Glycoprotein G of Herpes Simplex Virus 2 as a Novel Vaccine Antigen for Immunity to Genital and Neurological Disease

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The envelope glycoproteins of herpes simplex virus 1 (HSV-1) and HSV-2, with the exception of glycoprotein G, elicit cross-reactive B- and T-cell responses. Human vaccine trials, using the cross-reactive glycoproteins B and D, have shown no protection against genital HSV-2 infection or disease. In this study, the mature form of glycoprotein G (mgG-2) of HSV-2 was used for immunization of mice, either alone or in combination with adjuvant CpG, followed by an intravaginal challenge with a lethal dose of a fully virulent HSV-2 strain. Mice immunized with mgG-2 plus CpG showed low disease scores and a significantly higher survival rate (73%) than mice immunized with mgG-2 alone (20%) or controls (0%). Accordingly, limited numbers of infectious HSV-2 particles were detected in the spinal cord of mice immunized with mgG-2 plus CpG. The observed protection was associated with a gamma interferon (IFN-γ) response by splenic CD4+ T cells upon antigen restimulation in vitro and in vaginal washes 1 day postinfection. The majority of sera collected from mice immunized with mgG-2 plus CpG showed macrophage-mediated antibody-dependent cellular cytotoxicity and antibody-dependent complement-mediated cytolysis, while no neutralization activity was observed. In conclusion, we have shown that immunization with the type-specific mgG-2 protein in combination with CpG could elicit protective immunity against an otherwise lethal vaginal HSV-2 challenge. The mgG-2 protein may therefore constitute a promising HSV-2 vaccine antigen to be considered for future human trials.

Herpes simplex virus 2 (HSV-2) is one of the most common sexually transmitted infections worldwide (52). Epidemiological data from different countries support an increasing prevalence in the population (18). The first global estimate on HSV-2 infection, based on several studies from 12 regions, concluded that 536 million individuals were infected prior to 2003 and 23.6 million were infected during 2003 (35). HSV-2 infects the genital mucosa and establishes a latent infection in sensory dorsal root ganglia (DRG), from where the virus can reactivate, giving widespread genital lesions or, more commonly, no symptoms, i.e., asymptomatic shedding of virus. In newborns and in immunocompromised patients, HSV-2 can elicit severe and often fatal central nervous system (CNS) or disseminated infections. Further, genital HS-2 infection is associated with a 3-fold increased risk of HIV acquisition (19). Thus, development of intervention approaches to counter genital HSV-2 infections is of major public health importance.

Great efforts have been made to develop a vaccine against genital HSV-2 infection or disease (28, 29). Human vaccine trials have been performed using the HSV-2 glycoprotein B (gB-2) and/or glycoprotein D (gD-2) as antigens. Results from randomized double-blind placebo-controlled multicenter trials including >13,000 subjects have been discouraging, showing no protection against HSV-2 infection or disease (5, 13, 14, 53). HSV-1 and HSV-2 are closely related viruses with a high degree of similarity at the protein level. For the immunogenic envelope glycoproteins, all but glycoprotein G contain immunogenic regions which elicit cross-reactive B- and T-cell responses. An interesting observation is that a previous HSV-1 infection reduces only the severity of the clinical symptoms but does not confer protection against acquisition of HSV-2 (8, 14, 32). Thus, HSV-2 can infect the individual despite the existence of cross-reactive immune responses elicited after a prior HSV-1 infection. Since HSV-2 is sexually transmitted and usually infects persons at an older age than does HSV-1, it is obvious that HSV-2 escapes cross-reactive immune responses elicited from the HSV-1 infection. Deduced from these observations, there is a rationale to evaluate an HSV-2 type-specific protein as a vaccine candidate.

Glycoprotein G of HSV-2 (gG-2) was first described in 1984 as an envelope protein which was lacking a counterpart in HSV-1-infected cells (36, 51). The gG-2 protein is expressed as an unglycosylated precursor which is further N-glycosylated, generating a high-mannose precursor. This precursor protein is cleaved into a secreted amino-terminal portion (sgg-2) and to a carboxy-terminal membrane-anchored portion. The latter protein is further O-glycosylated, generating the mature portion of gG-2 (mgG-2) (3, 54). As mgG-2 elicits a type-specific antibody response, such antibodies have been used for several years as a serological marker of HSV-2 infection in clinical settings. Subsequently, this antigen was also shown to elicit a type-specific CD4+ T cell response (9, 17). The function of mgG-2 in the genital HSV-2 infection, however, remains elusive. Although mgG-2 is nonessential for viral replication in cell culture, we have shown earlier that an intact mgG-2 in clinical HSV-2 isolates is of importance for human infection, as mgG-2-negative HSV-2 isolates are rarely detected (33). In cell culture, an mgG-2-negative HSV-2 mutant was recently shown to spread mostly from cell to cell with an impaired capacity to produce extracellular infectious HSV-2 particles (1).
Altogether, these findings warrant studying the mgG-2 protein as a putative vaccine antigen to elicit protective immunity to genital HSV-2 infection or disease.

MATERIALS AND METHODS

Mice. Six- to 8-week-old female C57BL/6 mice were purchased from Scanbur BK AB, Sweden. Gamma interferon (IFN-γ)-gene knockout mice on a C57BL/6 background were a kind gift from Nils Lycke, MIVAC, University of Gothenburg. Mice were divided into groups (6 to 10 mice) and kept in separate cages for 1 week. Mice were anesthetized with 3% isoflurane (Baxter) throughout all injections, vaginal washings, and challenge with virus. All animal experiments were approved by the ethical board in Gothenburg (Dnr 46-2008).

Cells and viruses. African green monkey kidney (GMK-AH1) cells were cultured in Eagle’s minimal essential medium supplemented with 2% calf serum and antibiotics. Baby hamster kidney (BHK21) cells were propagated in Glasgow minimum essential medium (G-MEM) with 8% fetal bovine serum. Wild-type (wt) HSV-2 strain 333 and a local wild-type HSV-2 isolate, B4327UR (26), were used.

Production of mgG-2 protein. The mgG-2 protein is highly O-glycosylated, with coupling of N-acetylgalactosamine (GalNAc) residues to serine or threonine. This feature was utilized for purification by Helix pomatia lectin affinity chromatography as described earlier (42). Briefly, BHK21 virus-infected cell membranes (HSV-2 strain B4327UR) were solubilized in 1% NP-40 in 0.1 M glycine-NaOH, pH 8.8. Tris-buffered saline (TBS) was used as wash buffer, and 0.02 M GalNAc (Sigma-Aldrich) in TBS was used as elution buffer. The eluate was dialyzed at 4°C overnight to remove GalNAc before use. The protein concentration was measured by Bio-Rad protein assay.

Characterization of mgG-2 antigen. For several years, we have utilized Helix pomatia lectin affinity chromatography-purified mgG-2 as antigen in an enzyme-linked immunosorbent assay (ELISA) format in our clinical laboratory for detection of human anti-mgG-2 antibodies. In the production of the antigen, exclusively type-specific reactivity of the mgG-2 antigen is a sensitive marker of a lack of other cross-reactive viral proteins. The purified mgG-2 protein was therefore tested in ELISA by using human sera from isolation-positive HSV-1- or HSV-2-infected individuals as well as from HSV-negative subjects. In addition, mgG-2 antigen was tested in ELISA and Western blotting (WB) as described previously (34) using cross-reactive HSV MAbs directed against gB, gC, and gD (6) and an anti-gC-2 monoclonal antibody (MAb; kindly provided by Edward Trybula) produced at our laboratory (unpublished data). The anti-mgG-2 MAbs O1.C5.B2 and O3.G11.H7 were used as positive controls (34). The mgG-2 protein was also subjected to SDS-PAGE using a 4 to 12% NuPAGE bis-Tris gel, followed by silver staining with a Silver-Xpress silver staining kit (Invitrogen) according to the manufacturer’s description. Purified mgG-2 was tested for toxic effects on T cells derived from the spleens of three mice using the cell proliferation kit II [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-5-tetrazolium hydroxide (XTT) assay; Roche Applied Science]. Potential endotoxin contamination was analyzed by use of Endochrom-E K reagent (Charles River, Charleston, SC) at the Department of Bacteriology, University of Gothenburg.

Adjuvant. Synthetic oligodeoxynucleotides with a complete phosphorothioate backbone containing two optimal mouse CpG motifs (ODN1826, TCC ATG ACG TTC CTG ACG TT; Operon Biotechnologies GmbH, Germany) were used as adjuvant (7).

Immunization scheme. In this study, we first used a subcutaneous (s.c.) dose followed by two doses given intranasally (i.n.) with a 14-day interval. This immunization model was chosen to achieve systemic as well as mucosal immunity (20, 45). Four separate experiments were performed with, in total, 26 mice. The s.c. dose was given in a volume of 200 µl followed by two doses of 15 µl given i.n. Each dose contained 10 µg mgG-2 alone (mgG-2) or in combination with 20 µg CpG (mgG-2 plus CpG). A control group that received phosphate-buffered saline (PBS) was included in each experiment. A control group of 10 mice given CpG alone was included for the T-cell proliferation assay and for measurement of IFN-γ production in vaginal washes.

Serum samples. Serum samples were collected 18 days after the third immunization. In addition, serum samples were obtained for HSV-2 DNA analysis from vaccinated and control mice at day 6 postinfection (p.i.).

Detection of IgG antibodies against mgG-2. An indirect ELISA technique was used as described previously (55). Briefly, Helix pomatia lectin-purified mgG-2 (1 mg/ml) was coated at a 1:1,000 dilution in carbonate buffer (pH 9.6) on Maxisorp microtiter plates (Nalgé Nunc Int.) and incubated overnight at 4°C. Plates were blocked with 2% bovine serum albumin (BSA) in PBS. After dilution in PBS with 1% BSA and 0.05% Tween 20, sera were incubated for 1 h at 37°C. Peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at a 1:1,000 dilution was used as a conjugate, and o-phenylenediamine was used as the substrate. The reaction was stopped with 1 M sulfuric acid, and the absorbance was read at 490 nm. Sera were titrated in 3-fold dilution steps starting at a 1:30 dilution. The antibody titer was defined as the reciprocal value of the highest serum dilution giving an optical density (OD) greater than that for the blank plus 0.3 OD unit. Subclass-specific ELISAs for detection of IgG1 and IgG2c were performed in a similar way by using subclass-specific peroxidase-conjugated goat anti-mouse IgG1 as the conjugate (Southern Biotech) at a 1:2,000 dilution.

NT assay. The neutralization (NT) capacity of hyperimmune sera drawn from mice vaccinated with mgG-2 or mgG-2 plus CpG was tested in a microneutralization assay described previously (2). Briefly, 50 PFU of wt 333 was mixed with 2-fold dilutions of serum at a 1:20 start dilution. The mixtures were incubated in a 96-well microtiter plate at 37°C for 2 h, followed by addition of GMK-AH1 cells in suspension. The NT titer was defined as the reciprocal value of the highest serum dilution for which no cytopathic effect was detected. Complement-dependent NT activity was evaluated with the same protocol described above with 2.5% guinea pig serum as the source of complement (Sigma-Aldrich). In both assays, HSV-2-positive human serum was used as a positive control.

ADCC. The antibody-dependent cellular cytotoxicity (ADCC) assay was modified from the method described earlier (30). BHK21 target cells were infected with wt 333 at a multiplicity of infection of 4. The cells were labeled with 100 µCi Na2125I and 30 µCi /H9262-I-labeled human interleukin-2 (IL-2; Proleukin; Novartis). The IL-2 concentration was maintained throughout both assays. One hundred microliters of target cells (1 x 105) and serum samples, diluted in ADCCMEM, were added to a 96-well plate (Nalcon Surface; Nunc, Denmark) and incubated for 30 min at 37°C in a humid atmosphere with 5% CO2. Effector cells were added, followed by incubation for 18 h at 37°C in a humid atmosphere of 5% CO2. An effector/target (E/T) cell ratio of 100:1 was optimal for both assays. For detection of maximum release, 100 µl of 5% sodium dodecyl sulfate was added to wells containing 100 µl of target cells. Supernatants were collected by harvesting frames (Molecular Devices Corp.) and counted using a Cobra Auto-gamma counter (model 5002/5003; Packard Instrument Company). Results were presented as the mean value of tested triplicates. The percentage of specific release of 51Cr (ADCC%) was calculated by the following formula: [(counts per minute
of immune serum — counts per minute of nonimmune serum)/\(^{(\text{counts per minute of maximum release} - \text{counts per minute of nonimmune serum})} \times 100.

Heat-inactivated serum from one mouse immunized with thymidine kinase-negative virus was used as a positive control, and serum from a control mouse immunized with PBS was used as nonimmune serum. All serum samples were tested at a 1:20 dilution. Sera presenting an ACMMC% of >5 were considered ADCC positive.

**Antibody-dependent complement-mediated cytolysis (ACMC).** HSV-2-infected (wt 333) BHK21 cells labeled as described previously (37) were washed and resuspended in TBS supplemented with 10% IFS and 1% antibiotics and used as target cells. Target cells (6 × 10⁵) and 50 μl of heat-inactivated sera in 2-fold dilution steps starting at 1:25 were mixed into each well. After 1 h of incubation at 37°C, complement (serum from guinea pig) was added at a final 1:15 dilution. After an additional 2 h, supernatants were collected and specific release of 51Cr was measured. Maximum release from target cells was obtained by adding 100 μl sodium dodecyl sulfate to wells containing 100 μl of target cells. The percentage of cytolytic activity (ACMMC%) of tested sera was expressed with the following formula: \(\{(\text{counts per minute of immune serum and complement} - \text{counts per minute of negative serum and complement})/\text{counts per minute of maximum release}\} \times 100\).

The ACMC titer was defined as the reciprocal value of the serum dilution presenting an ACMC% of >10.

**Proliferation assay.** Three weeks after the last immunization, spleens and genital lymph nodes (gLNs) were removed from three mice per group and pooled. Single-cell suspensions were produced and, an EasySep mouse CD4⁺ enrichment kit and EasySep Magnet (Stemcell Technologies Inc.) were used to purify CD4⁺ T cells from spleens according to the manufacturer’s recommendations. A total of 2 × 10⁶ spleen cells, γLN-derived cells, or enriched CD4⁺ T cells, including 20% splenocytes, were added per well in a 96-well plate (Nunc). Cells were cultured either alone with 2.5 μg/ml concanavalin A (Sigma-Aldrich) as a positive control or together with 3 μg/ml mgG-2 in Iscove’s basal medium (Biochrom AG) containing 10% fetal bovine serum (Sigma-Aldrich) with 100 μg/ml gentamicin (Sigma-Aldrich), 50 μM 2-mercaptoethanol (Sigma-Aldrich), and 2 mM l-glutamine (Biochrome AG). Samples were added in triplicate. Cells were incubated at 37°C with 5% CO₂ for 96 h, after which 1 μCi of [6-3H]thymidine (Amersham Biosciences) was added, followed by an additional incubation overnight. The cells were harvested and run in a β-counter (1450 MicroBeta; Trilux) to measure the cell proliferation as counts per minute. Data were expressed as the arithmetic mean stimulation counts per minute of negative serum and complement)/(counts per minute of immune serum and complement)/(counts per minute of nonimmune serum). Samples were kept frozen at −70°C until analyzed. DRG, spinal cord, and brain samples were thawed and homogenized using a Dounce tissue grinder. The samples were diluted in HBSS, and infectious HSV-2 was estimated by a plaque assay. Briefly, samples were added to a monolayer of GMK-AH1 cells and incubated for 1 h at room temperature, before Iscove’s medium containing 1% methylcellulose (Sigma-Aldrich), 2% new born calf serum (Sigma-Aldrich), and 1% antibiotics was added. The cells were incubated at 37°C with 5% CO₂ for 72 h before viral plaques were stained with crystal violet (Sigma-Aldrich) and the number of PFU was counted using light microscopy.

**Detection of HSV-2 DNA by PCR.** HSV-2 DNA was quantified in sera at day 6 p.i., as well as in DRG and spinal cord samples collected from surviving mice at day 21 p.i. Samples were analyzed by real-time PCR. Viral DNA was extracted by a Magnapure LC robot (Roche). Subsequent amplification of a segment of the gB-2 gene was performed utilizing TaqMan probes labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) and an ABI Prism 7000 PCR instrument (Applied Biosystems). This assay shows high specificity and a similar sensitivity as a nested PCR system (41). A plasmid (pUC57) containing the target sequence was constructed (GenScript) and amplified in *Escherichia coli* XL-1 Blue, purified by a HiSpeed plasmid maxikit (Qiagen), and quantified by spectrophotometer analysis. A standard curve was included in each run and was based on six 5-fold dilutions of the plasmid using an initial concentration of 1 × 10⁶ HSV-2 genome copies per reaction. Due to inhibition of the PCR by the high content of cellular DNA, tissue samples from DRG, spinal cord, and brain were diluted 1:4 in PBS before extraction of HSV-2 DNA.

**Statistics.** SPSS (version 16) software for Microsoft Windows was used for statistical calculations. Fisher’s exact test was used for survival data, the Mann-Whitney (nonparametric) test was used for titer values, and the general linear model (univariate) was used for statistical calculations. Fisher’s exact test was used for survival data, the Mann-Whitney (nonparametric) test was used for titer values, and the general linear model (univariate) was used for statistical calculations. Fisher’s exact test was used for survival data, the Mann-Whitney (nonparametric) test was used for titer values, and the general linear model (univariate) was used for statistical calculations. Fisher’s exact test was used for survival data, the Mann-Whitney (nonparametric) test was used for titer values, and the general linear model (univariate) was used for statistical calculations. Fisher’s exact test was used for survival data, the Mann-Whitney (nonparametric) test was used for titer values, and the general linear model (univariate) was used for statistical calculations.

**RESULTS**

The mgG-2 antigen did not contain HSV-2 cross-reactive proteins. In order to rule out the possibility that the mgG-2 antigen contained other HSV-2 proteins, sera obtained from HSV-negative, HSV-1-positive, and HSV-2-positive individuals were subjected to ELISA using purified mgG-2 as the coating antigen. As expected, anti-mgG-2 MAb f HSV-1-positive sera were clearly reactive. Sera from HSV-negative and HSV-1-positive individuals were unreactive to mgG-2 antigen in the ELISA, indicating that no other immunogenic viral glycoproteins were present in the mgG-2 antigen preparation. Further, a lack of other major HSV-2 glycoproteins in the mgG-2 preparations was confirmed in Western blots and by ELISA using anti-HSV MAb directed against gB, gC, and gD (data not shown). Purified mgG-2 presented a band in WBs with an apparent molecular mass of 115 kDa and a weaker
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**FIG 1** The mgG-2 protein, at a concentration of 1 µg (A) or 10 µg (B), is visualized by silver staining after separation by SDS-PAGE. The O-glycosylated mgG-2 protein (115 kDa) is indicated with an arrow.

band with a molecular mass of 70 kDa using anti-mgG-2 MAbs and HSV-2-positive sera, as described earlier (12, 34). SDS-PAGE followed by silver staining showed a marked band corresponding to the O-glycosylated mgG-2 (115 kDa) (Fig. 1). The mgG-2 protein showed no toxic properties in the XTT assay, and the level of endotoxin was below the detection limit (<0.5 EU/ml; data not shown).

**mgG-2 induced antibody responses in vaccinated mice.** Sera collected from mice immunized with mgG-2 and mgG-2 plus CpG and from control mice 18 days after the third immunization were analyzed for anti-mgG-2 IgG, IgG1, and IgG2c antibodies. The mgG-2 antigen alone was immunogenic and elicited a marked antibody response. Mice immunized with mgG-2 plus CpG showed significantly higher IgG titers ($P < 0.007$) than the mgG-2 group (Fig. 2A). Serum samples were also analyzed for the presence of IgG1 and IgG2c subclass titers by using subclass-specific ELISAs. We found that mice immunized with mgG-2 alone presented generally higher IgG1 titers than IgG2c titers, while for sera from mice immunized with mgG-2 plus CpG, significantly higher IgG2c levels were detected ($P = 0.001$) (Fig. 2B).

**Immunization with mgG-2 plus CpG induced CD4$^+$ T cell proliferation and enhanced IFN-γ production.** The role of cell-mediated immunity in HSV-2 protection has been investigated by others, showing that CD4$^+$ as well as CD8$^+$ T cells are of importance in HSV-2 clearance after a vaginal infection (38, 39, 47). In addition, the importance of IFN-γ in protection against genital herpes infection in mice is well documented (22, 49). A proliferation assay was used to examine if mgG-2 elicited a cellular immune response. Whole spleen cells, gLNs, and enriched splenic CD4$^+$ T cells were collected from immunized mice and controls immunized with PBS. Spleen cells and enriched splenic CD4$^+$ T cells were also collected from mice immunized with CpG alone. Collected cells were restimulated with mgG-2 in vitro. Spleen cells from control mice showed low SI values and undetectable levels of IFN-γ, while splenocytes from both mgG-2-immunized groups showed significantly higher SI values than the controls ($P < 0.01$) (Fig. 3A). The IFN-γ production in spleen cells for the mgG-2 plus CpG group was significantly higher than that in spleen cells for the controls ($P = 0.04$). Although the IFN-γ production was higher in the mgG-2 plus CpG group than the mgG-2 group, the difference was not statistically significant ($P = 0.09$). For both vaccinated groups, the SI was significantly higher than that for the controls, with no significant difference between mice given mgG-2 or mgG-2 plus CpG (Fig. 3A and B). However, the level of IFN-γ production for CD4$^+$ T cells collected from mice immunized with mgG-2 plus CpG was significantly higher than that for cells from mice vaccinated with mgG-2 or controls ($P < 0.01$) (Fig. 3B). Proliferation from gLNs was low, with no IFN-γ production detected from any of the groups (data not shown). To confirm the enhanced IFN-γ production in restimulated spleen cells and enriched CD4$^+$ T cells, all samples were reanalyzed using the CBA Th1/Th2 cytokine assay. Similar to what was described with the ELISA technique, significantly higher IFN-γ levels were detected for the mgG-2 plus CpG group than the mgG-2 group. In addition, for the mgG-2 group, IL-5 was detected in supernatant from both spleen cells and splenic CD4$^+$ T cells, indicating a Th2-biased immune response. No significant levels of IL-2 or IL-4 could be detected for any of the groups (data not shown). Taken together, our results show that immunization with mgG-2 plus CpG can induce an antigen-specific Th1-type T-cell response.

**Immunization with mgG-2 plus CpG induces protection in a mouse model of genital herpes.** Groups of 6 to 10 mice were immunized 3 times with mgG-2 or mgG-2 plus CpG. Three weeks after the last immunization, blood samples were collected. Sera were subjected to an mgG-2-specific IgG antibody ELISA (A) and a subclass ELISA for detection of IgG1 and IgG2c antibodies (B). Data are from four experiments and presented as mean ± SEM.

**FIG 2** Immunization with mgG-2 elicits a B-cell response. Mice were immunized 3 times with mgG-2 or mgG-2 plus CpG. Three weeks after the last immunization, blood samples were collected. Sera were subjected to an mgG-2-specific IgG antibody ELISA (A) and a subclass ELISA for detection of IgG1 and IgG2c antibodies (B). Data are from four experiments and presented as mean ± SEM.
immunized with one s.c. injection of mgG-2 or mgG-2 plus CpG, followed by two intranasal instillations as booster doses. As shown in Fig. 4A, nonimmune mice showed progressive disease starting at day 4 after challenge, and all mice were euthanized due to neurological disease at day 10. The mgG-2 group showed signs of vaginal infection at day 4. Although a delay in disease progression was observed, the survival rate was not significantly different from that for the controls (Fig. 4B). Mice vaccinated with mgG-2 plus CpG showed mild or no genital inflammation. Immunization with mgG-2 plus CpG conferred a protection rate (73%) significantly higher than that for the controls (0%, \( P < 0.0001 \)) or that conferred by immunization with mgG-2 alone (20%, \( P = 0.0003 \)) (Fig. 4B).

Immunization with mgG-2 plus CpG fails to generate protection in IFN-\( \gamma \) gene-knockout mice. To confirm the importance of a Th1-type immune response in the protection, mice defective in the IFN-\( \gamma \) gene were immunized with mgG-2 plus CpG three times and challenged with a lethal dose of HSV-2 333, as described above. As shown in Fig. 5, no protection was observed and all mice developed severe disease and were euthanized at day 6 or 7 p.i. This result implies that IFN-\( \gamma \) plays an important role in the immunity induced following mgG-2 plus CpG immunization.

Reduction of HSV-2 load in vagina, spinal cord, and serum. Vaginal washes were collected at 3 days p.i., and infectious virus was measured by a plaque assay. The mgG-2 and mgG-2 plus CpG groups presented significantly lower HSV-2 titers (mean values, 394 PFU/sample \( P < 0.03 \) and 344 PFU/sample \( P = 0.005 \), respectively) than the control group (mean value, 762 PFU/sample) (Fig. 6A). Although the difference in survival rates between mice vaccinated with mgG-2 and mgG-2 plus CpG was significant, no statistically significant differences in the viral load in the vagina were detected between the groups. Next, we investigated the presence of infectious virus in neuronal tissue. Results for the mgG-2, mgG-2 plus CpG, and control groups at day 6 p.i. are summarized in Fig. 6B and C. For the mgG-2 group, infectious HSV-2 was isolated from DRG in 2/8 mice. For the mgG-2 plus CpG group, 1 mouse of 8 was isolation positive, presenting a value close to the detection limit of 20 PFU/3 ganglia (\( P = 0.01 \)). There was a significant difference (\( P = 0.01 \)) between the mgG-2 plus CpG group and controls. For the spinal cords, significantly lower levels of infectious virus were detected for the mgG-2 plus CpG group (mean value, 109 PFU/70 mg spinal cord) than for the mgG-2 group (mean value, 821 PFU/70 mg spinal cord; \( P = 0.004 \)) or controls (mean value, 2,314 PFU/70 mg spinal cord; \( P <
HSV-2 could not be isolated from brain tissue collected from any of the groups (data not shown). Viral DNA was also quantified in DRG and spinal cord from six surviving mice in the mgG-2 and mgG-2 plus CpG groups at day 21 p.i. A low DNA copy number per ganglion (close to the detection limit) was found in DRG from 3/6 (mean value, 120) and 4/6 (mean value, 36) mice in the mgG-2 and mgG-2 plus CpG groups, respectively. For the spinal cord, 5/6 mice from the mgG-2 group were positive (mean value, 930), compared with 4/6 mice (mean value, 73) from the mgG-2 plus CpG group (data not shown).

We also quantified the viral load in serum at day 6 p.i. by real-time PCR. Results are presented in Fig. 6D. The viral load in sera from the mgG-2 plus CpG group (mean value, 907 DNA copies/ml) was significantly lower than that in sera from the mgG-2 group (mean value, 2,554 DNA copies/ml; \( P = 0.04 \)) or controls (mean value, 6,898 DNA copies/ml; \( P = 0.0001 \)).

Vaginal IFN-γ response in immunized mice after challenge. Since protection in the mgG-2 plus CpG group correlated to IFN-γ production in CD4+ T cells, we also measured IFN-γ in vaginal washes collected at day 1 p.i. As shown in Fig. 7, the IFN-γ levels for the mgG-2 plus CpG group were significantly higher (mean value, 235; \( P = 0.05 \)) than those for the mgG-2 group (mean value, 89) and controls immunized with PBS or CpG alone, in which the values for all except one of the mice were below the detection limit.

Lack of neutralization capacity. As antibody-mediated NT has been considered important for protection against certain viruses after natural infection or after immunization, we investigated NT activity. Immune sera collected from mice belonging to the mgG-2 (\( n = 5 \)) and the mgG-2 plus CpG (\( n = 11 \)) groups were tested. None of the immune sera presented NT activity alone or in the presence of complement (titers \( < 1/20 \)) (Table 1).

Antibody-dependent cell-mediated cytotoxicity. Since MP as well as NK cells can mediate ADCC, we used enriched fractions of these cells as effectors in ADCC assays (MP-ADCC and NK-ADCC). Eleven of 19 serum samples (58%) collected from mice immunized with mgG-2 plus CpG showed MP-ADCC, and 4 of 9 serum samples (44%) showed NK-ADCC (Table 1). We also investigated if there was a correlation between IgG antibody response and ADCC by comparison of the magnitude of anti-mgG-2-specific IgG, IgG1, and IgG2c titers for sera presenting positive or negative ADCC. Sera which presented a positive MP-ADCC
showed significantly higher IgG titers (\( P = 0.02 \)) and IgG2c titers (\( P = 0.012 \)) than MP-ADCC-negative sera. There were no significant differences in IgG1 titers between MP-ADCC-positive and negative sera or between total IgG or subclass titers and NK-ADCC (data not shown).

![FIG 6](http://jvi.asm.org/) Significant reductions of viral load in vaginal washes, spinal cord, DRG, and serum. Viral loads are expressed as infectious HSV-2 (PFU) in vaginal washes at day 3 p.i. (data are from four experiments) (A). PFU were detected at day 6 p.i. in lumbosacral ganglia (data are from one experiment) (B) and spinal cord (two experiments) (C). HSV-2 DNA was detected in serum at day 6 p.i. by quantitative PCR (data are from two experiments) (D). Detection limits of 20 PFU/ml for vaginal washes, 7 PFU/ganglion, 20 PFU/70 mg spinal cord, and 50 DNA copies/ml for serum are indicated with dashed lines. All samples presenting negative results are plotted in groups below the detection limit. Arithmetic mean values (solid lines) ± SEM (dashed lines) are indicated in panels A, C, and D.

![FIG 7](http://jvi.asm.org/) Higher levels of IFN-\( \gamma \) in vaginal washes from mice immunized with mgG-2 plus CpG. IFN-\( \gamma \) was analyzed in vaginal washes at day 1 p.i. Results are from one experiment and presented as pg/ml. The detection limit of 10 pg/ml is indicated with a dashed line.

**TABLE 1** Results from serological assays for sera from mice immunized with mgG-2, mgG-2 plus CpG, or PBS (negative sera)

| Method      | mgG-2 | mgG-2+CpG | PBS |
|-------------|-------|-----------|-----|
| Neutralization* | 0/5   | 0/11      | 0/3 |
| MP-ADCC<sup>b</sup> | ND<sup>d</sup> | 11/19 | 0/2 |
| NK-ADCC<sup>c</sup> | ND   | 4/9       | 0/2 |
| ACMC<sup>c</sup> | 3/10 | 8/12      | 0/2 |

* An NT titer of \( \geq 20 \) was considered a positive result. Sera were tested alone and by addition of complement.

**<sup>b</sup>** Macrophage-mediated ADCC (MP-ADCC) and NK cell-mediated ADCC (NK-ADCC) titers of 20 were considered positive.

**<sup>c</sup>** Sera showing an antibody-dependent complement-mediated cytolysis (ACMC) titer of \( \geq 25 \) were considered positive.

**<sup>d</sup>** ND, not done.
Antibody-dependent complement-mediated cytolysis. We investigated if sera from immunized mice could mediate ACMC. Sera were tested at 2-fold dilutions starting at 1:25. Three of 10 serum samples collected from the mgG-2 group and 8/12 serum samples collected from the mgG-2 plus CpG group were positive (Table 1). All positive samples from both immunized groups showed titers of between 25 and 100.

**DISCUSSION**

We show that a combined systemic and mucosal immunization with mgG-2 plus CpG could elicit significant protection against vaginal challenge with an otherwise lethal dose of a fully virulent HSV-2 strain in mice. The observed protection induced by immunization with mgG-2 plus CpG was found to be associated with IFN-γ production from CD4+ T cells and by higher levels of IFN-γ in the vaginal washes at day 1 p.i. The importance of IFN-γ in the protection induced by immunization with mgG-2 plus CpG was further documented, in that immunization with mgG-2 plus CpG failed to elicit any appreciable level of protection in IFN-γ gene-knockout mice. Several studies using the murine model have shown that CD4+ T cells and IFN-γ production play important roles in protection against genital infection with HSV-2 (39, 47–49). Previous studies using a live attenuated thymidine kinase-deficient HSV-2 strain for immunization followed by a subsequent IVAG challenge have also shown that IFN-γ-secreting CD4+ T cells are crucial for protection (23, 24, 44, 49). Furthermore, vaccinated CD4+ T-cell-deficient mice, which rapidly die after a genital challenge, were shown to be rescued by treatment with exogenous IFN-γ (22).

Vaccination with mgG-2 alone or mgG-2 plus CpG significantly reduced the viral load in vaginal washes compared with that in controls. Although there was no significant difference in the numbers of infectious HSV-2 particles in the vaginal washes between the vaccinated groups, the significant reduction of HSV DNA in serum detected for mice immunized with mgG-2 plus CpG suggests that the overall viral replication was reduced. IFN-γ production locally has been shown to be effective at reduction of the infection in the genital tract. It is therefore reasonable to assume that the higher levels of IFN-γ detected in the vaginal washes in the mgG-2 plus CpG group contributed to clearance of the infection more efficiently. An important issue in HSV-2 vaccine development is to achieve protection from infection of the DRG, spinal cord, and CNS. The increased survival rate and the lower disease scores for mice immunized with mgG-2 plus CpG were associated with significantly lower titers of HSV-2 in the spinal cord. **In vitro** studies using an mgG-2-negative HSV-2 mutant described that mgG-2 is involved in the extracellular release of infectious particles (1). Our data presented here suggest that the induced immune responses could suppress infectious HSV-2 from infecting neurons. However, IFN-γ has been shown to inhibit viral replication in the nervous system via induction of nitric oxide synthase, leading to production of nitric oxide (10), and IFN-γ-secreting CD4+ T cells are able to clear HSV-1 from sensory ganglia and spinal cord in a murine model (27). An alternative explanation to the reduced viral load in the nervous system tissue described here might be that locally produced IFN-γ can clear infectious HSV-2 particles of already infected neurons.

Several studies using B-cell-knockout mice challenged with a lethal dose of wild-type HSV-2 IVAG have shown that antibodies have a limiting effect in early stages of the vaginal infection (<24 h), with a reduced local viral load (16, 22, 40, 46). mgG-2 induced a high specific IgG antibody response both alone and when used together with CpG, albeit with an IgG2c subclass bias when mgG-2 was used together with CpG. The majority of sera from mice immunized with mgG-2 plus CpG exerted MP-ADCC activity, and the activity correlated with the IgG2c titers. It is well-known that the mouse IgG2 subclass binds to FcγRI more efficiently than IgG1 (50). Protection induced by passive transfer of anti-HSV MAbs or immune sera in mice was shown to correlate with Fcγ-dependent mechanisms, such as ADCC (2, 11, 25). As a single mgG-2 MAb was earlier shown to present low ADCC activity (2), four of our own produced anti-mgG-2 MAbs (34) were tested. They were all negative (unpublished observation). The discrepancy between the ADCC function of immune sera from vaccinated mice and the MAbs may be explained by the fact that all MAbs tested are of the IgG1 subclass. Furthermore, it was stated that the relatively poor efficacy observed in clinical HSV-2 vaccine trials, using gD and gB as immunization antigens, may have partially been the result of failure to elicit high levels of antibodies capable of mediating ADCC (31). Taken together, ADCC is a potentially important mechanism of antibodies in the control of the HSV infection.

Another interesting finding was that the immune sera presented no NT activity. Although there is no clear correlation between protection and levels of NT antibodies in either humans or animal models, this study is to our knowledge the first to report that it is possible to induce protection without NT activity of serum. Two anti-mgG-2 MAbs and a rabbit hyperimmune serum have also been shown earlier to be devoid of neutralizing activity (2, 4, 43). Furthermore, our own produced anti-mgG-2 MAbs exerted no NT activity with or without complement in GMK-AH1 cells (unpublished observation). The neutralizing antibody response has previously been used as the only surrogate of antibody-mediated protection in animal and human trials. The lack of NT activity of anti-mgG-2 antibodies may explain why mgG-2 has not been evaluated earlier as a vaccine candidate. However, clones from a random phage peptide display library obtained by using an anti-mgG-2 MAb (34) were used as immunogens and administered subcutaneously in a vaccination model in mice. The selected phage clones, which contained short stretches of mgG-2 (3 to 4 amino acids), induced partial protection (≤60%) against challenge with HSV-2 (21).

Taken together, we report for the first time that a combined systemic and mucosal immunization with mgG-2 plus CpG can generate considerable protective immunity to a lethal vaginal challenge with a fully virulent HSV-2 strain in mice. The protection was found to be associated with the IFN-γ response by CD4+ T cells and in the vagina. These observations encourage further evaluation of mgG-2 as a vaccine candidate for induction of protective immunity to HSV-2-induced infection or disease.

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