Mucosal Administration of CpG Oligodeoxynucleotide Elicits Strong CC and CXC Chemokine Responses in the Vagina and Serves as a Potent Th1-Tilting Adjuvant for Recombinant gD2 Protein Vaccination against Genital Herpes

Sara Tengvall,¹ Annika Lundqvist,¹ Roselyn J. Eisenberg,² Gary H. Cohen,² and Ali M. Harandi^{1*}

Institute of Biomedicine, Department of Microbiology and Immunology, Sahlgrenska Academy at Göteborg University, Medicinaregatan 7A, Box 435, 413 46 Göteborg, Sweden,¹ and Department of Microbiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania²

Received 21 September 2005/Accepted 15 February 2006

Although sexually transmitted pathogens are capable of inducing pathogen-specific immune responses, vaginal administration of nonreplicating antigens elicits only weak, nondisseminating immune responses. The present study was undertaken to examine the potential of CpG-containing oligodeoxynucleotide (CpG ODN) for induction of chemokine responses in the genital tract mucosa and also as a vaginal adjuvant in combination with glycoprotein D of herpes simplex virus type 2 (HSV-2) for induction of antigen-specific immune responses. We found that a single intravaginal administration of CpG ODN in mice stimulates a rapid and potent response of CC chemokines macrophage inflammatory protein 1 α (MIP-1 α), MIP-1 β , and RANTES as well as of CXC chemokines MIP-2 and IP-10 in the vagina and/or the genital lymph nodes. Importantly, intravaginal vaccination with recombinant gD2 in combination with CpG ODN gave rise to a strong antigen-specific Th1-like immune response in the genital lymph nodes as well as the spleens of the vaccinated mice. Further, such an immunization scheme conferred both systemic and mucosal immunoglobulin G antibody responses as well as protection against an otherwise lethal vaginal challenge with HSV-2. These results illustrate the potential of CpG ODN for induction of potent chemokine responses in the genital tract and also as a vaginal adjuvant for generation of Th1-type mucosal and systemic immune responses towards a nonreplicating antigen derived from a sexually transmitted pathogen. These data have implications for the development of a mucosal vaccine against genital herpes and possibly other sexually transmitted diseases.

Development of mucosal vaccines for the female reproductive tract may have profound implications for the prevention of sexually transmitted diseases. Despite numerous efforts, no such vaccines are currently available, mainly due to lack of information on how best to activate a protective immune response in the female genital tract mucosa. The female genital tract is a part of the common mucosal immune system that has some unique characteristics. The female genital tract lacks organized lymphoepithelial structures resembling intestinal Peyer's patches where mucosal immune responses are initiated and disseminated to distant effector sites. Further, many aspects, including the distribution and properties of immunocompetent cells, the proportions of immunoglobulin (Ig) isotypes, and the permeability of the genital tract epithelium to proteins, are changed during the course of the estrus cycle. Whereas sexually transmitted pathogens, such as herpes simplex virus type 2 (HSV-2), induce pathogen-specific immune responses, local administration of nonreplicating antigens into the vagina elicits only modest, nondisseminating immune responses (16, 22, 23).

HSV-2 infects human genital tract mucosa and is the leading cause of genital ulcers worldwide (10, 27). Over the last decades, there have been numerous efforts to develop vaccines against genital herpes infection and disease. Several candidate vaccines involving inactivated viruses, protein subunit, recombinant glycoprotein, plasmid DNA, and attenuated live viruses have been tested experimentally in animals and found to confer variable levels of protection against genital herpes (11). Several protein subunit vaccines based on HSV-2 envelope glycoproteins have reached advanced-phase clinical trials. Recent such studies showed that systemic vaccination with recombinant HSV-2 glycoproteins B and D in MF59, an oil-in-water emulsion adjuvant, failed to confer protection against genital HSV-2 infection or disease (26). However, more-recent studies reported that systemic vaccination with gD2 formulated with alum-monophosphoryl lipid yielded limited protection but only in women who had never been infected with either of two HSV serotypes (25). Therefore, there remains a great need to develop an effective mucosal vaccine against genital herpes capable of eliciting more-potent immune effector and, hence, protective responses while remaining well tolerated.

Several recent reports have shown that synthetic oligodeoxynucleotides (ODNs) containing unmethylated cytidine-phosphate-guanosine with appropriate flanking regions (CpG motif), which act through activation of the Toll-like receptor 9 signaling pathway, serve as potent inducers of innate and ac-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Sahlgrenska Academy at Göteborg University Vaccine Research Institute (GUVAX), Göteborg University, Medicinaregatan 7A, Box 435, 413 46 Göteborg, Sweden. Phone: 46 31 7736229. Fax: 46 31 7736210. E-mail: ali.harandi@microbio.gu.se.

quired immune responses in both systemic and mucosal compartments (7, 12). We have recently demonstrated that vaginal administration of CpG-containing ODN (CpG ODN) triggers production of Th1-type cytokines gamma interferon (IFN- γ), interleukin-12 (IL-12), and IL-18 in the vaginal mucosa and/or the genital lymph nodes (gLN). Further, such treatment led to a dramatic increase in the total numbers of B cells, NK cells, NKT cells, and T cells in the genital lymph nodes (6). Interestingly, some of these immune components were demonstrated to play a significant role in innate immune protection against genital herpes (9, 20). We and others have shown that pretreatment of mice with a single vaginal dose of CpG ODN, without any exogenous viral antigen codelivery, affords significant innate immune protection against primary genital herpes infection and disease (1, 6, 21). The observed protection was found to induce the development of an HSV-2-specific memory response affording sterilizing immunity against reinfection (6).

Immunity against pathogens is orchestrated in part by the ordered release of different chemokines that act as chemoattractants and activators of various immune cells. The chemokine superfamily is divided into four subfamilies, CXC, CC, CX₃C, and C, based on the number and relative position of conserved cysteine residues (30). Of the different chemokines studied to date, members of the CC and CXC chemokine subfamilies appeared to have the greatest effects on the recruitment of various immune cells, such as natural killer (NK) cells, polymorphonuclear cells, dendritic cells, macrophages, and lymphocytes, to the site of vaccination/infection. Thus, the CC chemokine RANTES (regulated on activation, normal T cell expressed and secreted) is produced mainly by epithelial cells, endothelial cells, fibroblasts, and T lymphocytes, and by binding to CCR1, CCR3, or CCR5, it chemoattracts T cells, monocytes, NK cells, and dendritic cells. Other members of the CC chemokine subfamily are macrophage inflammatory protein 1 α (MIP-1 α) and MIP-1 β , which can induce the influx of NK cells, macrophages, and immature dendritic cells (14). Recent studies have shown that these CC chemokines are important factors in the development of a Th1-type immune response (24). The CXC chemokines IFN- γ -inducible protein 10 (IP-10) and MIP-2 are produced by a variety of cell types, including fibroblasts, endothelial cells, and mononuclear cells, and chemoattract monocytes and T cells (for IP-10) and neutrophils (for MIP-2) to the site of vaccination/infection (14).

The present study was undertaken to examine the impact of vaginally administered CpG ODN for the induction of CC and CXC chemokine responses in the female genital tract mucosae and the draining lymph nodes of inbred and outbred mice. Further, we investigated the efficacy of CpG ODN as a mucosal adjuvant coadministered with recombinant HSV-2 gD protein for induction of humoral and cell-mediated immunity both in the female genital tract mucosa and in the spleen and asked if such a vaccination scheme could elicit protective immunity against genital herpes in mice.

MATERIALS AND METHODS

Mice. Six- to 8-week-old female C57BL/6 (M&B) or NMRI (Charles River) mice were kept in ventilated cages under specific-pathogen-free conditions at the EBM Animal Facility, Sahlgrenska Academy, Göteborg University. All experi-

ments were carried out with the approval of the Ethical Committee for Animal Experimentation in Göteborg, Sweden. The studies were performed twice.

ODNs. The CpG ODN used in this study was 1826 (TCC ATG ACG TTC CTG ACG TT), a 20-mer containing two copies of a CpG motif known to have potent immunostimulatory effects on the murine immune system (29). The control ODN was TCC ATG ACG TTC CTG ACG TT, a 20-mer containing no CpG motif. All ODNs were used with a complete phosphorothioate backbone. The ODNs were purchased from Operon Biotechnologies GmbH (Germany).

CpG ODN administration and tissue saponin extraction. In the first study, 60 μ g of CpG ODN was administered intravaginally to groups of 33 mice. Three mice were sacrificed at different times after CpG ODN administration, and the vaginas and gLNs were excised and weighed before storage at -20°C in a phosphate-buffered saline (PBS) solution containing 2 mM phenylmethylsulfonyl fluoride, 0.1 mg of soybean trypsin inhibitor (Sigma) per ml, and 0.05 mM EDTA. The tissue samples were thawed and then permeabilized with saponin (Sigma) at a final concentration of 2% (wt/vol) in PBS at 4°C overnight. Tissue samples were centrifuged at 13,000 rpm for 10 min and supernatants collected for further analyses.

Production and purification of recombinant gD2. Sf9 (*Spodoptera frugiperda*) cells (Gibco BRL) were grown in Sf900II medium (Gibco BRL), infected with recombinant baculovirus expressing gD2 (306t) (28) at a multiplicity of infection of 4, and cultured at 27°C in a Celligen bioreactor (New Brunswick Scientific) for 48 h. Cells were removed by centrifugation, and the clarified medium was concentrated with a Pellicon cassette filter (Millipore) with a 10,000-molecular-weight cutoff. The concentrated supernatant was then exchanged with PBS. The exchanged medium was passed over a DL6 immunosorbent column and eluted as previously described (19). gD2 (306t) proteins were concentrated, dialyzed against PBS, and stored at -80°C .

Vaccination study. For mucosal vaccination studies, groups of 8 to 10 female C57BL/6 and NMRI mice were injected subcutaneously with 3.0 mg of Depo-Provera (Upjohn s.a., Puurs, Belgium) in PBS. Six days later, some of the mice were immunized with CpG ODN (30 μ g), recombinant gD2 (5 μ g), gD2 (5 μ g) plus CpG ODN (30 μ g), or gD2 (5 μ g) plus control ODN (30 μ g). All immunizations were performed once or twice at 10-day intervals. The studies were performed twice.

Proliferation assay. CD11c⁺ cells were purified from spleen cells of naïve mice by using MACS beads from Miltenyi Biotec (Gladbach, Germany) according to the manufacturer's recommendations and cultured overnight in the presence of recombinant gD2. The CD11c⁺ cells (10^4 /well) were cocultured with splenocytes, gLN cells, and splenic CD4⁺ T cells (purified by using MACS beads [Miltenyi Biotec, Gladbach, Germany]) isolated from the vaccinated animals 3 to 4 weeks after the last vaccination. Cells were seeded in triplicate wells of 96-well flat-bottomed plates, at a concentration of 1×10^5 cells per well in Iscove's medium supplemented with L-glutamine, 50 μ M 2-mercaptoethanol, gentamicin, and 10% fetal calf serum, and incubated at 37°C . After 96 h of incubation, culture supernatants were collected and assayed for cytokine contents. On day 4, cells were pulsed with 1 μ Ci of [^3H]thymidine (Amersham Pharmacia) for the last 6 h of incubation. The cellular DNA was harvested on glass-fiber filters and then assayed by liquid scintillation counting. Data are expressed as the mean counts per minute.

Cytokine and chemokine quantification. Concentrations of cytokines and chemokines in the tissue and/or cell culture supernatants were determined by using Dousset chemokine enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Abingdon, United Kingdom) according to the manufacturer's recommendations.

Analysis of antibody response. Maxisorp 96-well plates (Nunc) were coated with 100 μ l of recombinant gD2 (306t), diluted to a final concentration of 5 μ g/ml, in 50 mM carbonate buffer (pH 9.6) for 4 h at room temperature, followed by overnight incubation at 4°C . The plates were blocked with 2% bovine serum albumin in PBS for 30 min at 37°C . Serial dilutions of sera or vaginal extracts obtained 3 to 4 weeks after vaccination from the different vaccination groups were incubated for 1 h at 37°C . After being washed with 0.05% Tween 20, the plates were incubated for another 1 h at 37°C with horseradish peroxidase-conjugated goat anti-mouse IgA, IgG, IgG1 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), or IgG2c (Research Diagnostic, Inc., Flanders, NJ). The plates were then washed with 0.05% Tween 20 and developed using 100 μ l of 1 mg/ml o-phenylenediamine dihydrochloride (Sigma) in 0.1 M citrate buffer (pH 4.5) containing 0.04% H_2O_2 . After 10 to 50 min of incubation at room temperature, the absorbance was read at 450 nm.

Virus and virus challenge. HSV-2 strain 333 was grown in monolayers of African green monkey kidney cells (GMK-AH1) and prepared by one cycle of freezing and thawing followed by removal of cellular debris by centrifugation, and virus titers were determined. For virus challenge experiments, groups of five

FIG. 1. Time course of CC and CXC chemokine production in response to intravaginal CpG ODN administration. C57BL/6 mice were injected subcutaneously with 3.0 mg of Depo-Provera. Six days later (day 0), the mice were inoculated vaginally with CpG ODN 1826 (60 μ g per mouse). The vaginas (A) and the gLNs (B) were excised, and saponin was extracted at the indicated time points (two to four mice per time point). The tissue extracts were analyzed for their MIP-1 α , MIP-1 β , IP-10, and MIP-2 contents by ELISA. The data are expressed as increases (*n*-fold) over levels for naïve mice. The peak levels of chemokines were significantly higher than those of the respective control groups at *P* of <0.01 for vaginal MIP-1 α and MIP-1 β as well as gLN MIP-1 α and at *P* of <0.05 for the others by the Student *t* test.

to six vaccinated as well as naïve mice were injected subcutaneously with 3.0 mg of Depo-Provera (Upjohn s.a., Puurs, Belgium) in PBS. Six days later, the mice were anesthetized with isoflurane (Baxter Medical AB) and challenged by an intravaginal administration of 9×10^4 PFU (200 50% lethal doses [LD₅₀]) of HSV-2 strain 333 in 10 μ l of Hank's balanced salt solution.

Monitoring of infection. (i) Virus replication. Following intravaginal HSV-2 challenge, vaginal fluids were collected by pipetting 40 μ l of sterile Hank's balanced salt solution in and out of the vagina until a discrete clump of mucus was retrieved. A second wash was then performed. The two washes were pooled and stored at -70°C . HSV-2 titers were determined by plaque assay of GMK-AH1 cell monolayers by using the standard method.

(ii) Inflammation and disease. Mice were examined daily for vaginal inflammation, neurological illness, and death after HSV-2 challenge. The severity of the disease was graded as healthy (score of 0), genital erythema (score of 1), moderate genital inflammation (score of 2), severe and purulent genital lesions (score of 3), hind limb paralysis (score of 4), and death or sacrifice due to paralysis (score of 5).

Statistical analyses. The Student *t* test was used for statistical analyses with Graph Pad Prism 4 software (Calif.).

RESULTS

Mucosal administration of CpG ODN induces CC and CXC chemokine responses in the vagina and the draining lymph nodes of C57BL/6 mice. Given the importance of CC and CXC chemokines in the development of immune responses, we sought to examine if a mucosal administration of CpG ODN can elicit production of CC chemokines MIP-1 α and MIP-1 β and CXC chemokines IP-10 and MIP-2 in the vagina and the draining gLNs. To this end, the chemokine levels were measured at various intervals after a single administration of CpG ODN in the vagina and the gLNs. Low levels of MIP-1 α and MIP-1 β were detected in the vagina and the gLNs of naïve C57BL/6 mice. The vaginal level of MIP-1 α increased 100-fold over levels in the control animals within 8 h after CpG ODN administration, declined to 15-fold within 48 h, and

FIG. 2. Kinetics of vaginal CC and CXC chemokine production after vaginal CpG ODN administration in outbred NMRI mice. The data are expressed as increases (n -fold) in concentrations of RANTES, MIP-1 α , MIP-1 β , IP-10, and MIP-2 over those for naïve mice. The experiment was performed as described in the legend for Fig. 1. The peak levels of chemokines were significantly higher than those of the respective control groups at P of <0.01 for RANTES and MIP-1 β and at P of <0.05 for MIP-1 α by the Student t test.

stayed at this level for up to 14 days. The MIP-1 α level in gLN extracts rose 52-fold over that of naïve mice within 8 h and then decreased within 48 h (5-fold) (Fig. 1B). Likewise, the vaginal level of MIP-1 β increased 110-fold, remained high for 24 h (70-fold), and waned by day 14 (3- to 14-fold) (Fig. 1A). The gLN levels of MIP-1 β increased 19-fold by 8 h and decreased by day 2 (2-fold), followed by a second increase after 3 to 4 days (5-fold) (Fig. 1B).

The vaginal and gLN levels of CXC chemokines IP-10 and MIP-2 were very low in untreated C57BL/6 mice. The vaginal level of IP-10 rose 12-fold within 8 h, declined to 4-fold by day 2, and remained at this level for 14 days (Fig. 1A). Interestingly, the IP-10 levels in gLN extracts showed a 27-fold increase within 8 h but declined rapidly to 2-fold by day 2 (Fig. 1B). The level of MIP-2 in the vagina increased 26-fold within 8 h and then waned by day 4, followed by a second smaller wave of MIP-2 production on day 5 (Fig. 1A). The level of MIP-2 in the gLN slightly increased within 8 h (threefold) and then declined to the basal level followed by a second peak on day 8 (threefold) (Fig. 1B). Thus, mucosal administration of CpG ODN stimulates a rapid and potent rise in CC and CXC chemokine levels in both the vagina and the regional lymph nodes.

Mucosal administration of CpG ODN induces CC and CXC chemokine responses in the vaginas of outbred mice. Having shown that a vaginal administration of CpG ODN induces potent CC and CXC chemokine responses in the genital tracts of inbred C57BL/6 mice, we next asked whether CpG ODN could induce production of the CC and CXC chemokines in the vaginas of outbred mice. To this end, we determined the levels of the CC and CXC chemokines in the vaginas and the gLNs of NMRI mice at various intervals after a single vaginal administration of CpG ODN. The level of RANTES in the vagina increased 15-fold over that of naïve mice within 4 h, peaked at 65-fold by 8 h, and then declined to 25-fold by day 2. A second peak in the vaginal level of RANTES was observed on day 4 (45-fold), but that level waned within 14 days (Fig. 2). The level of MIP-1 α rose by 2 h, peaked at 1 day (15-fold), and then gradually declined. The level of MIP-1 β in the vagina

displayed a trend comparable to that of MIP-1 α (Fig. 2). The levels of IP-10 and MIP-2 increased within 8 h (3.5- and 4-fold, respectively) and then decreased to the levels observed for the naïve control mice (Fig. 2). While the level of RANTES in the gLNs of NMRI mice rose 10-fold within 8 h and remained high for 24 h (data not shown), the gLN levels of other chemokines remained unaltered throughout the course of the experiment. Hence, vaginal administration of CpG ODN stimulates rapid production of RANTES, MIP-1 α , MIP-1 β , IP-10, and MIP-2 in the female genital tract mucosae of outbred mice.

Vaginal vaccination with HSV-2 gD protein plus CpG ODN elicits antigen-specific mucosal and systemic immune responses. Vaginal immunization with nonreplicating antigen was shown to induce poor local and systemic immune responses (16). Having shown that CpG ODN can elicit a potent Th1 cytokine and chemokine milieu in the vagina and the draining lymph nodes, we next asked whether coadministration of CpG ODN with recombinant gD2 protein could elicit mucosal and systemic immune responses. To this end, female C57BL/6 mice were immunized vaginally twice at 10-day intervals with CpG ODN, gD2, gD2 plus CpG ODN, or gD2 plus control ODN. Four weeks after the last immunization, humoral and cell-mediated immune responses in gLNs, vaginas, and spleens were analyzed.

We first examined the impact of CpG ODN on the induction of a gD-specific T-cell response. To this end, CD11c⁺ dendritic cells pulsed with gD2 protein were cocultured with either gLN or splenic CD4⁺ T cells purified from the vaccinated mice. The gD-specific proliferative responses as well as the gD-specific production of IFN- γ and MIP-1 β were analyzed after 96 h in gLN and CD4⁺ T-cell culture supernatants by use of ELISA. As expected, the gLNs from naïve control and CpG ODN-vaccinated mice did not show any gD-specific proliferative response (Table 1). The gLNs isolated from the gD-vaccinated group showed a moderate level of gD-specific proliferation. However, the gD plus CpG ODN group displayed significantly higher proliferation levels than those displayed by the groups that received gD alone ($P < 0.001$). The gLN cells from naïve and CpG ODN-vaccinated mice produced no detectable levels

TABLE 1. gD-specific proliferative and cytokine responses in splenic CD4⁺ T cells and gLN cells isolated from vaginally vaccinated mice^a

Control group or vaccine	Result for splenic CD4 ⁺ cells			Result for gLN cells		
	Proliferation (cpm)	IFN- γ (pg/ml/10 ⁶ cells)	MIP-1 β (pg/ml/10 ⁶ cells)	Proliferation (cpm)	IFN- γ (pg/ml/10 ⁶ cells)	MIP-1 β (pg/ml/10 ⁶ cells)
Control	12,876 $\pm$ 4,627	UD	1,675 $\pm$ 49	1,567 $\pm$ 1,301	UD	2,930 $\pm$ 71
CpG ODN	588 $\pm$ 169	UD	545 $\pm$ 49	193 $\pm$ 47	UD	130 $\pm$ 0
gD	20,768 $\pm$ 4,661	UD	4,490 $\pm$ 311	12,431 $\pm$ 3,529	250 $\pm$ 14	1,430 $\pm$ 99
gD plus CpG ODN	63,844 $\pm$ 7,741**	8,880 $\pm$ 198**	10,269 $\pm$ 594**	36,795 $\pm$ 8,176**	1,495 $\pm$ 35**	5,360 $\pm$ 14*

^a Splenic CD4⁺ T cells or gLN cells (10⁶ cells/ml) isolated from C57BL/6 mice immunized intravaginally with CpG ODN, gD, or gD plus CpG ODN were cocultured with CD11c⁺ cells (10⁵ cells/ml) pulsed with recombinant gD for 96 h. The data are expressed as means $\pm$ standard deviations. UD, undetectable. *, $P < 0.01$; **, $P < 0.001$ (statistical significance as determined by the Student *t* test, compared with results for the gD group).

of IFN- γ . A low gD-specific IFN- γ response was detected in the culture of the gLNs from the gD-vaccinated group. In contrast, the gD-plus-CpG-vaccinated group showed a sixfold increase in IFN- γ production over that of the gD2 group ($P < 0.001$). Similarly, gLNs from the gD-plus-CpG-vaccinated group produced significantly higher levels of MIP-1 β than the gD-vaccinated group (Table 1).

We next tested the impact of mucosal vaccinations with gD or gD plus CpG ODN on induction of cell-mediated immune responses in the splenic CD4⁺ T cells. Mice vaccinated with CpG alone showed no changes. Splenic CD4⁺ T cells from the gD plus CpG ODN group developed significant proliferative and IFN- γ responses, whereas these responses were not detected in CD4⁺ T cells of mice from the gD group. The splenic CD4⁺ T cells from the gD-vaccinated group produced an intermediate level of MIP-1 β . Likewise, MIP-1 β production was significantly greater in the gD-plus-CpG-vaccinated group than in the gD group (Table 1).

Finally, we examined the levels of gD-specific IgA and/or IgG in the sera and saponin-extracted vaginas of vaccinated mice. Interestingly, only the gD plus CpG ODN group had significant levels of gD-specific IgG in their sera and vaginas (Fig. 3A). We also tested for IgG1 (an indicator of Th2 response) and IgG2c (an indicator of Th1 response in C57BL/6 mice) (15) isotypes in the sera of vaccinated animals. As shown in Fig. 3B, low levels of IgG1 and IgG2c were detected in the gD-vaccinated as well as the gD-plus-control ODN-vaccinated mice. In contrast, the gD-plus-CpG ODN-vaccinated group displayed considerable amounts of IgG1 and IgG2c, with IgG2c being the predominant isotype (Fig. 3B). However, none of the vaccinated groups had any detectable level of IgA in their vaginal extracts (data not shown). Altogether, these results imply that vaginal immunization of mice with gD and CpG ODN elicits Th1-skewed mucosal and systemic immune responses.

Mucosal vaccination with HSV-2 gD protein plus CpG ODN confers protection against genital herpes in both inbred and outbred mice. Given the potential of vaginal CpG ODN administration, without any viral antigen coadministration, in inducing strong innate immune protection against genital herpes (1, 6), we examined whether a gD plus CpG ODN vaccination protocol could confer an acquired protective immunity against a subsequent vaginal challenge with a lethal dose of HSV-2. Therefore, 3 to 4 weeks after the last immunization, groups of vaccinated mice were intravaginally challenged with 200 LD₅₀ (9×10^4 PFU) of HSV-2 strain 333. Following the virus challenge, vaginal titers of virus as well as the outcome of

disease were examined. Three days after viral challenge, all mice except those inoculated with gD plus CpG ODN showed significant levels of viral replication in the vagina (Fig. 4A). Likewise, all animals except those given gD plus CpG ODN developed a rapidly progressing disease requiring euthanization within 9 to 10 days after challenge (Fig. 4B). The gD plus CpG group, on the other hand, had very low titers of HSV-2 in their vaginal fluids ($P < 0.01$), and 80% of the animals were protected against HSV-2 challenge (Fig. 4A and C).

Next, we addressed the question of whether the gD plus CpG ODN vaccination protocol could confer protection against a subsequent HSV-2 challenge in outbred mice. To this

FIG. 3. gD-specific IgG antibody response in vaginally vaccinated mice. Groups of female C57BL/6 mice ($n = 5$) were intravaginally vaccinated two times, 10 days apart, with CpG ODN, gD, gD plus CpG ODN, or gD plus control ODN. Four weeks after the last immunization, the mice were sacrificed and the gD-specific IgG levels in their sera and their vaginal extracts (A) as well as IgG1 and IgG2c antibody levels in their sera (B) were determined using an HSV-2 gD-specific ELISA. The data are expressed as the log₁₀ means $\pm$ standard errors of the means of the optical density (OD) values of 1 of 10 prediluted samples measured at 450 nm. ND, not done.

FIG. 4. Mucosal vaccination of C57BL/6 mice with gD plus CpG ODN confers strong protection against genital herpes. Groups of female C57BL/6 mice were intravaginally vaccinated twice, 10 days apart, with CpG ODN, gD, gD plus CpG ODN, or gD plus control ODN. Four weeks after the last vaccination, the mice were challenged vaginally with a lethal dose of HSV-2. The animals were examined for vaginal viral titer on day 3 (A). The data are expressed as means + standard errors of the means of virus loads (PFU/sample) from groups of 7 to 10 mice. The virus titers for mice immunized with gD plus CpG ODN were significantly lower, as determined by Student's *t* test, than those of the gD group (**, $P < 0.01$). Animals were monitored daily for survival (B) and signs of disease (C). The results are pooled from two independent experiments.

end, groups of NMRI mice were immunized vaginally twice with CpG ODN, gD, or gD plus CpG ODN. Four weeks after the last immunization, the immunized mice and the naïve control mice were challenged with 200 LD₅₀ (9×10^4 PFU) of HSV-2 strain 333. As depicted in Fig. 5, all control mice and mice immunized with CpG ODN showed significant levels of

FIG. 5. Vaginal vaccination with gD plus CpG ODN elicits protection against genital herpes in outbred NMRI mice. Groups of female NMRI mice ($n = 5$) were intravaginally immunized twice, 10 days apart, with CpG ODN, gD, or gD plus CpG ODN. Four weeks after the last vaccination, the mice were challenged vaginally with a lethal dose of HSV-2. The animals were examined for vaginal viral titer on day 3 (A). The results are expressed as means + standard errors of the means of virus loads (PFU/sample). *, $P < 0.5$ by Student's *t* test, compared with results for the gD group. Animals were monitored daily for survival (B) and signs of disease (C).

HSV-2 in their genital secretions, rapidly developed signs of the disease, and died. Mice immunized with gD plus CpG ODN showed significantly lower vaginal viral titers than gD-immunized mice ($P < 0.05$) (Fig. 5A). Further, the gD plus CpG ODN group showed higher survival and lower pathology

scores following vaginal HSV-2 challenge than mice immunized with gD alone (Fig. 5B and C). Taken together, these data imply that double vaginal immunization of mice with gD plus CpG ODN is capable of generating protection against vaginal HSV-2 challenge in both inbred and outbred mice.

We then tested whether a single vaginal immunization with gD plus CpG ODN could give rise to protection against subsequent HSV-2 challenge. To this end, groups of C57BL/6 mice were immunized intravaginally with a single dose of gD plus CpG ODN. Four weeks after the immunization, the immunized mice and naïve mice were intravaginally challenged with a lethal dose of HSV-2. Similarly to control animals, all mice immunized once with gD plus CpG ODN had high levels of vaginal HSV-2 titers (Fig. 6A). Additionally, all immunized mice developed signs of the disease and died as a result of neurological illness within 10 days after HSV-2 challenge (Fig. 6B and C). Thus, a single vaginal immunization of mice with gD plus CpG does not confer protection against genital HSV-2 infection and disease. We also compared the efficacies of high and low doses of CpG ODN for induction of protection. Groups of C57BL/6 mice were vaccinated twice with gD plus either 30 μ g or 2 μ g of CpG ODN and then were challenged with a lethal HSV-2 dose. The result shows a diminished protection in the group vaccinated with a low dose (2 μ g) of CpG ODN (data not shown). Thus, these results indicate that a dose of 30 μ g of CpG ODN can serve as a potent mucosal adjuvant for protein vaccination against genital HSV-2 infection in mice.

DISCUSSION

We have recently shown that mucosal administration of CpG ODN into the murine vaginal mucosa elicits strong Th1-associated cytokine responses in the vagina and also mobilizes the influx of immunocompetent cells into the genital lymph nodes. Further, a single vaginal dose of CpG ODN confers potent innate immune protection against genital herpes (1, 6). We show in the present study that the local mucosal administration of CpG ODN is associated with a rapid and strong increase in the CC chemokines MIP-1 α , MIP-1 β , and RANTES and the CXC chemokines IP-10 and MIP-2 in both the vagina and the draining lymph nodes. This finding is in line with our previous results that local administration of CpG ODN elicits production of the Th1-associated cytokines IFN- γ , IL-12, and IL-18 and the Th1-associated chemokine RANTES in the vagina (6). MIP-1 α , MIP-1 β , and RANTES play an essential role in trafficking of immunocompetent cells, including macrophages, dendritic cells, NK cells, and activated T cells, to the site of immunization or infection. The production of these chemokines is associated with development of a Th1-type immune response (24). The rapid production of MIP-1 α , MIP-1 β , and RANTES in both the vagina and the draining lymph nodes is of fundamental importance for immunity against human immunodeficiency virus type 1 (HIV-1) infection in the vagina, as these are the major HIV-suppressive factors (3).

We also showed that within 8 h following mucosal delivery of CpG ODN, the levels of CXC chemokine IP-10 markedly increased in both the vagina and the gLNs and stayed elevated for 2 days. IP-10/CXCL10 attracts monocytes and activated T lymphocytes at inflammatory foci and promotes selective en-

FIG. 6. Single vaginal immunization with gD plus CpG ODN failed to generate protection against genital herpes in mice. Groups of female C57BL/6 mice ($n = 5$) were intravaginally immunized with a single dose of gD plus CpG ODN [gD + CpG ($\times 1$)] and were challenged 4 weeks later with a lethal dose of HSV-2. The results are expressed as means $\pm$ standard errors of the means of virus loads (PFU/sample). The mice were examined for vaginal viral titer on day 3 (A) and were also monitored daily for survival (B) and signs of disease (C).

hancement of Th1 responses and increased IFN- γ gene expression (2, 4, 5). Thus, CpG-induced IP-10 responses in the vagina and the gLNs may contribute to both induction of a local Th1-skewed milieu and recruitment of immunocompetent cells into the vaginal mucosa and the gLNs. We and others have documented the critical importance of Th1-type immunity, including IFN- γ response for immune protection against genital herpes infection in mice (8, 9, 18). Within 8 h following vaginal

delivery of CpG ODN, the levels of MIP-2 increased in both the vagina and the gLNs. MIP-2, a homologue of human IL-8, possesses a strong chemotactic activity for neutrophils and can therefore contribute to the development of immunity in the genital tract. It is noteworthy that neutrophils were shown to play an important role in resolution of HSV-2 infection of the mouse vagina (17). Interestingly, our results indicate that vaginal administration of CpG ODN elicited rapid CC and CXC chemokine responses in the vaginas of inbred as well as outbred mice. Nonetheless, the gLN chemokine responses observed to occur in inbred C57BL/6 mice were almost absent in NMRI outbred animals.

Because Th1 immune response was shown to be associated with protective immunity in the murine genital herpes model, we examined whether the Th1-tilted milieu induced by mucosal administration of CpG ODN potentiated the immune response against a coadministered recombinant gD protein. We found that vaginal immunization of mice with gD protein plus CpG ODN elicited a strong antigen-specific Th1-type cell-mediated immunity, both in the gLNs and the spleen, as evidenced by a potent gD-specific proliferative and IFN- γ response by gLN and splenic CD4⁺ T cells. Of note, IFN- γ is the most important immune correlate in immunity against genital herpes (20). The vaccinated animals were significantly protected against an otherwise lethal vaginal challenge with HSV-2. Conversely, vaccination of mice with CpG ODN or gD alone failed to elicit any appreciable gD-specific IgG antibody, T-cell, or IFN- γ response. Not surprisingly, these mice succumbed rapidly to genital HSV-2 challenge. The CpG motif was found to be critical for an adjuvant effect of CpG ODN, as groups of mice vaccinated with gD plus control ODN did not resist a lethal challenge dose. These results corroborate and expand the recent finding by Kwant and Rosenthal (13) that vaginal immunization with CpG ODN plus recombinant gB protein induced gB-specific antibody response as well as protection against a vaginal HSV-2 challenge in mice. Interestingly, our results showed that vaginal immunization with gD plus CpG ODN could generate protection against genital herpes in outbred NMRI mice, which are genetically undefined. We also demonstrated that double vaginal immunization with gD plus CpG ODN is required to generate a considerable level of protection, as C57BL/6 mice immunized with a single dose of gD plus CpG ODN did not show any appreciable level of protection against HSV-2 challenge.

In summary, the present study demonstrates the efficacy of CpG ODN for the generation of potent CC and CXC chemokine responses in the vagina and the gLNs and also as a mucosal/vaginal adjuvant for generation of Th1-skewed T-cell response towards a nonreplicating antigen in both the local genital lymph nodes and the spleen. These findings highlight the value of CpG ODN as a potent mucosal adjuvant for the induction of protective Th1-tilted immune responses against genital herpes and justify further investigation of the use of CpG ODN for the development of mucosal vaccines against other sexually transmitted pathogens, such as HIV.

ACKNOWLEDGMENTS

We are grateful to Karolina Thörn for skilled technical assistance. This study was supported by the Swedish Research Council for Medicine (VR); the Swedish International Development Cooperation

Agency (Sida); the Göteborg University Vaccine Research Institute (GUVAX); the Swedish Foundation for Strategic Research; the Mucosa, Immunity and Vaccine Center (MIVAC); the Royal Society of Arts and Sciences in Göteborg, and the Wilhelm and Martina Lundgren Foundation. This research was also supported in part by Public Health Service Grant AI 18289 to Gary H. Cohen and grants AI 056045 and NS 36731 to Roselyn J. Eisenberg.

REFERENCES

- Ashkar, A. A., S. Bauer, W. J. Mitchell, J. Vieira, and K. L. Rosenthal. 2003. Local delivery of CpG oligodeoxynucleotides induces rapid changes in the genital mucosa and inhibits replication, but not entry, of herpes simplex virus type 2. *J. Virol.* **77**:8948–8956.
- Bonecchi, R., G. Bianchi, P. P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P. A. Gray, A. Mantovani, and F. Sinigaglia. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* **187**:129–134.
- Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* **270**:1811–1815.
- Farber, J. M. 1997. Mig and IP-10: CXC chemokines that target lymphocytes. *J. Leukoc. Biol.* **61**:246–257.
- Gangur, V., F. E. Simons, and K. T. Hayglass. 1998. Human IP-10 selectively promotes dominance of polyclonally activated and environmental antigen-driven IFN-gamma over IL-4 responses. *FASEB J.* **12**:705–713.
- Harandi, A. M., K. Eriksson, and J. Holmgren. 2003. A protective role of locally administered immunostimulatory CpG oligodeoxynucleotide in a mouse model of genital herpes infection. *J. Virol.* **77**:953–962.
- Harandi, A. M., and J. Holmgren. 2004. CpG DNA as potent inducer of mucosal immunity: implications for immunoprophylaxis and immunotherapy of mucosal infections. *Curr. Opin. Investig. Drugs* **5**:141–145.
- Harandi, A. M., B. Svennerholm, J. Holmgren, and K. Eriksson. 2001. Differential roles of B-cells and IFN- γ -secreting CD4⁺ T-cells in innate and adaptive immune control of genital HSV-2 infection in mice. *J. Gen. Virol.* **82**:845–853.
- Harandi, A. M., B. Svennerholm, J. Holmgren, and K. Eriksson. 2001. Interleukin-12 (IL-12) and IL-18 are important in innate defense against genital herpes simplex virus type 2 infection in mice but are not required for the development of acquired gamma interferon-mediated protective immunity. *J. Virol.* **75**:6705–6709.
- Kinghorn, G. R. 1994. Epidemiology of genital herpes. *J. Int. Med. Res.* **22**:14A–23A.
- Koelle, D. M., and L. Corey. 2003. Recent progress in herpes simplex virus immunobiology and vaccine research. *Clin. Microbiol. Rev.* **16**:96–113.
- Krieg, A. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* **20**:709–760.
- Kwant, A., and K. L. Rosenthal. 2004. Intravaginal immunization with viral subunit protein plus CpG oligodeoxynucleotides induces protective immunity against HSV-2. *Vaccine* **13**:3098–3104.
- Luster, A. D. 2002. The role of chemokines in linking innate and adaptive immunity. *Curr. Opin. Immunol.* **14**:129–135.
- Martin, R. M., J. L. Brady, and A. M. Lew. 1998. The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice. *J. Immunol. Methods* **212**:187–192.
- Mestecky, J., and P. N. Fultz. 1999. Mucosal immune system of the human genital tract. *J. Infect. Dis.* **179**:S470–S474.
- Milligan, G. N. 1999. Neutrophils aid in protection of the vaginal mucosae of immune mice against challenge with herpes simplex virus type 2. *J. Virol.* **73**:6380–6386.
- Milligan, G. N., and D. I. Bernstein. 1997. Interferon-gamma enhances resolution of herpes simplex virus type 2 infection of the murine genital tract. *Virology* **229**:259–268.
- Nicola, A. V., S. H. Willis, N. N. Naidoo, R. J. Eisenberg, and G. H. Cohen. 1996. Structure-function analysis of soluble forms of herpes simplex virus glycoprotein D. *J. Virol.* **70**:3815–3822.
- Parr, M. B., and E. L. Parr. 2003. Vaginal immunity in the HSV-2 mouse model. *Int. Rev. Immunol.* **22**:43–63.
- Pyles, R. B., D. Higgins, C. Chalk, A. Zalar, J. Eiden, C. Brown, G. Van Nest, and L. R. Stanberry. 2002. Use of immunostimulatory sequence-containing oligonucleotides as topical therapy for genital herpes simplex virus type 2 infection. *J. Virol.* **76**:11387–11396.
- Russell, M. W. 2002. Immunization for protection of the reproductive tract: a review. *Am. J. Reprod. Immunol.* **47**:265–268.
- Russell, M. W., and J. Mestecky. 2002. Humoral immune responses to microbial infections in the genital tract. *Microbes Infect.* **4**:667–677.
- Schrum, S., P. Probst, B. Fleischer, and P. F. Zipfel. 1996. Synthesis of the CC-chemokines MIP-1alpha, MIP-1beta, and RANTES is associated with a type 1 immune response. *J. Immunol.* **157**:3598–3604.
- Stanberry, L. R., S. L. Spruance, A. L. Cunningham, D. I. Bernstein, A.

- Mindel, S. Sacks, S. Tying, F. Y. Aoki, M. Slaoui, M. Denis, P. Vandepapeliere, and G. Dubin. 2002. Glycoprotein-D-adjuvant vaccine to prevent genital herpes. *N. Engl. J. Med.* **347**:1652–1661.
26. Straus, S. E., A. Wald, R. G. Kost, R. McKenzie, A. G. Langenberg, P. Hohman, J. Lekstrom, E. Cox, M. Nakamura, R. Sekulovich, A. Izu, C. Dekker, and L. Corey. 1997. Immunotherapy of recurrent genital herpes with recombinant herpes simplex virus type 2 glycoproteins D and B: results of a placebo-controlled vaccine trial. *J. Infect. Dis.* **176**:1129–1134.
27. Whitley, R. J. 2002. Herpes simplex virus infection. *Semin. Pediatr. Infect. Dis.* **13**:6–11.
28. Willis, S. H., A. H. Rux, C. Peng, J. C. Whitbeck, A. V. Nicola, H. Lou, W. Hou, L. Salvador, R. J. Eisenberg, and G. C. Cohen. 1998. Examination of the kinetics of herpes simplex virus glycoprotein D binding to the herpesvirus entry mediator, using surface plasmon resonance. *J. Virol.* **72**:5937–5947.
29. Yi, A.-K., M. Chang, D. Peckham, A. Krieg, and R. Ashman. 1998. CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. *J. Immunol.* **160**:5898–5906.
30. Zlotnick, A., and O. Yoshie. 2000. Chemokines: a new classification system and their role in immunity. *Immunity* **12**:121–127.