resulting expression of encoded exogenous proteins offers the theoretical benefit of antigen presentation in the context of class I major histocompatibility complex (MHC) molecules, potentially resulting in the generation of a powerful cell-mediated immune response [5]. Recent reports have described the use of nucleic acid vaccination to protect from influenza virus challenge in mice and chickens [6–9].

Herpes simplex virus type 2 (HSV-2) gD is an important herpes envelope glycoprotein involved in attachment and pene-

tration of the virus into host cells. Investigations of the natural immune response to HSV-2 infection in humans reveal that serum antibodies against gD develop in all patients by 6–12 months after primary infection [10]. Cervical antibodies to gD develop even faster, appearing within 2 weeks of primary HSV-2 infection [11]. T cells that respond specifically to gD have been isolated from HSV-infected mice and from a lesion in 1 human with recurrent genital herpes [12, 13]. Passive immunization with monoclonal antibodies directed against gD protects mice from live HSV challenge [14, 15]. HSV subunit vaccines containing gD2 have recently been studied for the prevention of genital herpes in animal models and in humans [16, 17]. We were interested in studying whether a DNA plasmid encoding gD2 might also confer immunity in the murine vaginitis model of genital herpes.

Topical 1,25-dihydroxyvitamin-D3 (D3) administered to rodents changes the profile of cytokines elicited from underlying lymphoid tissue [18, 19]. Topical and intramuscular administration of this compound as an adjuvant to protein immunogens in mice resulted in enhanced serum and mucosal immune responses. We hypothesized that incorporation of D3 might enhance the proposed immunogenicity of an existing plasmid containing the sequence for HSV-2 gD [20]. The present experiments sought evidence that immunizations with a plasmid construct (pWW65) containing a Rous sarcoma virus promoter and the sequence for gD2 are immunogenic and provide some protection from severe disease due to HSV-2 infection in the murine vaginitis model.

Materials and Methods

Animals. BALB/c mice (female, 6–8 weeks old; Sasco, Wilmington, MA) were used in all groups.

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Animal research was performed after written review and approval of the protocol by the University of Utah Institutional Animal Care and Use Committee in accordance with US Department of Agriculture (USDA) guidelines. Animals were housed in a USDA-approved vivarium.

B.A.A. is employed by Paradigm Biosciences as Vice President of Research and has an appointment at the University of Utah. R.A.D. is employed by the University of Utah, Department of Pathology, and is a consultant to Paradigm Biosciences. J.D.K. is employed by the University of Utah School of Medicine, Division of Infectious Diseases, and receives salary and research support from Paradigm Biosciences.

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Reprints or correspondence: Dr. John D. Kriesel, University of Utah School of Medicine, Division of Infectious Diseases, Room 4B-322, 50 N. Medical Dr., Salt Lake City, UT 84132.

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Virus and cultures. HSV-2 strain MS originally obtained from E. Kern (Department of Medicine, University of Alabama at Birmingham) was grown in mink lung cells, aliquoted, and stored at -70°C until ready for use in challenge experiments. Quantitative viral cultures were obtained from the mice 1, 2, 4, and 6 days after challenge by vaginal washing with 50 μL of PBS and mixing into 500 μL of Eagle MEM. To avoid disrupting the genital epithelium before viral challenge, vaginal washing specimens (for ELISA) were not taken before challenge but were delayed until postchallenge day 1. Vaginal washing virus titers were obtained using plaque assays on Vero cells.

Vaccine preparation. Plasmids pWW65 and pRSVnt have been described [20]. pWW65 contains the HSV-2 gD gene expressed under the control of a Rous sarcoma virus promoter. pRSVnt lacks the gD2 sequence but otherwise is identical to pWW65. The plasmids were amplified using transformed *Escherichia coli* DH5a and purified using Wizard Megapreps (Promega, Madison, WI) to a concentration of 1.0 mg of DNA/mL.

Vaccinations. Preliminary experiments were done with a single intramuscular immunization in the left thigh with 3–100 μg of pWW65 or pRSVnt, followed by live viral challenge 6 weeks later. No alum and no proteins were included in the vaccine. In the subsequent study of multiple nucleic acid vaccinations, 10 mice received pWW65 alone 2, 3, and 4 weeks before challenge; 10 received pWW65 and 0.1 μg of D3 (gift of Hoffman-La Roche, Nutley, NJ) 4 weeks before challenge, then pWW65 alone 2 and 3 weeks before challenge (pWW65 + D3 group); and 10 mice received pRSVnt alone 2, 3, and 4 weeks before challenge.

Pretreatment and inoculation. Preliminary experiments were done using concentrated virus (6.3×10^6 pfu/mL) without medroxyprogesterone pretreatment. To synchronize the estrus cycle in the mice and produce uniform susceptibility to HSV-2 challenge, the animals were treated with subcutaneous medroxyprogesterone (Depo-Provera; Upjohn, Kalamazoo, MI) 4 days before challenge [21]. Microscopic examination of vaginal washings 3 days after medroxyprogesterone injection confirmed that all 30 animals were in the diestrus phase of the menstrual cycle. Mice were anesthetized before inoculation with 2.0 mg of intraperitoneal ketamine. About 100 μL of virus suspension was adsorbed onto Dacron pledgets and placed in the vagina for 30 min.

Assessment of vaginitis. Mice were scored daily by an unblinded observer for the severity of external genital inflammation (0 = no vaginitis, 1 = mild swelling or redness, 2 = moderate swelling or erosions, and 3 = severe genital maceration) [22]. Animals were defined as having "severe disease" if they developed urinary retention or hind limb paralysis at any time or external genital maceration on 2 consecutive days. Such animals were promptly sacrificed.

Serology. Neutralizing antibodies were determined on 96-well plates as described [23]. Briefly, 1:20 initial serum dilutions, run in quadruplicate, were serially diluted 1:2 and incubated with 24 pfu of HSV-2 at 37°C for 60 min. Vero cells (10^4) were added and incubated at 37°C for 5 days. Neutralizing titers are expressed as the inverse of the highest dilution at which no cytopathic effect was detected. Ninety-six-well plates were prepared for ELISA by coating with 3 $\mu\text{g}/\text{mL}$ baculovirus-expressed (for serum determinations) or purified (for vaginal washing determinations) gD2. Non-specific binding was prevented by incubating the plates for 2 h at

37°C with a solution of 10% normal goat serum and 0.4% Tween in PBS (NGS/Tween/PBS). Serial 1:2 dilutions of clinical samples in NGS/Tween/PBS were done starting at an initial dilution of 1:100 for vaginal washings and 1:1000 for serum. Binding of anti-gD2 antibodies was detected by incubating the serum dilutions for 90 min at 37°C with goat anti-IgG, -IgA, or -IgM antiserum (Pharmigen, San Diego) tagged with horseradish peroxidase, developing with chromogen substrate, and reading optical densities (ODs) at 405 nm. Log-transformed ODs were plotted against log-transformed dilutions, and linear regression analysis was used to determine end-point titers. End points were designated as 3-fold over binding observed from control mouse sera.

Statistical analysis. Development of severe disease was compared between groups using Fisher's exact and the χ^2 tests for discrete data. Clinical severity scores through day 8 and HSV titers from vaginal secretions through day 6 were compared by area under the curve (AUC) analysis using the Mann-Whitney test [24]. Serum, vaginal washing, and neutralizing antibody titers were compared using the Mann-Whitney or Kruskal-Wallis test. *P* values expressed are two-tailed.

Results

Preliminary experiments using single 30- to 100- μg pWW65 immunizations at least 4 weeks before live viral challenge resulted in partial protection from HSV-2-induced disease (data not shown). In an attempt to enhance immunity, groups of 10 animals were given three vaccinations at weekly intervals with 100 μg of pRSVnt or pWW65. A third group received 100 μg of pWW65 + 0.1 μg of D3 with the first vaccination and 100 μg of pWW65 alone with the subsequent two vaccinations (pWW65 + D3 group). Pretreatment of the animals with medroxyprogesterone allowed genital infection to occur in all animals, based on vaginal washing viral cultures 1 and 2 days after challenge. The viral inoculum (5.3×10^5 pfu/mL) following medroxyprogesterone pretreatment was less than that used in previous experiments but produced severe infection in all 10 nonimmune animals (data not shown).

All 10 animals immunized with pRSVnt developed severe disease, requiring sacrifice by day 8 (figure 1). Six of 10 animals immunized with pWW65 developed severe disease by day 14 ($P = .04$ vs. pRSVnt group). No animals immunized with pWW65 + D3 developed severe disease by day 13 ($P < .001$ vs. pRSVnt group). Animals immunized with pWW65 or pWW65 + D3 had much lower vaginitis AUC scores than those given pRSVnt ($P < .001$, data not shown). The pWW65 + D3 group showed a trend toward lower vaginitis AUC scores than the pWW65 group ($P = .08$). Animals in the pWW65 and pWW65 + D3 groups had significantly lower vaginal washing virus titers (AUC scores) than those in the pRSVnt group ($P < .001$, both comparisons; figure 2). No difference in vaginal washing virus titers was detected between the pWW65 and pWW65 + D3 groups.

Low levels of anti-gD2 IgG antibodies were detected in the prechallenge serum of a few of the animals that received

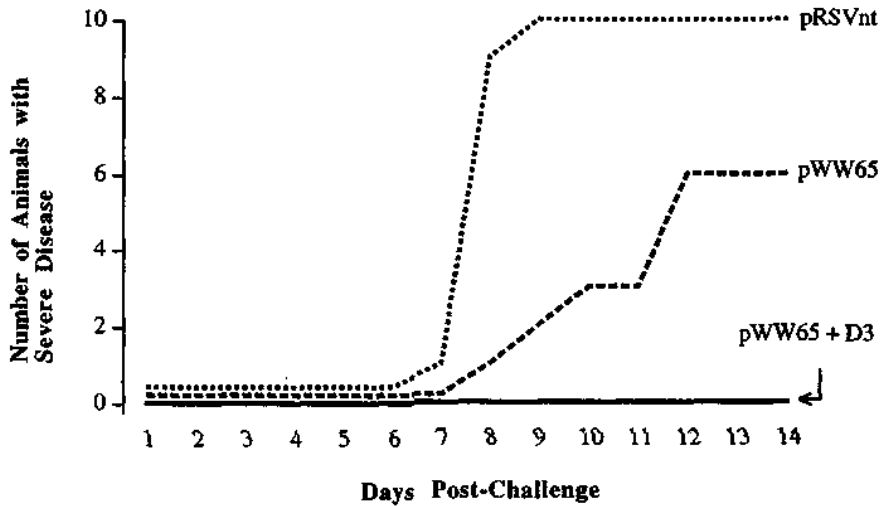


Figure 1. Time course of severe disease development after HSV-2 intravaginal challenge among animals multiply immunized with pWW65, pWW65 + vitamin D3, or pRSVnt. Significantly, more animals that received pRSVnt developed severe disease than did those that received either pWW65 or pWW65 + D3 ($P = .04$ and $P < .001$, respectively). Animals that received pWW65 alone were also more likely to develop severe disease than those that received pWW65 + D3 ($P = .04$).

pWW65 or pWW65 + D3 (figure 3). Rising levels of anti-gD2 IgG antibodies were detected 2 and 6 days after viral challenge in the serum of these animals. No anti-gD2 antibodies were detected in serum samples from the pRSVnt group at any time. Anti-gD2 antibodies also were detected by ELISA in vaginal washings from pWW65 and pWW65 + D3 animals on postchallenge days 1 and 6 (figure 4). No anti-gD2 antibodies were detected by ELISA in vaginal washings from pRSVnt animals at these time points. Both serum and vaginal antibody responses appeared to be exclusively IgG, with predominance of IgG1 and IgG2a. No significant IgA or IgM production has been observed following immunization with these plasmids.

Significant serum neutralizing activity was not detected in any of the groups before intravaginal viral challenge. Animals

in the pWW65 and pWW65 + D3 groups had detectable serum neutralizing activity 6 days after challenge (geometric mean titers [GMTs], 1:31 and 1:27, respectively). Serum from animals that received pRSVnt did not have detectable neutralizing activity ($P < .001$ vs. the other groups). Quantitative ELISA (figure 4) and neutralizing antibody titers were similar between the pWW65 and pWW65 + D3 groups. Among the 10 animals that received only pWW65, levels of serum anti-gD and neutralizing antibodies did not correlate with protection from severe disease. However, vaginal washing antibody levels were lower in the animals that developed severe disease ($n = 6$) than in those that were protected ($n = 4$) at postchallenge day 1 (GMT = 1.75 vs. 3.49, $P = .02$) and day 6 (GMT = 2.47 vs. 3.07, $P = .03$).

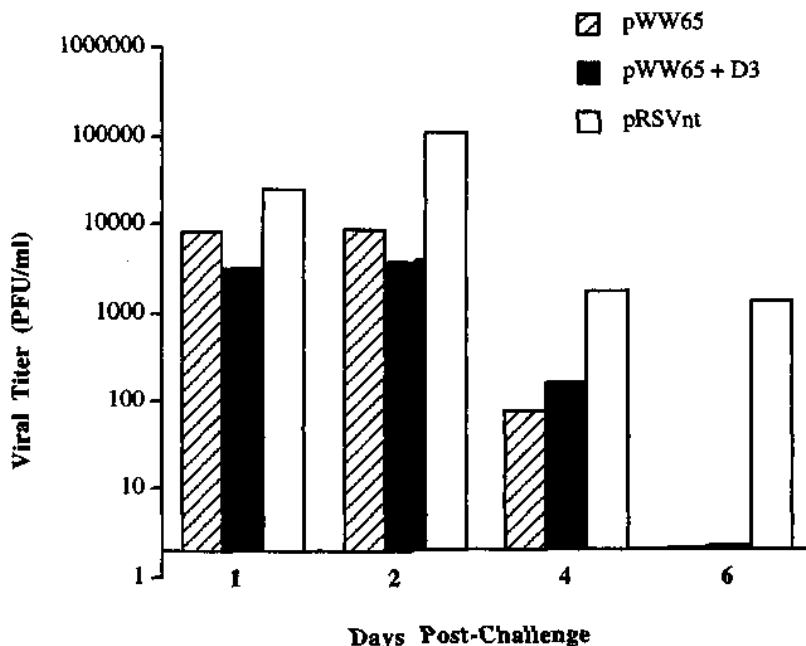
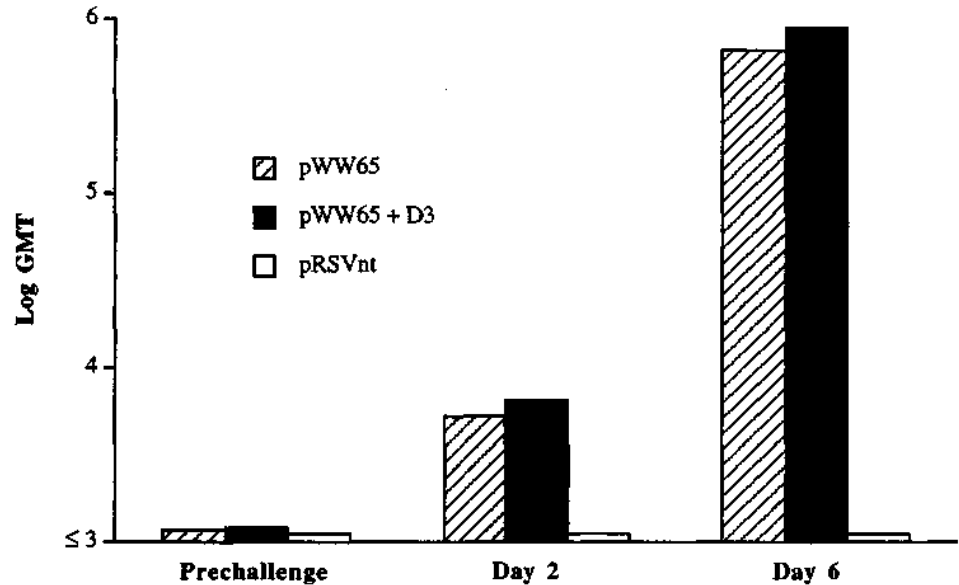


Figure 2. Postchallenge geometric mean vaginal washing virus titers by vaccine group and time after live viral challenge. Vaginal washing virus titer area under the curve (AUC) values (see Materials and Methods) for animals that were immunized with pRSVnt were significantly higher than for those that received either pWW65 or pWW65 + vitamin D3 ($P < .001$). No difference in virus titer AUC was detected between the pWW65 and pWW65 + D3 groups.

Figure 3. Serum geometric mean antibody titers (GMTs) against HSV-2 gD before and 2 and 6 days after challenge. Antibody levels were measured by ELISA. There was no difference between groups before challenge ($P = .10$). Animals immunized with pRSVnt had undetectable levels (<3 ELISA U) of serum antibodies at each time point, significantly lower vs. other 2 groups at postchallenge days 2 and 6 ($P < .001$). Serum antibody levels from pWW65 and pWW65 + vitamin D3 groups were not significantly different at any measured time point.



Perigenital ulcerations appeared 7–11 days after challenge in 1 of 4 animals that received pWW65 alone and in 6 of 10 animals that received pWW65 + D3 (P not significant). Swab and tissue cultures for HSV taken from these lesions on postchallenge day 13 were negative. Histologic sections taken from 3 representative erosions showed skin necrosis with a mixed subcutaneous infiltrate that included neutrophils and lymphocytes. Three of the 4 remaining perigenital erosions healed completely in 2–4 weeks, and 1 animal died of an unknown cause.

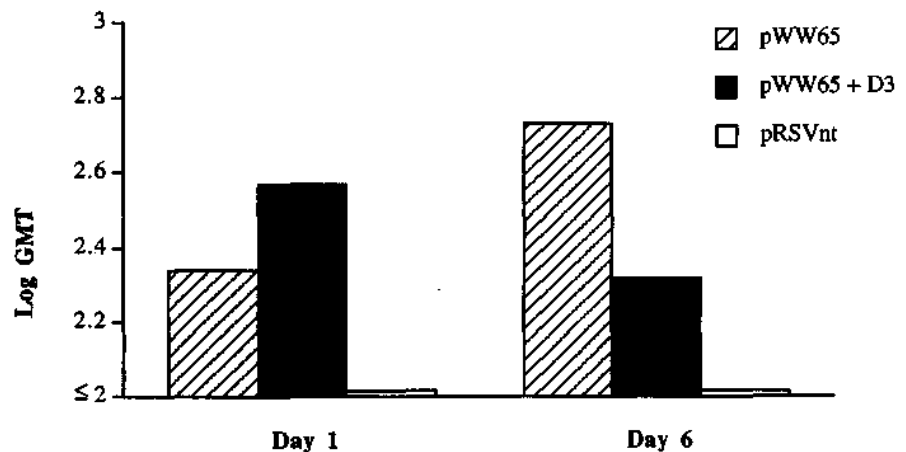
Discussion

In this study, three immunizations with a DNA vaccine encoding HSV-2 glycoprotein D provided partial protection from the development of severe disease in the murine vaginitis model. pWW65-vaccinated animals had lower virus titers from vaginal secretions and produced gD2-specific serum antibodies

and serum HSV-2 neutralizing antibodies after challenge. pRSVnt-vaccinated (control) animals did not produce measurable antibody in serum or vaginal secretions and rapidly developed severe disease due to HSV-2 infection. Addition of a single injection of 0.1 μg of vitamin D3 at the beginning of the vaccination series appeared to enhance protection from severe disease but did not significantly enhance levels of antibodies in serum or vaginal washings.

Humoral immune responses to gD (protein) have been associated with prevention of disease [15, 24, 25]. We believe that contamination of our plasmid preparations with gD2 cannot account for the immunogenicity of pWW65. The Rous sarcoma virus promoter included in pWW65 and pRSVnt is active only in eukaryotic systems, making production of gD2 theoretically impossible during plasmid replication in bacteria. We have shown that after precipitation of DNA in cold ethanol, no detectable protein is left in our plasmid preparations. Western blotting has also failed to detect gD2 in our nucleic acid vaccine

Figure 4. Vaginal washing geometric mean antibody titers (GMTs) against HSV-2 gD on days 1 and 6 after challenge. Animals immunized with pRSVnt had undetectable levels of vaginal washing antibodies at both time points, significantly lower vs. other 2 groups ($P < .001$). Vaginal washing antibody levels among animals in pWW65 and pWW65 + vitamin D3 groups were not significantly different at either time point. Measured levels of vaginal washing antibodies within each group did not change significantly between postchallenge days 1 and 6 (i.e., there was no significant rise or fall within pWW65 or pWW65 + D3 group).



preparations. We infer, on the basis of the presence of gD2-specific antibodies exclusively in the pWW65-vaccinated animals, that plasmid transfection of murine cells and expression of gD2 occurred in our system.

No significant serum antibody activity was detected before challenge by either ELISA or neutralization techniques. Although we cannot exclude the presence of low levels of anti-gD antibodies in the prechallenge sera, the rapid rise in detectable antibodies after challenge suggests that immunologic memory was induced by pWW65 vaccinations. The lack of detectable serum neutralizing activity or anti-gD antibodies in the pRSVnt controls at postchallenge day 6 is not surprising, as these animals developed severe disease and were sacrificed by day 8. This was probably too soon for a humoral immune response to primary HSV-2 infection to be measured.

A different pattern of vaginal washing antibody activity was observed, in which somewhat lower, more stable levels of antibodies were measured (figure 4). Transudation of serum antibodies or low-level production and secretion of antibodies by the vaginal mucosa may account for this observation. A subanalysis of 10 animals that received only pWW65 showed that those producing higher levels of vaginal antibodies were more likely to be protected from severe disease, suggesting a protective role of vaginal washing antibodies in this system, whatever their origin. We did not detect IgA or IgM in vaginal secretions or serum following vaccinations with pWW65. Other groups investigating nucleic acid vaccines have also detected primarily IgG and low or undetectable levels of IgM and IgA [4, 6, 9, 26].

The mechanism of protection resulting from immunizations with pWW65 includes humoral and likely cellular immune responses. Plasmid immunization in other animal systems has resulted in cytotoxic lymphocyte responses to HIV [4, 26], influenza virus [6, 27], and *Plasmodium* species [28]. Rouse et al. [29] have shown that antigen-presenting cells transfected with DNA encoding for HSV-specific proteins generate antigen-specific CD8 cytotoxic lymphocytes. These findings by other groups provide support for the hypothesis that nucleic acid immunization results in transfection of host cells, transcription of foreign DNA, production of the antigen within host cells, and HSV antigen presentation to T lymphocytes in the context of MHC class I. The presence of serum antibodies in our experiments suggests that antigen-presenting cells, CD4 T lymphocytes, and B lymphocytes are involved in the induced immune response to pWW65. The detection of anti-gD2 antibodies also suggests presentation of gD2 antigen in the context of MHC class II in our system.

Vitamin D3 dramatically enhances mucosal immune responses to protein antigens [18] and contrasts with the prevailing view that this compound is an immunosuppressive agent [30]. The mechanism of D3 enhancement of immunity in these systems is not completely understood but may relate to enhancement of a Th2 immune response via locally increased

interleukin (IL)-4, IL-5, and IL-10 production [18, 19]. We did not find enhancement of serum or vaginal antibody levels among the pWW65 + D3 animals compared with the pWW65-alone group. However, the groups were relatively small and the animals were given D3 only once during the immunization regimen. It is conceivable that using larger groups, D3 with each immunization, or a different dose of D3 might enhance antibody levels in the serum or at mucosal surfaces. Work to clarify this is in progress.

Addition of D3 to the pWW65 vaccination schedule appeared to have a beneficial effect on the course of HSV-2-induced disease in this system. We consider this to be a preliminary observation, which needs to be carefully confirmed using larger groups. We have considered the possibility that D3 may not have acted as an immunomodulator but could have had a direct effect on the host target epithelium, rendering it less susceptible to HSV-2 infection. However, we believe that D3 given along with a plasmid vaccination 4 weeks earlier is unlikely to be active at the time of challenge [31], and subsequent experiments have shown that addition of D3 to the control vaccine does not provide protection from vaginitis or severe disease in our system.

The presence of perigenital ulceration in some animals that were vaccinated and survived challenge may be related to a vigorous local cellular immune response or persistent viral replication (or both). Negative viral cultures and histologic sections support the hypothesis that these lesions are the result of a vigorous local immune response. More work is needed to characterize the cells contained in these inflammatory infiltrates and to determine whether very low levels of virus or viral antigens might be persisting within the lesions.

In summary, the nucleic acid vaccine encoding gD2 was immunogenic and attenuated HSV-2-induced vaginitis in a murine model. This approach offers a theoretical benefit over vaccination with proteins, because transfection of muscle with plasmid DNA may result in both MHC class I and II presentation of antigen, resulting in a broader, perhaps more effective immune response. Future studies may include vaccination with new plasmid constructs containing more than one HSV-2 gene, examining the efficacy of this vaccine in animal models of recurrent herpes, and measuring cellular immune responses following nucleic acid vaccinations against HSV.

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