Prime-Boost Immunization with DNA and Modified Vaccinia Virus Ankara Vectors Expressing Herpes Simplex Virus–2 Glycoprotein D Elicits Greater Specific Antibody and Cytokine Responses than DNA Vaccine Alone

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Several reports have indicated that prime-boost strategies of vaccination can enhance the level of specific immunity induced by nucleic acid vaccines. The present report describes such a strategy with herpes simplex virus (HSV)-2 glycoprotein D (gD), using combinations of plasmid vector that expresses gD (pgD2) and a recombinant modified vaccinia virus Ankara vector that expresses gD (MVA-gD2). The IgG antibody response to gD and the HSV-2 neutralizing antibody response were greatest when the MVA-gD2 vector was used as the priming immunization and then was boosted with either pgD2 or MVA-gD2. Determination of the isotype profile of MVA-gD2–primed mice revealed a much broader distribution of isotypes than that seen after DNA vaccination. In addition, antigen-stimulated spleen cells from mice primed with MVA-gD2 and boosted with either MVA-gD2 or pgD2 produced higher levels of interleukin-2 and interferon-γ than did those from pgD2-primed mice, indicating that a prime-boost immunization strategy that uses the MVA and plasmid DNA vector dramatically enhances and diversifies the humoral and cellular immune response to HSV-2 gD.

Several reports have described DNA immunization as a potential vaccination strategy for immunization against herpes simplex virus (HSV) [1–8]. DNA vaccination is an attractive approach to HSV vaccine development for several reasons. Like other subunit vaccines, DNA vaccines have a potential safety advantage over live herpesvirus vaccines; live herpesvirus vaccines are considered to be problematic because of the ability of the virus to establish latency and reactivate. Even attenuated and replication-defective viruses are capable of establishing latency in the host and of being reactivated by subsequent superinfection [9, 10]. Compared with traditional subunit vaccines made from purified proteins, development of a DNA vaccine is considerably easier and less expensive, eliminating the purification of recombinant proteins. In addition, unlike protein subunit vaccines and inactivated virus vaccines, DNA vaccines may effectively stimulate cell-mediated, as well as humoral, immunity [11]. In spite of these potential advantages, as well as the numerous reports of their effectiveness in preclinical studies, the effectiveness and practicality of DNA immunization for human pathogens still has to be demonstrated. In particular, it is not clear that the levels of specific immunity induced by DNA immunization are sufficient for protection against subsequent infection and disease.

Recently, several vaccination strategies have been reported to greatly enhance the immunogenicity of plasmid-delivered antigens [12]. One of the most promising strategies is the sequential delivery of encoded antigens by different types of vectors, a strategy generically referred to as a “prime-boost” [13]. Results from several studies, notably those from simian immunodeficiency virus animal models, have demonstrated that the combination of a plasmid DNA priming step followed by a nonreplicating poxvirus vector boost generated high frequencies of pathogen-specific T cells and resulted in superior levels of protection against challenge [14, 15]. In most studies, the combination of DNA priming followed by recombinant vector boosting appeared to be superior to other schemes of vaccination, such as DNA priming followed by protein boosting. Furthermore, by using a nonreplicating viral vector, the prime-boost vaccination strategy retains some of the safety advantages associated with subunit vaccination.

The aim of the present study was to determine whether a prime-boost strategy of immunization enhances the quality of the immune response to a candidate subunit DNA vaccine for genital herpes. To explore this strategy, we constructed a recombinant modified vaccinia virus Ankara (MVA) vector that expresses the HSV-2 glycoprotein D (gD2) and used this vector for immunization in combination with a plasmid vector that also expresses gD2. gD2 was chosen as the HSV-2 antigen to
be investigated because of the extensive body of data available concerning its use as a potential vaccine antigen for genital herpes [9, 10]. The choice of MVA as a vector in a prime-boost strategy of immunization was dictated by several considerations, including its inability to replicate in human cells and its prior use during the later stages of the smallpox eradication campaign [16]. We show here that a MVA-delivered gD2 boost enhanced the antibody response to plasmid-delivered gD2, compared with a plasmid DNA boost. Surprisingly, the antibody response to gD2 was even higher and of more diverse isotype distribution if the MVA-gD2 vector was used as a priming immunization and the plasmid DNA followed as a boost. Similarly, this combination of priming with MVA vector followed by plasmid DNA boost produced greater cellular immune responses than either the reverse combination or the DNA immunization alone. These results indicate that a combination of different delivery vectors may be advantageous for maximizing the immune response to this HSV glycoprotein and suggest that the optimal order of delivery vectors may depend on the antigen being delivered.

Materials and Methods

Cells and viruses. Vero cells and baby hamster kidney (BHK)-21 cells were obtained originally from the American Type Culture Collection. Primary chick embryo fibroblast (CEF) cells were a kind gift of Norman Cooper (National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH], Bethesda, MD). Vero and BHK-21 cells were grown and maintained in Dulbecco’s minimal essential medium (MEM), and CEF cells were grown in MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 µg/mL gentamicin. HSV-2 (strain MS) was obtained from the American Type Culture Collection. Primary chick embryo fibroblast (CEF) cells were a kind gift of Linda Wyatt and Bernard Moss (Laboratory of Viral Diseases, NIAID, National Institutes of Health [NIH]), was propagated and titered on Vero cells. MV A was a gift of Linda Wyatt and Bernard Moss (Laboratory of Viral Diseases, NIAID, National Institutes of Health [NIH]), was propagated and titered on Vero cells. MV A was a gift of Linda Wyatt and Bernard Moss (Laboratory of Viral Diseases, NIAID, National Institutes of Health [NIH]), was propagated and titered on Vero cells. MV A was a gift of Linda Wyatt and Bernard Moss (Laboratory of Viral Diseases, NIAID, National Institutes of Health [NIH]), was propagated and titered on Vero cells. MV A was a gift of Linda Wyatt and Bernard Moss (Laboratory of Viral Diseases, NIAID, National Institutes of Health [NIH]), was propagated and titered on Vero cells.

Plasmid constructions. First, for pcDNA3-gD2 (pgD2), the U6 open-reading frame encoding gD was amplified from genomic HSV-2 (strain MS) DNA by polymerase chain reaction (PCR). Viral DNA was isolated as described elsewhere [18]. The PCR product was cloned into Topo Zero Blunt vector (Invitrogen), using protocols described by the manufacturer. The gene was then subcloned as an EcoRI-Xhol fragment in frame with the sequence of an influenza hemagglutinin (HA) epitope that had previously been inserted into pcDNA3 (Invitrogen) to generate a carboxy HA-tagged gD2 gene, pgD2.

Second, for pLW22-gD2, the plasmid pLW22 (a gift of Linda Wyatt and Bernard Moss, NIAID, NIH) was an MVA insertion vector consisting of a strong synthetic promoter with unique Pmel and AscI sites for insertion of foreign coding sequences, a lacZ marker gene, and flanking MVA sequences that allow the insertion of both genes into the deletion II site in MVA [19]. A pair of complementary oligonucleotides, 5′-TAATAGTACGTTCAATGGCATAGTCC-3′ and 5′-GGAGTACGTTCAATTGCTAGCAG-3′, containing Nhel, Muni, and SpeI (in bold), were annealed and inserted at the Pmel site in pLW22 to generate plasmid pLW22-SMN. The sequences 5′-AATTTAGTACGTTCAATGGCATAGTCC-3′ (which contains an Nhel site) and 5′-AATTTAGTACGTTCAATGGCATAGTCC-3′ were annealed and inserted at the EcoRI site in pgD2 to obtain pgD2E. Similarly, the pair 5′-CTAGGGGACATGCGCAATGGCATAGTCC-3′ (which contains an SpeI site) and 5′-CTAGGGGACATGCGCAATGGCATAGTCC-3′ were annealed and inserted at the XhoI site in pgD2E to generate pgD2EX. The latter was digested with Nhel and SpeI to obtain a Nhel-SpeI fragment of gD2, which was inserted in the SpeI-Nhel sites in pLW22-SMN to obtain pLW22-gD2. Plasmids were purified using the Qiagen maxi or endo-free plasmid purification kits, according to protocols described by the manufacturer (Qiagen).

Construction of recombinant virus MV A-gD2. To construct a recombinant MVA expressing HSV-2 gD, we first added an Nhel and an SpeI site to the sequences immediately flanking the 5′ and 3′ ends, respectively, of the gD2 sequence in pgD2. We then modified the MVA plasmid insertion vector pLW22, as described above, by insertion of a 26-bp polylinker that contained SpeI-Muni-Nhel sites to the vector at its unique Pmel site to produce pLW22-SMN. This enabled us to subclone gD2 as an Nhel-SpeI fragment into the Nhel-SpeI sites in pLW22-SMN, to produce pLW22-gD2. The plasmid pLW22-gD2 (3 µg) was transfected into MVA-infected BHK-21 cells (MOI, 0.05). Recombinant viruses were identified by expression of lacZ, and isolated plaques were screened for gD2 expression by Western blot analysis. Plaques that expressed the full-length glycosylated gD2 were purified further by additional plaque assay on BHK-21 cells. A recombinant plaque consistently expressing normal gD2 (MVA-gD2) was expanded in BHK-21 cells and purified as described elsewhere [17] for subsequent use.

Western blotting. Recombinant plasmid-transfected or virus-infected cells were resuspended in a lysis buffer that contained 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% sodium desoxycholate, and 1% Triton-100. After 3 cycles of freeze-thaw, cell lysates were clarified for 30 s at 13,000 g in a refrigerated bench-top centrifuge. Proteins were resolved in a 4–12% Bis-Tris electrophoresis gel. Proteins were transferred onto a nitrocellulose membrane and incubated for 1 h in a 1:1000 dilution of a mouse anti-gD monoclonal antibody (Advanced Biotechnologies) prepared in blocking buffer (5% wt/vol nonfat milk dissolved in Tris-buffered saline [TBS]). The membrane was washed twice (15 min each wash) in TBS and incubated with a 1:7500 dilution of an alkaline phosphatase-conjugated goat anti-mouse antibody (Promega) for 1 h. The membrane was washed as described above and visualized by addition of Western blue–stabilized substrate for alkaline phosphatase (Promega). The reaction was stopped after 3–5 min by the addition of distilled water.

Immunizations and virus challenge of animals. BALB/cByJ mice were purchased from Jackson Laboratories and housed in cages at a core facility in the Center for Biologics Evaluation and Research, Food and Drug Administration (Bethesda, MD). Sterile food and drinking water were supplied ad libitum. Mice used in these experiments were 8–12 weeks old and were age-matched within an experiment. For DNA immunization, plasmid DNA was coated on 1-µg gold beads and delivered directly into the epidermis of the mouse with a helium-driven Helios gene-gun system (Bio-Rad Laboratories), according to protocols described by the manufacturer. MVA vectors were administered subcutaneously (in 100...
µL of sterile, endotoxin-free PBS) by injection under the skin at the base of the tail. For virus challenge, each mouse was injected with 25 LD₅₀ (5 × 10⁸ pfu) of HSV-2 (MS) into the peritoneum and monitored for >4 weeks for the development of disease symptoms and death.

ELISA for HSV-specific antibody. Detection of antibody to HSV glycoprotein was performed by ELISA as described elsewhere [7, 20], using 96-well Immulon-1² microtiter plates (Dynex Technologies) coated with 0.5 µg/mL of an HSV-2 glycoprotein preparation. The end-point titer was defined as the highest dilution of serum that gave an absorbance at 405 nm value that was greater than that of the matched dilution of normal prebleed mouse serum and was also >0.050.

Cell-mediated immune response and cytokine ELISAs. Cytokine levels (interleukin [IL]-2, IL-4, and interferon [IFN]-γ) in supernatants of cultured splenocytes, stimulated in vitro with inactivated HSV-2, were determined by comparison with a recombinant protein standard in an antibody-capture ELISA. Spleen cell suspensions were prepared by aseptic removal of the spleen into a 100-mm petri dish that contained 10 mL of PBS–2% fetal bovine serum (FBS), preparation of single-cell suspension, and passage through a 0.7-mm cell strainer (Becton-Dickenson). Erythrocytes were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker). Cells were washed twice in PBS–2% FBS, diluted to (IL-2 assays) or were eliminated by lysis with ACK (Biowhittaker).

Serum neutralization. Neutralizing antibody assays were performed as described elsewhere [7], using 96-well plates that contained 5 × 10⁴ Vero cells and 5 × 10⁵ pfu of HSV-2 per well.

Statistical analysis. Significant differences between groups, defined as P < .05, were analyzed using an unpaired, 2-tailed Student’s t test with InStat software (GraphPad Software).

Results

Construction of plasmid and MVA vectors expressing HSV-2 gD. The HSV-2 U₆ open-reading frame, encoding the viral gD, was cloned into plasmid and MVA vectors for the expression of gD in vivo. Sequencing of pgD2 (data not shown) and Western blot analysis of pgD2-transfected Vero cells using a gD-specific monoclonal antibody (figure 1A) confirmed the authenticity of the plasmid expression vector. Similarly, Western blot analysis demonstrated the expression of gD2 in both BHK-21 (lane 1) and CEF (lane 2) cells infected with MVA-gD2 (figure 1B). The 47–48-kDa protein detected by a gD monoclonal antibody appeared to be identical in size to gD2 in HSV-2–infected cells (lane 3). There was no detectable gD2 expression in BHK-21 or CEF cells infected with the parent MVA virus (data not shown). Thus, the introduction of pLW22-gD2 into MVA generated a recombinant virus that expresses a full-length HSV-2 gD.

MVA-gD2 boost of the antibody response to plasmid-delivered gD. In initial experiments, we demonstrated that pgD2 elicited an HSV-specific antibody response when used to immunize BALB/c mice by either intramuscular injection or gene-gun delivery. In our hands, gene-gun delivery routinely resulted in the higher and more consistent antibody titers, even though much less DNA was delivered by the gene-gun procedure than by intramuscular injection (a maximum of 1 µg by gene gun, compared with 50 µg intramuscularly). Immunization by gene-gun

Figure 1. Western blot analysis of plasmid and modified vaccinia virus Ankara (MVA)–expressed herpes simplex virus (HSV)–2 glycoprotein D (gD). A, Vero cells were transfected with either plasmid gD2 (lane 1) or were mock transfected (lane 2). Cell extracts were analyzed by Western blotting using a monoclonal antibody to HSV-2 gD. Lane 3, Cell extract from HSV-2–infected Vero cells. B, Baby hamster kidney–21 cells (lane 1) or chick embryo fibroblast cells were infected with MVA-gD2 (lane 2). Cell lysates were analyzed by Western blotting. Lanes 3 and 4, Uninfected Vero cell lysate and affinity-purified gD2, respectively. M, Marker lane.
delivery of pgD2 produced antibody of an exclusively IgG1 isotype, whereas intramuscular delivery also yielded IgG2a and, occasionally, IgG2b (data not shown). In the subsequent experiments described here, which were designed to investigate the potential of a prime-boost vaccination strategy using pgD2 and MVA-gD2, we delivered 1 μg of pgD2 by gene gun.

In vivo experiments that used the MVA-gD2 vector were designed to determine the immunogenicity of vector-delivered gD, its potential to enhance the response to plasmid-delivered gD, and feasible doses of MVA-gD2 to be used in further experiments. Groups of mice were immunized with 1 μg of either control plasmid DNA or pgD2 (table 1). Three weeks later, groups were boosted with either control MVA (106 pfu) or increasing doses of MVA-gD2 (106, 107, or 108 pfu); serum samples were obtained 3 weeks later. Although a single inoculation with pgD2, followed by immunization with control MVA, resulted in readily detectable HSV-specific IgG, the antibody response to pgD2 was significantly enhanced (P < .05) when MVA-gD2 was used for the booster immunization at any of the 3 concentrations tested (tables 1 and 2). Similarly, the levels of neutralizing antibody that resulted from a booster immunization with MVA-gD2 were significantly higher (P < .05) than those obtained from pgD2 followed by control MVA. There was no detectable HSV-specific IgG or neutralizing antibody in these or any other experiments in which only control plasmid DNA and/or control MVA was used for immunization. In a second experiment, groups of mice were immunized with the 3 doses of MVA-gD2 used in the experiment described above. Serum samples were obtained 2 weeks later, and a second immunization using the same doses was administered at 3 weeks. Serum samples were again obtained 3 weeks later and analyzed for HSV-2–specific antibody (table 2). Although no antibody was detectable after 1 immunization with 108 pfu of MVA-gD2, 60% and 80% of animals that received 106 and 107 pfu of MVA-gD2, respectively, produced detectable anti-gD antibodies with IgG titers ranging from 1:40 to 1:640. Three weeks after boosting, all mice in each of the 3 groups seroconverted with mean IgG titers (log10) of 3.53, 3.77, and 3.95, respectively (table 2).

To further investigate the strength and diversity of the antibody response resulting from prime-boost combinations of MVA-gD2 and pgD2, we delivered 1 μg of pgD2 by gene gun.

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Of interest, the IgG titers after 2 immunizations were similar for all groups, even though there had been no detectable antibody after the first immunization in the group that had received 108 pfu of MVA-gD2. Further analysis of the serum samples obtained after 2 immunizations revealed that there were high levels of IgG1 and IgG2a, as well as occasionally detectable IgG2b, but no detectable IgG3 (titers <1:40). Taken together, these initial experiments indicated that MVA-gD2 expresses gD2 in vivo at sufficient levels to elicit a specific antibody response; in subsequent experiments, we chose to deliver 107 pfu of MVA or MVA-gD2. Moreover, the antibody response to either MVA-delivered or plasmid-delivered gD could be boosted by a second inoculation of MVA-gD2.

Antibody response to prime-boost combinations of plasmid- and MVA-delivered gD2. Having established that both plasmid- and MVA-delivered gD elicited specific antibody responses in mice, we investigated the antibody response resulting from various delivery combinations of the 2 vectors in a prime-boost strategy of vaccination. Here, the first immunization is referred to as the “priming” dose, whereas the second immunization is referred to as the “booster” dose. Ten groups of mice were immunized 2 times at 3-week intervals with various combinations of control DNA, control MVA, pgD2, or MVA-gD2. Serum samples were obtained for analysis of total anti-gD–specific IgG 3 weeks after the booster immunization. There was no measurable HSV-specific IgG response in animals that received either a control DNA priming dose followed by a control MVA booster dose or 2 control MVA immunizations (data not shown); similarly, there was no measurable response in animals that received a control MVA priming dose followed by a control DNA booster dose (figure 2, column 1). Mice that were primed with pgD2 and boosted with either pgD2 or MVA-gD2 had levels of specific IgG antibody that were similar to those of animals that received a single immunization with MVA-gD2, but these levels of specific IgG antibody were substantially higher than those of animals that only received one immunization with pgD2 (figure 2, compare columns 2–4 with columns 7–8). Of interest, priming with the recombinant MVA-gD2 virus and boosting with either MVA-gD2 or pgD2 (figure 2, columns 5 and 6) elicited significantly higher levels of IgG than either pgD2-primed group (P < .05), which suggests that this order of vector immunization might be advantageous for enhancing the antibody response to gD.

To further investigate the strength and diversity of the antibody response resulting from prime-boost combinations of MVA-gD2 and pgD2, we delivered 1 μg of pgD2 by gene gun.

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<tr>
<th>Group</th>
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Note: Data are mean log10 titer ± SD. Limit of detection for each isotype, 1:40.

Table 1. Effect of varying doses of modified vaccinia virus Ankara (MVA)–glycoprotein D2 (gD2) immunization on the antibody response to gD2 after priming with plasmid (p)– or MVA-expressed gD2: experiment 1.

Table 2. Effect of varying doses of modified vaccinia virus Ankara (MVA)–glycoprotein D2 (gD2) immunization on the antibody response to gD after priming with plasmid- or MVA-expressed gD2: experiment 2.
primed with pgD2 produced measurable neutralizing antibody significantly higher levels (P < .05) of specific IgG (figure 3). As a result, the IgG1 response that resulted when mice were primed with gene-gun delivery of pgD2 (figures 2 and 3) suggested an immune response skewed toward a humoral Th2-type response. Correspondingly, the IgG1 and IgG2a response elicited when mice were primed with MVA-gD2 suggested a response that might include both Th1-type humoral and Th2-type cellular immune responses. As a measure of the cellular immune response elicited in the prime-boost immunization strategies described above, we quantified the production of the cytokines released from splenocytes from immunized mice that were restimulated with HSV-2 antigen in vitro. Individual splenocyte cultures were prepared from each mouse of the immunized groups described in figure 2, stimulated in culture with HSV-2 antigen, and the supernatants assayed for the production of IL-2 and IFN-γ (Th1 type) and IL-4 (Th2 type) (figure 4). There was no detectable IL-4 among the various groups (limit of detection, 15 pg/mL). The quantity of IL-2 released from splenocytes derived from mice primed with pgD2 was not substantially above background (either unstimulated or mock-infected extract stimulated) or above levels observed in control immunized animals. On the other hand, mice primed with MVA-gD2 (figure 4A, columns 4–6) produced significantly higher levels (P < .05) of detectable IL-2 in splenocytes after stimulation with HSV-2 antigen, compared with control stimulation (figure 4A). Similarly, the levels of IFN-γ that were produced in the groups primed with MVA-gD2 in the prime-boost experiments were distinctly higher than those produced in any of the other immunized groups (figure 4B, columns 5 and 6). Among the various other immunized groups in the present study, only mice immunized with a single injection of MVA-gD2, with a mock booster of control DNA vector, had detectable IFN-γ levels above background (figure 4B, column 4). Again, these results suggest that priming with the recombinant MVA-gD2 virus qualitatively alters the immune response to gD relative to DNA immunization with plasmid-delivered gD.

**Protection of mice from lethal HSV-2 challenge.** As a comparison of the protective capacity of immune responses elicited by immunization with the gD2-expressing vectors, we used a mouse intraperitoneal challenge model to evaluate the protective capacity of the response engendered by these vaccination strategies. Five groups of mice were immunized as in the experiment described in figure 3. Antibody responses, as determined in parallel ELISA assays, were similar in titers and pattern to that reported in the previously described experiment (data not shown). All mice were challenged by intraperitoneal injection of 25 LD₅₀ of HSV-2 (strain MS) and monitored for 4 weeks for symptoms of virus infection and death (table 3). The group of mice vaccinated with control vectors succumbed to virus infection within 9–12 days, with no survivors (group A). With the exception of group B (pgD2 prime and pgD2 boost), in which the survival rate was 80%, all mice in all of the other groups immunized with gD2-expressing vectors survived the challenge (i.e., 100% survival in groups C, D, and E).
Figure 3. Antibody response to combinations of plasmid (p) glycoprotein D2 (gD2) and modified vaccinia virus Ankara (MVA)–gD2 prime-boost. Mice were immunized as described in figure 2 with the indicated combinations of plasmid and MVA vectors. Herpes simplex virus (HSV)-2-specific antibody was analyzed by ELISA for HSV-2-specific IgG (A), antibody isotype (B), and by HSV-2 neutralization assay for neutralizing antibody (C).

Discussion

The development of safe and effective herpes simplex vaccines has proved to be difficult. Indeed, there have been several recently reported failures of HSV-2 glycoprotein subunit vaccines in both preventative and therapeutic clinical trials [21]. These results underscore the difficulties inherent in a simple protein subunit approach to genital herpes vaccination. Future development of a subunit vaccine for genital herpes may require more complex vaccine candidates that include multiple viral antigens, such that several stages of the interaction of the virus and the host can be targeted. Regardless of the composition, however, a successful vaccination strategy will probably need to invoke a vigorous broad-based immune response. Here, we describe such a strategy that enhances the response to HSV-2 gD while retaining the safety advantages of subunit vaccination. In the present article, we have demonstrated that a prime-boost immunization strategy that uses an MVA vector expressing the HSV-2 gD gene followed by a gD plasmid-expression vector produces potent humoral and cellular immune responses. This strategy was superior to a 2-dose DNA immunization scheme or to a strategy that used DNA immunization followed by MVA vector immunization. Of note, the immunization strategy that used 2 doses of MVA-gD, rather than as part of a prime-boost strategy, also resulted in high anti-gD antibody titers of several IgG isotypes, as well production of IFN-γ by antigen-stimulated splenocytes in vitro. The magnitude of these responses was comparable to that observed after MVA-gD was used as the priming dose and pgD2 as the booster dose. There may well be circumstances in which the use of MVA-delivered antigens, without involving plasmid DNA delivery, is advantageous as well. In our experiments, a second immunization with MVA-gD2 was very effective in boosting the response to plasmid- or MVA-delivered gD. However, at least in some model systems, 2 immunizations with a recombinant MVA vector were reported to be less effective than a DNA vector–MVA vector prime boost combination in generating protective immunity [22]. Thus, potential benefits of multiple MVA vector immunizations
in one vaccination protocol will have to be considered in the context of a possible broader use of MVA in various other vaccines, including the possibility of a new-generation smallpox vaccine.

HSV-2 gD has been used extensively as a protein subunit vaccine in clinical trials [23], including the ones noted earlier [21], and the gene for gD has been investigated as a potential DNA vaccine candidate [4–6, 24]. The prime-boost strategy for vaccination, which consists of the sequential delivery of a vaccine antigen by 2 fundamentally different methods, has been reported to generate extremely potent humoral and cellular immune responses [13, 25]. We chose to examine a combination of delivery vectors that maintains some of the safety features of subunit vaccines. Both vectors, plasmid DNA and nonreplicating MVA, have been evaluated to some extent for safety in humans [26]. In addition, MVA can be grown in CEF cells, a cell substrate that probably would be acceptable for vaccine production. Because simplicity and feasibility are such strong considerations for the success of any vaccination strategy, we examined a simple 2-dose delivery regimen that used relatively small amounts of each vector.

There have been a limited number of studies to date that have investigated the use of prime-boost vaccination strategies for protection against HSV. To date, none have used the combination of DNA and nonreplicating poxvirus vectors described in the present article. In one reported study, DNA priming with HSV-2 gD expression plasmid followed by boosting with recombinant gD protein increased the antibody response and cellular immune responses in mice, compared with 2 immunizations with gD plasmid DNA [27]. In a more recent study, DNA priming with an HSV-1 gB expression plasmid followed by boosting with a gB-expressing replication-competent vaccinia virus resulted in increased antibody and T cell responses [28]. In that system, however, the reverse combination of a gB-expressing vaccinia virus priming inoculation followed by a gB plasmid boost resulted in optimal immune responses when both vectors were delivered via a mucosal route. Taken together, those results, as well as those from the present study, indicate that prime-boost strategies of antigen delivery enhance the immunogenicity of HSV antigens compared with plasmid DNA alone and qualitatively alter the immune response after vaccination in potentially beneficial ways.

On the basis of results reported in other systems, we initially examined the ability of MVA-expressing gD vector to boost the immune response to plasmid-delivered gD. Indeed, although a single inoculation with the MVA-gD2 vector was capable of stimulating a strong specific antibody response, a second inoculation with MVA-gD2 significantly boosted the response to an initial plasmid or MVA-gD2 immunization. Although an MVA-gD2 boost following a plasmid gD priming

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<td>A</td>
<td>Control DNA</td>
<td>Control MVA</td>
<td>5/5</td>
<td>10.4 ± 1.3</td>
</tr>
<tr>
<td>B</td>
<td>pgD2</td>
<td>pgD2</td>
<td>1/5</td>
<td>14</td>
</tr>
<tr>
<td>C</td>
<td>pgD2</td>
<td>MVA-gD2</td>
<td>0/5</td>
<td>—</td>
</tr>
<tr>
<td>D</td>
<td>MVA-gD2</td>
<td>MVA-gD2</td>
<td>0/5</td>
<td>—</td>
</tr>
<tr>
<td>E</td>
<td>MVA-gD2</td>
<td>pgD2</td>
<td>0/5</td>
<td>—</td>
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</tbody>
</table>

NOTE. MVA, modified vaccinia virus Ankara; pgD2, plasmid glycoprotein D2.
immunization routinely appeared to result in a higher level of specific IgG, IgG1, and virus-neutralizing antibody than a plasmid gD boost, significantly higher levels of specific antibody were obtained when MVA-gd2 was used as a priming dose and then boosted with either plasmid gD or a second immunization with MVA-gd2. Most interestingly, the combination of MVA-gd2 priming immunization followed by either MVA-gd2 or pgd2 boost resulted in the most diverse isotype distribution and the highest level of cellular immune responses, as measured by cytokine production from antigen-stimulated spleen cells. It may be that the superior responses that resulted when MVA-gd2 was used as a priming immunization were due to a better initial expression of gD from the MVA vector, compared with the plasmid vector. Future experiments will address this possibility by evaluating alternative plasmid delivery mechanisms or multiple plasmid vector immunizations.

Results somewhat similar to those presented here were reported in a study that examined the mucosal response to HSV-1 gB expressed by plasmid and replicating vaccinia virus vectors [28]. In that study, the mucosal delivery of vaccinia gB followed by plasmid gB resulted in optimal mucosal and systemic antibody and T cell responses, compared with other delivery combinations of the vectors. However, when delivery was by intramuscular injection, the sequence of plasmid vector followed by vaccinia vector produced the greatest systemic antibody and T cell responses. In our hands, enhanced systemic humoral and cellular immunity to HSV-2 gD was obtained with the opposite delivery sequence. We did not evaluate mucosal immunity, because, in most studies, parenteral administration of plasmid or vaccinia vectors results in a minimal mucosal IgA response [5, 28]. Thus, at present, it is not clear whether the best prime-boost strategy for enhancing humoral and cellular immunity to a particular antigen is antigen-specific or due to other factors such as the means of delivery (gene gun vs. intramuscular injection), the route of delivery (systemic or mucosal), or the nature of the viral vector (replicating vs. nonreplicating).

Although evaluation of its safety and effectiveness is still in the early stages, there are several characteristics of MVA that make it an attractive vaccine vector. Because it does not replicate in most mammalian cells, it may provide a safer alternative than a replication-competent vaccinia virus vector [29]. Furthermore, studies have indicated that MVA vectors are as immunogenic as replicating vaccinia virus vectors [30], and there is evidence that the immune response to an MVA-expressed antigen, compared with that of a replication-competent vaccinia-expressed antigen, is less affected by preexisting immunity to vaccinia virus [31]. In fact, as was already noted, in the experiments reported here, 2 immunizations with MVA-gd2 produced high levels of both antibody and T cell responses.

The experiments described in the present article were all based on gene-gun-delivered DNA and subcutaneously injected MVA. Our choice of the gene gun for plasmid delivery was based on the efficiency and reproducibility of the technique, as determined in our own experiments, as well as those reported by others [32, 33]. It is clear, however, that this type of delivery has an influence on the resulting immune response [34]. Not only does gene-gun delivery of gD result in an exclusive IgG1 response, it may be the reason why our pgd2 prime/MVA-gd2 boost protocol also produced only IgG1. An IgG1 or limited isotype distribution in specific antibody response is not necessarily undesirable in all circumstances; in fact, it is not known whether a more diverse isotype profile response is actually important for HSV immunity. Nevertheless, it is important to understand how each of the parameters of the delivery system affects the final response so that, as the correlates of protection are defined, rational decisions may be made in the development of vaccines. Conflicting results have been reported regarding the effectiveness of gene-gun delivery of DNA vaccines as part of prime-boost vaccination protocols. For example, in one study, gene-gun delivery was reported to be ineffective in producing protective cell-mediated immune responses [15]. On the other hand, others have reported that gene-gun delivery is extremely efficient in enhancing CD8+ T cell immunity and inducing protective immunity [35]. Thus, future work will evaluate and compare alternative delivery mechanisms for both plasmid and MVA vector delivery.

Although the primary focus of the present study was to evaluate the relative immune responses elicited by the various vector delivery combinations, we also assessed the strength of protection using a lethal challenge model. Although such a challenge study is obviously not a natural route of infection, the demanding outcome is useful for assessing the systemic immune response to the delivered gD antigen. In the vector delivery combinations used here, there was little difference in the ability of each tested combination to protect mice from a lethal HSV-2 challenge regardless of the measured antibody levels and isotype profile (table 3). These results are not particularly surprising, because HSV-2 gD delivered by several types of vectors is protective in different types of animal models. Whether differences in protective effect in a lethal challenge model would have been apparent with higher challenge doses is not known but might be technically difficult because of the large amounts of virus required. Unfortunately, successful protection in animal models of HSV infection is not necessarily a good indicator of success in human clinical trials [10, 21]. The correlation between enhanced immune responses as a result of vaccination strategy and successful protection in animal models would arguably be more important for antigens that are weakly immunogenic and/or protective in some models.

In conclusion, our results indicate that a prime-boost protocol for immunization with HSV-2 gD that uses plasmid and nonreplicating vaccinia virus vectors is capable of enhancing the antibody and T cell responses, compared with DNA immunization alone. Such strategies of immunization for gD and other HSV-2 antigens may prove to be especially useful for invoking the vigorous diverse immune response necessary for the development of protective immunity to HSV-2.
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References