

# Induction of Mucosal Immunity against Herpes Simplex Virus by Plasmid DNA Immunization

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**The ability of mucosally delivered plasmid DNA encoding glycoprotein B (gB) of herpes simplex virus type 1 (HSV-1) to generate systemic as well as distal mucosal immunity was evaluated. BALB/c mice were immunized intranasally (i.n.) with gB DNA or DNA expressing  $\beta$ -galactosidase ( $\beta$ -Gal). Two days following immunization, gB and  $\beta$ -Gal gene expression was detected by reverse transcription (RT)-PCR in lungs and cervical lymph nodes (CLN). Histological analysis showed that  $\beta$ -Gal protein was expressed in vivo in the lungs and the CLN of animals immunized with i.n. administered  $\beta$ -Gal DNA. The immune responses generated by i.n. administration of gB DNA with or without cholera toxin (CT) were compared to those generated by intramuscular (i.m.) gB DNA and i.n. live HSV administration. Three i.n. doses of gB DNA over a 3-week period resulted in a distal mucosal immunoglobulin A (IgA) response. In addition, the mucosal IgA response was enhanced by coadministration of CT with gB DNA. The i.m. route of immunization induced a strong IgG response in the serum and vagina but was inefficient in generating a mucosal IgA response. Antigen-specific cytokine ELISPOT analyses as well as the serum IgG1/IgG2a ratio indicated induction of stronger Th2 responses following the additional i.n. administration of CT compared to i.n. or i.m. gB DNA or i.n. live HSV immunization. In addition, mucosal immunization with gB DNA induced anti-HSV cell-mediated immunity in vivo as measured by delayed-type hypersensitivity. Although i.n. DNA immunization was an effective means of inducing mucosal antibody, it was inferior to i.m. DNA delivery in providing protection against lethal HSV challenge via the vaginal route. In addition, both i.m. and i.n. plasmid immunizations failed to generate an immune barrier to viral invasion of the mucosa.**

Herpes viruses are among the most successful of all pathogens. The majority of human hosts are infected by several herpesviruses, and the viruses may cause diseases that are troublesome although rarely lethal. There is a need to develop suitable vaccines, particularly against herpes simplex viruses (HSV) (25). Currently, none are licensed for use in the United States. The most vulnerable sites for infection by HSV are abraded skin or intact mucosal surfaces. Effective immunity at such sites could conceivably contain infection. Mucosal immunity against HSV has received limited study, but evidence that mucosal defense plays a role in controlling experimental genital infection with HSV has been documented (4–6, 13, 14). One issue is how best to induce such immunity. A candidate approach is the use of plasmid DNA encoding viral genes, but whether such an approach will achieve acceptable levels of immunity requires exploration.

The large majority of studies on DNA vaccines employ systemic immunization and fail to record if responses additionally occur at mucosal sites (1, 18, 27). In the present report, we compare the intranasal (i.n.) route of DNA administration with the intramuscular (i.m.) route for their effectiveness at inducing several components of immunity at both systemic and distal mucosal sites. i.n. immunization with plasmid DNA encoding an HSV major glycoprotein (glycoprotein B [gB]) does induce both systemic and distal mucosal responses. Systemic responses following i.n. DNA immunization were inferior to those resulting from i.m. immunization, but distal mucosal responses to DNA given i.n. were increased by additionally

administering cholera toxin (CT) i.n. The CT adjuvant was most effective at enhancing the Th2 pattern of responsiveness. Although notable levels of mucosal immunity were induced by i.n. DNA immunization, this proved insufficient to provide barrier protection against vaginal challenge with HSV.

## MATERIALS AND METHODS

**Mice.** Female BALB/c mice (*H-2d*), 3 to 4 weeks old (Harlan Sprague-Dawley, Indianapolis, Ind.), were used. During the experimental procedures, the investigators adhered to guidelines proposed by the Committee on the Care of Laboratory Animal Resources, Commission of Life Sciences, National Research Council. The animal facilities of the University of Tennessee are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

**Virus.** Herpes simplex virus type 1 (HSV-1) (McKrae) or HSV-1 (KOS) was grown on Vero cell monolayers (ATCC CCL81) and was stored in aliquots at  $-80^{\circ}\text{C}$  until used. Titers were measured in Vero cells and expressed as PFU per milliliter (22).

**Plasmid DNA preparation.** Plasmid DNA encoding gB of HSV-1 (KOS) with the cytomegalovirus promoter was described previously (11). Plasmids were purified by polyethylene glycol precipitation according to Sambrook et al. (21) with some modifications. Cellular proteins were precipitated with 1 volume of 7.5 M ammonium acetate followed by isopropanol precipitation of the supernatant. After polyethylene glycol precipitation, plasmids were phenol-chloroform extracted (three times) and precipitated with 2 volumes of pure ethanol. The quality of DNA was checked by electrophoresis on 1% agarose gels.

**Immunization of mice.** For i.m. administration, 100  $\mu\text{g}$  of gB DNA was injected into the quadriceps muscles of 3- to 4-week old female BALB/c mice three times at weekly intervals (day 0, 7, and 14). i.n. immunizations were performed three times at weekly intervals (day 0, 7, and 14) with 100  $\mu\text{g}$  of gB DNA with or without 2  $\mu\text{g}$  of CT (Sigma Chemical Co., St. Louis, Mo.; no. C-3012) in a total volume of 50  $\mu\text{l}$  of Hanks balanced salt solution administered into the nares of deeply anesthetized mice. As controls, mice were given vector plasmid DNA mixed with CT or  $10^5$  PFU of HSV-1 (KOS) i.n. In some experiments mice were immunized with  $10^5$  PFU of HSV-1 KOS i.m.

**RNA expression following i.n. DNA immunization.** Lungs and cervical lymph nodes (CLN) were removed two days after plasmid DNA administration from the animals (2 or 3 animals per group). RNA was isolated as previously reported (9) and was treated with DNase (20 U/50  $\mu\text{l}$ ). After phenol-chloroform extraction and ethanol precipitation, the absence of plasmid DNA was confirmed by

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PCR. Total cellular RNA was reverse transcribed, and aliquots of cDNA were amplified by PCR to detect  $\beta$ -actin,  $\beta$ -galactosidase ( $\beta$ -Gal), and gB. The following primer sequences were used:  $\beta$ -actin, GTG GGG CGC CCC AGG CAC CA (sense) and CTC CTT AAT GTC ACG CAC GAT (anti-sense);  $\beta$ -Gal, TTG CAG TGC ACG GCA GAT AC (sense) and TTT GAG ACC AGA CCA ACT GG (anti-sense); and gB, GCG GCC TTC TTC GCC TTT TAC (sense) and CAG GTC GTC GCG GTC GGC GTC ACC (anti-sense).

**$\beta$ -Gal staining and histology.** Four days after i.n. administration of plasmid DNA encoding  $\beta$ -Gal or vector DNA, lungs and CLN from different mice were removed and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH = 7.2) for 1 h at 4°C. Subsequently, the tissues were washed several times with PBS and reacted overnight in substrate solution containing 20 mM potassium ferrocyanide, 20 mM potassium ferricyanide, 2 mM MgCl<sub>2</sub>, 2 mg of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal) per ml, and 120  $\mu$ l of 10% Nonidet P-40 and 100  $\mu$ l of 1% sodium deoxycholate per 20 ml. After the reaction, the whole tissues were washed with PBS and fixed with 3.7% formaldehyde in PBS. Tissues were embedded in paraffin, sectioned at 5  $\mu$ m, and stained with nuclear fast red.

**Vaginal challenge.** In order to synchronize the estrus cycle at the progesterone-dominated stage as described by Parr et al. (19), the immunized mice were injected subcutaneously with Depo-Provera (DP) (Upjohn Co., Kalamazoo, Mich.) at a concentration of 2 mg per mouse in 50  $\mu$ l of distilled H<sub>2</sub>O. Five days after the administration of DP, the animals were challenged intravaginally with 10<sup>4</sup> (5 50% lethal doses [5 LD<sub>50</sub>]) or 10<sup>6</sup> (500 LD<sub>50</sub>) PFU of HSV-1 McKrae. These mice were examined daily for vaginal inflammation, neurological illness, and death and were scored 1 to 5 depending on the severity of disease (0, no change; 1, mild inflammation; 2, moderate swelling; 3, severe inflammation; 4, paralysis; 5, death).

**ELISA.** Serum was collected by retroorbital bleeding. Vaginal washings were collected with a micropipette by introducing 100  $\mu$ l of PBS into the vaginal cavity, and 50 to 70  $\mu$ l were recovered per animal. The vaginal lavage sediments were subsequently removed by centrifugation, and 20- $\mu$ l volumes of each individual sample (6 or 8 mice per group) were pooled. Fecal samples were weighed and suspended in PBS containing 0.1% sodium azide at a concentration of 100 mg/ml. All samples were stored at -80°C until used. For detection of antibodies against gB, enzyme-linked immunosorbent assay (ELISA) plates (Dynatech Laboratories Inc., Chantilly, Va.) were coated with gB protein at a concentration of 2  $\mu$ g/ml (kindly provided by Rae Lyn Burke, Chiron Corporation Emeryville, Calif.). A standard ELISA was performed to determine the gB-specific antibodies in the samples, and the concentration of antibodies was determined as previously described (16). Briefly, after overnight incubation at 4°C with gB protein or anti-mouse immunoglobulin G (IgG), the plates were washed three times with PBS containing 0.05% Tween 20, pH 7.2 (PBST), and blocked by using PBS (pH 7.2) with 3% dehydrated milk for 2 h at 37°C. A total of 200  $\mu$ l of serum samples (prediluted in PBST) were added in duplicate and serially diluted twofold to a final 100- $\mu$ l final volume. Wells coated with 2  $\mu$ g of goat anti-mouse IgG or IgA (Southern Biotechnology Associates [SBA], Birmingham, Ala.; no. 1030-01) per ml were treated with serially diluted standard mouse IgA (SBA; no. 107-01), mouse IgG (SBA; no. 10701), mouse IgG2a (Pharmingen; no. 03021D), or IgG1 (Pharmingen; no. 03001D). Plates were incubated for 2 h at 37°C. After being washed, 100  $\mu$ l of goat anti-mouse IgG-conjugated horseradish peroxidase (IgGHRP) (SBA; no. 1030-05) was added and wells were incubated for 1 h. For dilution of IgG isotypes, goat anti-mouse IgGHRP or goat anti-mouse IgG2aHRP (SBA; no. 1070-05 and 1080-05, respectively) was added. After three washes 2,2-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid substrate was added. Antibody concentrations were determined from the standard curve. Mice serum responses were measured individually. The standard deviations (SD) of the serum antibody concentrations are based on individual serum samples from seven mice per group.

**Antibody ELISPOT assay.** The numbers of iliac lymph node (ILN) cells producing anti-HSV IgA and IgG antibodies were measured 5 days post-virus challenge by using the ELISPOT assay as described by Milligan and Bernstein (16). Briefly, 96-well nitrocellulose plates (Millipore) were coated with 100  $\mu$ l of carbonate buffer containing 2 to 4  $\mu$ g of gB protein and incubated overnight at 4°C. For determination of the total amounts of IgG and IgA spot-forming cells (SFC), the plates were coated with 2  $\mu$ g of rabbit anti-mouse IgG1 or IgA (Zymed, San Francisco, Calif.; no. 62-6500 or 61-6700, respectively) per ml. After blocking with RPMI medium with 10% fetal bovine serum (FBS), the ILN cells were added in 100- $\mu$ l volumes and incubated for 48 h at 37°C. The plates were then washed with PBS and PBST and incubated with biotinylated goat anti-mouse IgA or IgG (1:1,000) (Zymed; no. 62-6740 or 61-6540) for 1 h at 37°C. After being washed, alkaline phosphatase-conjugated streptavidin (Jackson Immuno Research; no. 016-050-084) was added at a 1:1,000 dilution, and wells were incubated at 37°C for 1 h. Subsequently, the spots were developed with the substrates nitro blue tetrazolium (NBT) (Sigma Chemical Co.; no. N5514) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma Chemical Co.; no. B-0274). The spots were counted by using a dissecting microscope (Leica Inc., Buffalo, N.Y.).

**Quantification of cytokine-producing cells after in vitro restimulation.** Fifteen days after the last i.n. immunization, the animals (6 per group) were sacrificed and the spleens were aseptically removed. Single-cell suspensions were made from the spleens and subsequently treated with ammonium chloride-containing

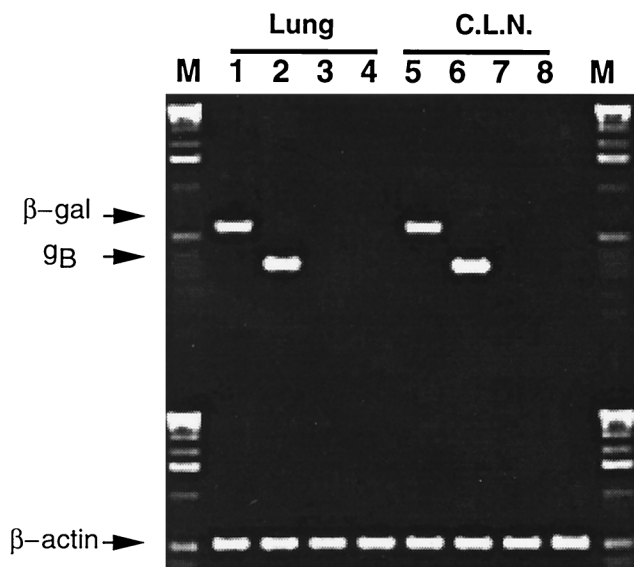


FIG. 1. Expression of mRNA for gB and  $\beta$ -Gal in lungs and CLN. Lungs and CLN were removed 2 days following i.n. administration of 100  $\mu$ g of plasmid DNA encoding gB,  $\beta$ -Gal, or vector DNA (2 or 3 animals per group). RNA was isolated as described in Materials and Methods and reverse transcribed, and cDNA was used for PCR detection of  $\beta$ -Gal and gB. PCR products were resolved on a 2% agarose gel and stained with ethidium bromide. Lanes: M, 1.0-kb DNA ladder; 1 and 5,  $\beta$ -Gal; 2 and 6, gB; 3 and 7,  $\beta$ -Gal negative control; 4 and 8, gB negative control.

Tris buffer for 3 min at 37°C to deplete the erythrocytes. These cells were used as effectors. Enriched dendritic cell (DC) populations obtained as described by Nair et al. (17) were used as stimulators. Briefly, splenocytes from naive female retired breeders were depleted of erythrocytes, and 3 ml of 10<sup>7</sup> cells were layered over 2 ml of metrizamide gradient (Nycomed Pharma A, Oslo, Norway; analytical grade, 14.5 g added to 100 ml of PBS, pH 7.0). The cells were then centrifuged at 600  $\times$  g for 10 min, and the cell interface was collected. The enriched DC population was pulsed for 3 h with UV HSV at a multiplicity of infection of 5 (prior to inactivation). Subsequently, the cells were washed and counted. The effector cells and the DC (naive or pulsed with UV HSV) were added at responder-to-stimulator ratios of 50:1, 25:1, 12.5:1, and 6.25:1 in 200  $\mu$ l of RPMI medium with 10% FBS per well into the coated and blocked ELISPOT plates. ELISPOT plates were pre-coated with the anticytokine antibodies anti-interleukin 5 (IL-5), anti-IL-4, and anti-gamma interferon (IFN- $\gamma$ ) (Pharmingen; no. 18051D, 18031D, and 18111D, respectively) at a concentration of 2  $\mu$ g/ml in 100  $\mu$ l in sterile carbonate buffer at 4°C overnight, and the plates were subsequently blocked with RPMI-10% FBS medium for 1 h at 37°C. After a 72-h incubation of the effector and stimulator cells (in a vibration-free incubator), the ELISPOT plates were washed with PBS (3 times) and PBST (3 times), and 100  $\mu$ l each of biotinylated anti-IL-5, biotinylated anti-IL-4, and biotinylated anti-IFN- $\gamma$  (Pharmingen; no. 18062D, 18042D, and 18112D, respectively), diluted 1:1,000 in PBST with 1% FBS, were added to the plates. After a 1-h incubation at 37°C, 100  $\mu$ l of 1:1,000-diluted alkaline phosphatase-conjugated streptavidin in PBST with 1% FBS was added, and the plates were incubated for another hour at 37°C. The spots were developed with the substrates NBT and BCIP and counted 24 h later with a dissecting microscope.

Levels of cytokine-producing cells were measured from vaginal-draining ILN 5 days after intravaginal challenge with HSV. At day 60 from the beginning of the experiment, the mice were challenged with HSV McKrae. Four days later the ILN were removed, and the cells were cultured without further restimulation for 72 h in ELISPOT plates pre-coated with anticytokine antibodies. Cells from animals immunized i.m. with gB or vector DNA were used as positive or negative controls, respectively.

**Viral titration.** Vaginal washings were collected at different time points after intravaginal challenge by pipetting 100  $\mu$ l of PBS into the vaginal cavity. The samples were stored at -80°C until used. Individual samples (50  $\mu$ l from each sample) were further diluted, and viral titers were obtained by using a plaque assay performed on Vero cells as described elsewhere (22).

**Delayed-type hypersensitivity (DTH).** The antigens were injected in 20- $\mu$ l volumes in the right ear pinna, and ear thickness was measured with a screw-gauge meter (Oditest; M. C. Kroepelin GmbH, Schluechtern, Germany) 48 h later as described in detail elsewhere (10). Test antigens included UV-inactivated HSV-1 (KOS) with a titer of 10<sup>5</sup> (prior to UV inactivation), and Vero cell extract was injected in the left ear pinna as a negative control. Results are expressed as

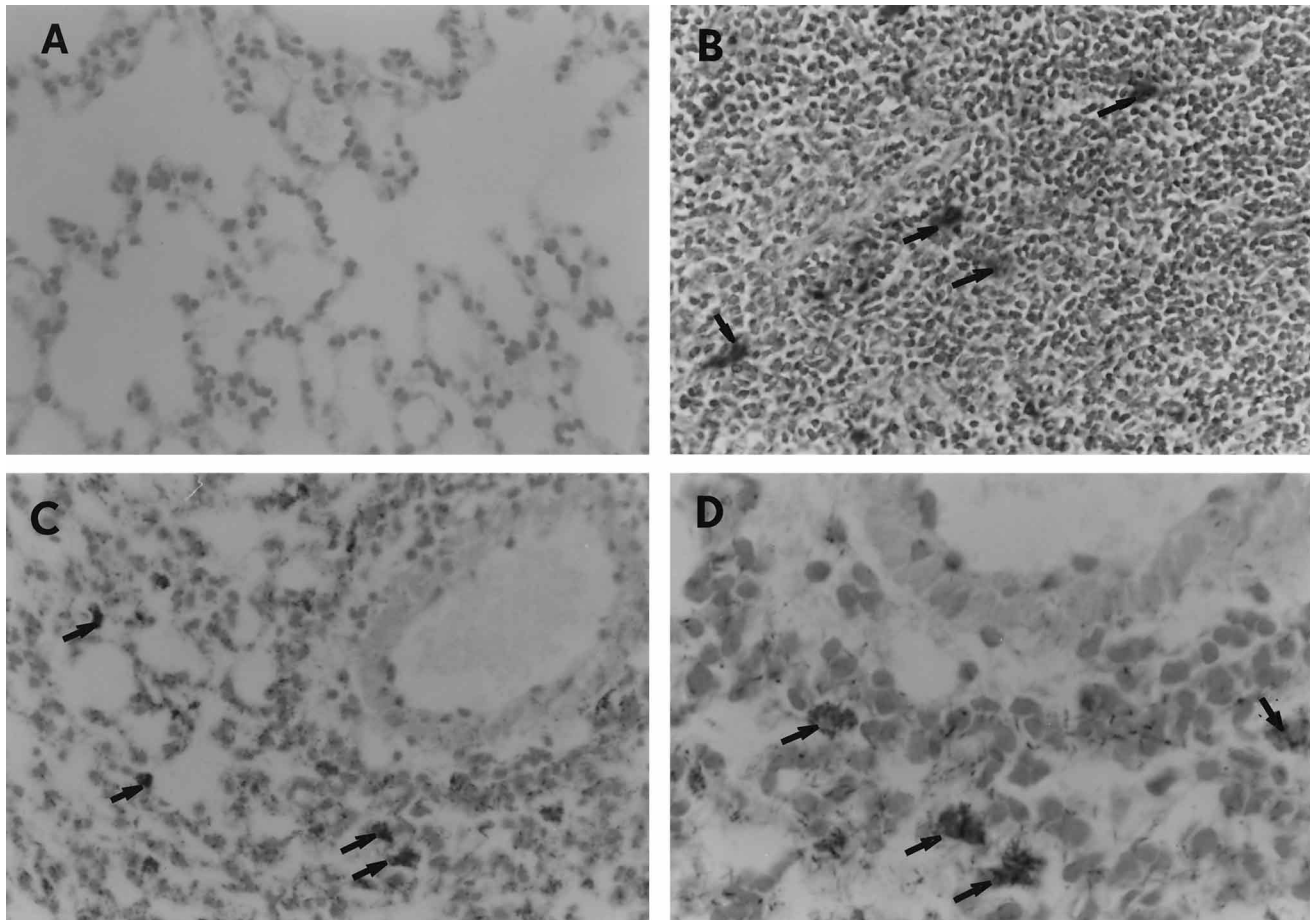


FIG. 2. Expression of  $\beta$ -Gal protein in tissue sections. Four days after i.n. administration of 100  $\mu$ g of plasmid DNA encoding  $\beta$ -Gal or vector DNA, lungs and CLN were removed, fixed with 4% paraformaldehyde, and incubated overnight with X-Gal substrate at 37°C. After overnight incubation, tissues were fixed in 3.7% formaldehyde in PBS and embedded in paraffin. Tissues were sectioned at 5  $\mu$ m and stained with nuclear fast red. (A) Vector-treated lung. Magnification,  $\times 170$ . (B) CLN from  $\beta$ -Gal-treated mouse. Magnification,  $\times 170$ . (C) Lung from  $\beta$ -Gal-treated mouse. Magnification,  $\times 170$ . (D) Lung from  $\beta$ -Gal-treated mouse. Magnification,  $\times 340$ .

mean increases in ear thickness at 48 h following ear pinna injection over the prechallenged thickness. Five mice per group were used, and the values obtained were analyzed for statistical significance.

**Statistics.** Wherever specified, data obtained were analyzed for statistical significance by Student's *t* test.

## RESULTS

**$\beta$ -Gal and gB mRNA expression in lungs and CLN following i.n. plasmid DNA administration.** To determine where the i.n. administered gB or  $\beta$ -Gal DNA plasmid was expressed, the lungs and CLN of the immunized mice were lysed and tested for the presence of gB- and  $\beta$ -Gal-specific mRNA by reverse transcription (RT)-PCR. gB or  $\beta$ -Gal mRNA expression was detected at day 2 in both lungs and CLN tissues following gB or  $\beta$ -Gal DNA administration, respectively, whereas no expression was observed in the mice immunized i.n. with vector (Fig. 1).

**$\beta$ -Gal enzymatic reaction and histology.** Little is known as to where the antigen is produced after mucosal plasmid DNA administration. To address this issue, plasmid DNA encoding  $\beta$ -Gal was i.n. administered to mice, and tissues were examined for  $\beta$ -Gal protein expression 4 days thereafter by a sensitive enzymatic approach on paraffin-embedded sections which preserved cellular architecture.  $\beta$ -Gal-expressing cells were readily demonstrable in alveolar epithelial cells and the bron-

chi as well as in the CLN. In the latter tissue,  $\beta$ -Gal-positive cells were present in the cortical regions. In addition, positive cells were apparently more frequent in CLN than in lung tissue (Fig. 2).

**Serum and mucosal antibody responses.** The efficacy of i.n. immunization with plasmid DNA encoding gB was compared at both mucosal and systemic sites with plasmid DNA injected i.n. and with infectious virus given i.n. or i.m. One group of mice immunized i.n. with DNA also received CT, a molecule well known as a mucosal adjuvant when given orally or i.n. (12, 23, 24). The i.n. immunization approach induced serum IgG antibody responses, and levels were slightly, but not significantly, increased in the group which was also given the CT adjuvant (Table 1). However, the serum antibody levels which resulted from i.n. immunization with gB DNA were less than those observed either in the i.m. gB DNA group or in those exposed i.n. to live HSV. Mucosal as well as i.m. DNA immunization resulted predominantly in anti-gB IgG2a serum antibody responses (a Th1 pattern of response), but coadministration of CT and gB DNA i.n. did push the response toward the Th2 pattern.

Distal mucosal antibody responses were measured in the feces and vaginal tract. As indicated in Table 1, i.n. immunization with gB DNA led to low vaginal and fecal IgA induction, but both responses were enhanced by the CT adjuvant. Re-

TABLE 1. Serum and distal mucosal antibody responses in virus- and DNA-immunized mice<sup>a</sup>

| Immunization        | Anti-gB serum antibody |                  | Mucosal anti-gB antibody |          |                     |          |
|---------------------|------------------------|------------------|--------------------------|----------|---------------------|----------|
|                     | IgG (ng/ml)            | IgG1/IgG2a ratio | Vaginal IgA              |          | Fecal IgA           |          |
|                     |                        |                  | gB specific (ng/ml)      | % gB IgA | gB specific (ng/ml) | % gB IgA |
| HSV i.m.            | 63,721 ± 3000          | 1/12             | 74 ± 61                  | 0.3      | ND                  | ND       |
| HSV i.n.            | 20,419 ± 451           | 1/8              | 2,754 ± 1,431            | 3.0      | 945 ± 75            | 1.0      |
| gB DNA i.m.         | 2,172 ± 891            | 1/10             | 11 ± 7.8 <sup>b</sup>    | 0.03     | 86 ± 59             | 0.07     |
| gB DNA i.n.         | 131 ± 60 <sup>c</sup>  | 1/4              | 27 ± 20 <sup>d</sup>     | 0.08     | 190 ± 139           | 0.02     |
| gB DNA plus CT i.n. | 210 ± 132              | 1/1              | 190 ± 93                 | 0.2      | 323 ± 136           | 0.2      |
| Vector plus CT i.n. | 10 ± 3                 | 0                | 1.8 ± 1.7                | 0        | 13 ± 3              | 0        |

<sup>a</sup> BALB/c mice were immunized either i.m. or i.n. with 100 µg of DNA or 10<sup>5</sup> live HSV. The immunization was repeated three times at 7 day intervals, samples (serum, fecal, and vaginal washings) were collected 15 days following the last immunization, and the levels of gB antibodies were measured by ELISA. Serum antibody levels were measured for individual mice (7 per group). The SD was calculated on the basis of serum level from individual mice in each group. Vaginal IgA responses were measured from pooled (5 or 6 mice per group) samples. The values represent averages from four independent experiments. Fecal samples were resuspended at a concentration of 100 mg/ml in PBS-0.1% sodium azide, and individual samples (5 or 6 per group) were tested for gB-specific as well as total IgA responses individually. % gB IgA ([gB-specific IgA/total IgA] × 100) was determined from fecal and vaginal samples of mice immunized with HSV and gB DNA.

<sup>b</sup> Significantly different from values obtained for mice immunized i.n. with vector DNA plus CT ( $P = 0.015$ ).

<sup>c</sup> Not significantly different from values obtained for mice immunized i.n. with gB DNA plus CT ( $P > 0.05$ ).

<sup>d</sup> Significantly different from values obtained for mice immunized i.n. with gB DNA plus CT ( $P = 0.025$ ).

sponses were less than those which occurred after i.n. live virus infection, but they were superior to the IgA responses which resulted from i.m. immunization with gB DNA. In contrast, vaginal IgG responses were higher in the group immunized with gB DNA i.m. compared to those immunized i.n., with or without CT (data not shown).

To measure humoral responses quantitatively at the cellular level, the ELISPOT approach was used to enumerate gB-specific IgA- and IgG-producing cells in the ILN draining the distal mucosal genital tract. All mice were challenged vaginally with 10<sup>6</sup> PFU of virus 4 days prior to ILN collection; otherwise the nodes were too small to permit analysis. Once again, it was evident that i.n. immunization with gB DNA along with CT was an effective means of inducing gB-specific IgA producers. Responses in the vaginal-draining ILN were greater than those which occurred with gB DNA given i.m., although they were severalfold less than those which occurred following i.n. infection with HSV. Although the gB DNA i.m. immunization approach induced a lower IgA SFC response than the i.n. protocol, the IgG SFC responses were far superior following the i.m. compared to the i.n. immunization route (Table 2).

Thus, as judged by the SFC assay, i.n. immunization with gB DNA along with CT proved to be more effective than the i.m. gB DNA approach at inducing common mucosal IgA responses, but it was inferior regarding humoral IgG induction.

**Pattern of CMI induction.** Cell-mediated immune (CMI) responses in the immunization groups mentioned above were measured by enumerating the number of cells in the spleen which produced Th1- or Th2-type cytokines upon secondary stimulation *in vitro* with DC exposed to inactivated virus. Positive ELISPOT responses were noted in the groups immunized with gB DNA i.m. or i.n. as well as in the i.n. virus-infected

group (Table 3). The i.n. gB DNA immunization approach resulted in a greater Th2 response than occurred following i.m. immunization. The incorporation of CT as an adjuvant mainly enhanced the Th2 cytokine responses, a pattern of events often noted with protein antigens when CT is used as a mucosal adjuvant (12).

Responses were also measured in ILN draining the vaginal mucosa, but in this instance animals in each group were challenged vaginally with HSV McKrae (10<sup>6</sup> PFU) 4 days prior to ILN collection. The results show that whereas i.m. immunization with gB DNA induced low Th2 responses (both IL-4 and IL-5 responses), the i.n. approach, when CT was used as an adjuvant, induced higher frequencies of IL-4 and IL-5 SFC (Table 4). Interestingly, pursuant to the HSV vaginal challenge, all groups, including the vector control group, mounted impressive IFN-γ (SFC) responses.

To determine whether antigen-specific cell-mediated responses could be detected *in vivo*, DTH reactions were measured at day 35 after the primary i.n. DNA immunization. Significant DTH responses were induced by i.n. gB DNA immunization ( $P \leq 0.01$ ). However, when CT was coadministered with gB DNA i.n., the DTH response was decreased compared to animals immunized with i.n. administered gB DNA ( $P \leq 0.01$ ) (Table 5).

**Resistance to vaginal challenge following immunization.** The i.n. immunization approach with gB DNA was compared to the control immunization systems mentioned previously for their efficiency at inducing protection against vaginal challenge by HSV. Prior to challenge, mice were given progesterone injections to synchronize the estrus cycle, since the stage of estrus can markedly affect susceptibility to HSV infection (19). Mice were challenged either with a high (10<sup>6</sup> PFU; 500 LD<sub>50</sub>) or low (10<sup>4</sup> PFU; 5 LD<sub>50</sub>) dose of a strain of virus (HSV-1

TABLE 2. Frequencies of IgA- and IgG-producing cells following virus and DNA immunization<sup>a</sup>

| Immunization and expt | IgA SFC/10 <sup>6</sup> cells |                    | IgG SFC/10 <sup>6</sup> cells |                    |
|-----------------------|-------------------------------|--------------------|-------------------------------|--------------------|
|                       | Total                         | gB specific (± SD) | Total                         | gB specific (± SD) |
| HSV i.n.              |                               |                    |                               |                    |
| 2                     | 970                           | 204 ± 44           | 6,923                         | 723 ± 40           |
| gB DNA i.m.           |                               |                    |                               |                    |
| 1                     | ND <sup>b</sup>               | 4 ± 2              | ND                            | 625 ± 13           |
| 2                     | 1,120                         | 13 ± 4             | 7,552                         | 432 ± 17           |
| gB DNA i.n.           |                               |                    |                               |                    |
| 1                     | ND                            | 0                  | ND                            | 15 ± 7             |
| 2                     | 1071                          | 11 ± 3             | 7,840                         | 36 ± 5             |
| gB DNA plus CT i.n.   |                               |                    |                               |                    |
| 1                     | ND                            | 32 ± 5             | ND                            | 120 ± 30           |
| 2                     | 784                           | 63 ± 7             | 5,125                         | 144 ± 21           |
| Vector i.n.           |                               |                    |                               |                    |
| 1                     | ND                            | 0                  | ND                            | 0                  |
| 2                     | 992                           | 2                  | 5,192                         | 18 ± 5             |

<sup>a</sup> BALB/c mice (6 per group) were immunized i.m. or i.n. with 100 µg of gB DNA or were immunized i.n. with 100 µg of gB DNA or 100 µg of gB DNA with or without 2 µg of CT. Control mice were given vector plasmid DNA or live HSV i.n. Forty-five days following the last immunization, the mice were challenged intravaginally with 10<sup>6</sup> PFU of HSV-1 McKrae. Four days following intravaginal challenge, the ILN were removed and pooled from each group. The frequencies of total and antigen-specific IgG and IgA SFC were determined by ELISPOT assay. SD is based on the number of SFC from three replicates per group.

<sup>b</sup> ND, not done.

TABLE 3. Frequencies of splenic cytokine-forming cells following virus or gB DNA immunization<sup>a</sup>

| Immunization and expt | Splenic SFC/10 <sup>6</sup> cells |              |                 |                     |                     |
|-----------------------|-----------------------------------|--------------|-----------------|---------------------|---------------------|
|                       | IL-5                              | IL-4         | IFN- $\gamma$   | IFN- $\gamma$ /IL-5 | IFN- $\gamma$ /IL-4 |
| HSV i.n.              |                                   |              |                 |                     |                     |
| 1                     | 20 $\pm$ 10                       | 168 $\pm$ 35 | 1,015 $\pm$ 125 | 50                  | 6                   |
| 2                     | 28 $\pm$ 10                       | ND           | 889 $\pm$ 87    | 31                  | ND                  |
| gB DNA i.m.           |                                   |              |                 |                     |                     |
| 1                     | 12 $\pm$ 5                        | 13 $\pm$ 5   | 135 $\pm$ 7.5   | 11                  | 10.3                |
| 2                     | 21 $\pm$ 3                        | ND           | 402 $\pm$ 28    | 19                  | ND                  |
| gB DNA i.n.           |                                   |              |                 |                     |                     |
| 1                     | 52 $\pm$ 24                       | 14 $\pm$ 7   | 83 $\pm$ 3      | 1.5                 | 5.9                 |
| 2                     | 32 $\pm$ 10                       | ND           | 168 $\pm$ 195   | 5.2                 | ND                  |
| gB DNA plus CT i.n.   |                                   |              |                 |                     |                     |
| 1                     | 185 $\pm$ 40                      | 147 $\pm$ 45 | 55 $\pm$ 10     | 0.2                 | 0.3                 |
| 2                     | 82 $\pm$ 7                        | ND           | 108 $\pm$ 30    | 1.3                 | ND                  |
| Vector i.n.           |                                   |              |                 |                     |                     |
| 1                     | 15 $\pm$ 2                        | 22 $\pm$ 7   | 5               |                     |                     |
| 2                     | 3 $\pm$ 3                         | ND           | 11 $\pm$ 8      |                     |                     |

<sup>a</sup> BALB/c mice (5 to 6 per group) were immunized with gB DNA, vector, or live HSV. The immunization was repeated three times at 7-day intervals. Fifteen days following the last immunization, the splenocytes from the immunized mice were in vitro-restimulated with an enriched DC population either naive or pulsed with UV HSV (multiplicity of infection = 5 before inactivation). Frequencies of cytokine-producing cells were measured by ELISPOT assay. Data from two experiments are presented. SD is based on three replicates per group. The numbers of SFC after naive DC restimulation is subtracted from the values of DC HSV-stimulated splenocytes.

McKrae) that in unimmunized mice is highly lethal. The vaginal mucosa was examined daily for signs of inflammation, and vaginal wash samples were assayed daily for 6 days or until death to measure levels of virus. The results are recorded in Tables 6 and 7.

It is apparent that mice immunized i.n. with live HSV-1 KOS resisted vaginal challenge by either high or low doses of HSV-1 McKrae. In addition, animals showed no signs of vaginal inflammation and virus could not be recovered from vaginal washings collected at 24 h (data not shown) and 48 h (Table 7) post-low-dose challenge. Some mice in the group immunized i.m. with gB DNA also resisted high-dose challenge (Table 6), and all survived challenge at low dose (Table 7). However, in mice immunized i.m. with gB DNA, some showed evidence of vaginal inflammation, and virus could be recovered for up to 5 days in some animals challenged at the high-dose level (Table 6). After low-dose challenge no signs of inflammation were observed in mice immunized i.m. with gB DNA (Table 7). The results of i.n. immunization with gB DNA indicated inferior levels of protection compared to the two other immunization protocols.

All i.n. immunized animals succumbed to high-dose challenge, although the average day of death was prolonged in comparison to the vector-immunized group (Table 6). Against low-dose challenge some protection was evident, particularly in the group which additionally received CT. Accordingly, in the i.n. plus CT group, over the 2- to 6-day period, average levels of virus in vaginal washings were significantly lower than in either the vector control group ( $P \leq 0.01$ ) or the group i.n. immunized with gB DNA ( $P \leq 0.01$ ) (Table 6). Although immunity was evident in terms of protection against challenge by low doses of virus, with the exception of mice immunized

TABLE 4. Cytokine-producing cells in ILN measured by ELISPOT<sup>a</sup>

| Immunization        | Cytokine SFC/10 <sup>6</sup> cells |              |               |
|---------------------|------------------------------------|--------------|---------------|
|                     | IL-5                               | IL-4         | IFN- $\gamma$ |
| gB DNA i.m.         | 7.3 $\pm$ 3                        | 22 $\pm$ 2   | 460 $\pm$ 80  |
| gB DNA i.n.         | 16 $\pm$ 4                         | 22 $\pm$ 7   | 444 $\pm$ 48  |
| gB DNA plus CT i.n. | 31 $\pm$ 10                        | 187 $\pm$ 47 | 537 $\pm$ 59  |
| Vector i.n.         | 3 $\pm$ 1                          | 21 $\pm$ 7   | 520 $\pm$ 59  |

<sup>a</sup> BALB/c mice (6 per group) were immunized with gB DNA or vector control at days 0, 7, and 14. Forty-five days after the last immunization the mice were challenged intravaginally with 10<sup>6</sup> PFU of HSV McKrae. Four days following challenge the ILN cells were removed and the cells were pooled from each group and analyzed for cytokine-producing cells without in vitro restimulation. The SD is based on three replicates per group.

with live virus, there was no evidence that immunity was a barrier to infection. Accordingly, in the mice which survived challenge with a low dose of virus, substantial increases in several immune parameters were observed (Fig. 3). Such secondary increased responses were not observed in animals challenged vaginally with 10<sup>4</sup> PFU of inactivated virus, indicating that viral replication had occurred and was responsible for the increased immune response.

## DISCUSSION

Immunization with naked DNA encoding various genes has proven to be a valuable means of inducing immunity (3, 11, 18). The great majority of studies have used the i.m. or gene gun delivery approach to administer the DNA and have emphasized systemic immunity. One report does demonstrate that i.n. administered DNA encoding the influenza virus NP does provide protection against mucosal challenge, but the nature of immunity engendered at mucosal sites was not measured (3). In the present report, we have established that i.n. immunization with DNA leads to mRNA and protein expression in the lung and CLN and provides systemic immune responses as well as immunity at distal mucosal sites. We also demonstrate that the adjuvant CT given along with the i.n. administered DNA markedly enhanced the mucosal IgA response. Animals immunized i.n. with DNA were immune to vaginal challenge by HSV, although not to the level achieved by live virus immunization. Moreover, the type of protection

TABLE 5. Development of DTH in DNA- or HSV-immunized mice<sup>a</sup>

| Immunization        | Mean ( $\pm$ SD) increase in ear thickness over prechallenge thickness at 48 h (10 <sup>-2</sup> mm) |               |
|---------------------|--|---------------|
|                     | UV HSV   | Vero extract  |
| HSV i.n.            | 13 $\pm$ 2.4   | 2 $\pm$ 0.8   |
| gB DNA i.n.         | 8 $\pm$ 0.5 <sup>b,c</sup>   | 2.7 $\pm$ 1.4 |
| gB DNA plus CT i.n. | 3 $\pm$ 2 <sup>d</sup>   | 1.2 $\pm$ 2.5 |
| Vector plus CT i.n. | 1 $\pm$ 0.4  | 2 $\pm$ 2.4   |

<sup>a</sup> Each group consisting of five mice was immunized with one of the immunogens at days 0, 7, and 14. As a positive control, 10<sup>6</sup> PFU of HSV-1 (KOS) was given i.n. For the DTH assay, each mouse was injected with either 10<sup>5</sup> PFU of UV HSV (titrated before inactivation) in the right ear pinna or Vero extract in the left ear pinna.

<sup>b</sup> Significantly different from values obtained for mice immunized i.n. with gB DNA and CT ( $P = 0.011$ ).

<sup>c</sup> Significantly different from values obtained for mice immunized i.n. with vector and CT ( $P = 0.00001$ ).

<sup>d</sup> Not significantly different from values obtained for mice immunized i.n. with vector and CT ( $P = 0.051$ ).

TABLE 6. Resistance to high-dose vaginal challenge in virus- and gB DNA-immunized mice<sup>a</sup>

| Immunization <sup>b</sup>        | No. of mice survived/no. of mice challenged | Avg time of death $\pm$ SD (days) | Log <sub>10</sub> of viral titer at day postchallenge <sup>c</sup> |                       |                       |                       |                       |
|----------------------------------|---|-----------------------------------|--|-----------------------|-----------------------|-----------------------|-----------------------|
|                                  |   |                                   | 2  | 3                     | 4                     | 5                     | 6                     |
| i.n. HSV <sup>d</sup>            | 4/4   | ND <sup>e</sup>                   | 1.9 $\pm$ 1 (3/4)  | 0 (0/4)               | 0 (0/4)               | 0 (0/4)               | 0 (0/4)               |
| i.m. gB DNA <sup>f</sup>         | 3/4   | 9.0                               | 4.6 $\pm$ 0.5 (4/4)  | 3.7 $\pm$ 0.2 (4/4)   | 2.5 $\pm$ 0.1 (4/4)   | 1.3 (1/4)             | 0 (0/4)               |
| i.n. gB DNA <sup>g</sup>         | 0/12  | 7.4 $\pm$ 1.7                     | 4.9 $\pm$ 0.2 (10/10)  | 4.3 $\pm$ 0.3 (10/10) | 3.5 $\pm$ 0.2 (10/10) | 3.4 $\pm$ 0.3 (10/10) | 2.7 $\pm$ 0.6 (10/10) |
| i.n. gB DNA plus CT <sup>h</sup> | 2/10  | 8.2 $\pm$ 1.3                     | 4.1 $\pm$ 0.4 (10/10)  | 3.4 $\pm$ 0.4 (10/10) | 2.8 $\pm$ 0.3 (10/10) | 2.1 $\pm$ 0.2 (10/10) | 1.5 $\pm$ 0.6 (7/10)  |
| i.n. vector DNA <sup>i</sup>     | 0/6   | 5.3 $\pm$ 0.4                     | 5.9 $\pm$ 0.3 (4/4)  | 4.6 $\pm$ 0.3 (4/4)   | 4.7 $\pm$ 0.4 (4/4)   | 4.3 $\pm$ 0.3 (4/4)   | 4.0 $\pm$ 0.2 (3/3)   |

<sup>a</sup> BALB/c mice were immunized i.n. with gB DNA (100  $\mu$ g), gB DNA (100  $\mu$ g) plus CT (2  $\mu$ g), or live HSV (10<sup>5</sup> PFU). The control group was given 100  $\mu$ g of gB DNA i.m. The immunization was repeated three times at 7-day intervals. Thirty days after the last immunization, the mice were injected with 2 mg of DP per mouse. Five days following the administration of DP, the mice were challenged intravaginally with 10<sup>6</sup> PFU of HSV McKrae. Vaginal viral titers were determined for each individual mouse at days 2, 3, 4, 5, and 6 following infection. At day 6 following challenge, vaginal samples were collected from all mice, and the paralyzed animals were terminated.

<sup>b</sup> *d*, *f*, *g*, and *h* versus *i*,  $P \leq 0.01$ , except for i.n. immunization with gB DNA versus i.n. immunization with vector DNA at day 3 postchallenge. *g* versus *h*,  $P \leq 0.01$ .

<sup>c</sup> Data in parentheses are number of vaginal washings positive for virus/total number of vaginal samples tested.

<sup>e</sup> ND, no death was observed until the end of the experiment.

induced by i.n. immunization did not provide a barrier to invasion upon viral challenge.

CT is a well-known mucosal adjuvant, although it is usually employed orally (8). Our report, that CT acts as a valuable adjuvant when used i.n., confirms the recent observation of a similar effect with peptide and protein antigens (23, 24). When given along with i.n. administered DNA, the CT adjuvant was particularly effective at enhancing the Th2-dependent nature of the immune response. Accordingly, the ratio of the IgG1/IgG2a antibody response noted in serum following CT coadministration was increased compared to that following i.n. or i.m. DNA immunization. At mucosal sites the level of specific IgA was significantly elevated, and the pattern of cytokine production by vaginal-draining ILN cells measured by the ELISPOT approach mainly reflected the type 2 cytokine response. Additionally, the DTH responses of mice i.n. immunized with gB DNA and CT were decreased compared to those of mice immunized with gB DNA or live HSV. This observation underscores the notion of Marinario et al. (12) that CT acts predominantly as a mucosal adjuvant which pushes the response towards the Th2 pattern. How this outcome is achieved is unresolved, but the effect usually appears to require the simultaneous presence of CT and antigen (24a). Whether this actually occurs with our DNA immunization studies is currently under investigation. Conceivably, adjuvant effects may become even more apparent if CT is given at an appropriate time following DNA administration when optimal protein expression is occurring. We are currently investigating this issue.

Our results demonstrate that i.n. administration of DNA induces both systemic and mucosal immunity at the genital and enteric sites. However, the levels of immunity induced were less than ideal and did not approach those resulting from live virus infection. As regards systemic immunity, the i.m. immunization route with DNA also proved superior. However, as noted previously in protein and peptide systems, i.m. immunization was less effective than i.n. immunization at inducing mucosal IgA. One aim of mucosal administration of antigen is to exploit the well-known common mucosal defense mechanisms (23) and induce barrier levels of immunity at multiple mucosal surfaces. This becomes an important concept for preventing invasion by pathogens which enter the body via mucosae and cause damage at the mucosal site. The DNA approach certainly engaged the common mucosal immune system and did induce detectable immunity at the two distal mucosal sites investigated. However, as regards defense against the test pathogen, HSV administered by the vaginal route induced levels of protection that were less than ideal and clearly were not a barrier to viral invasion. Hence, even in animals with readily detectable virus-specific IgA responses at the time of challenge, the virus appeared to persist and replicate in the vaginal tract. Moreover, such animals generated secondary systemic immune responses indicating that viral invasion had likely occurred. Indeed, at high levels of virus challenge none of the animals i.n. immunized with DNA survived. It appears, as was documented previously by the McDermott group (14), that immunity to HSV in the genital tract may depend more on the T-cell defense system functioning in the mucosal tissues

TABLE 7. Resistance to low-dose vaginal challenge in virus- and gB DNA-immunized animals<sup>a</sup>

| Immunization <sup>b</sup>        | No. of mice survived/no. of mice challenged | Avg time of death $\pm$ SD (days) | Clinical severity at day postchallenge |               |               |               | Log <sub>10</sub> of vaginal viral titer at day postchallenge <sup>c</sup> |                     |
|----------------------------------|---|-----------------------------------|--|---------------|---------------|---------------|--|---------------------|
|                                  |   |                                   | 6                                      | 7             | 8             | 9             | 2  | 6                   |
| i.n. HSV <sup>d,e</sup>          | 4/4   | ND <sup>f</sup>                   | 0                                      | 0             | 0             | 0             | 0 (0/4)  | 0 (0/4)             |
| i.m. gB DNA <sup>g</sup>         | 5/5   | ND                                | 0                                      | 0             | 0             | 0             | 2.8 $\pm$ 0.6 (5/5)  | 0 (0/5)             |
| i.n. gB DNA <sup>h</sup>         | 3/7   | 8.3 $\pm$ 0.4                     | 1.4 $\pm$ 1                            | 1.5 $\pm$ 1.1 | 2.1 $\pm$ 2   | 3.2 $\pm$ 2   | 3.5 $\pm$ 0.8 (7/7)  | 2.3 $\pm$ 1 (5/7)   |
| i.n. gB DNA plus CT <sup>i</sup> | 4/7   | 9 $\pm$ 0.4                       | 1.4 $\pm$ 1.5                          | 1.2 $\pm$ 1.5 | 1.8 $\pm$ 1.3 | 2.0 $\pm$ 2.4 | 2.8 $\pm$ 0.6 (5/5)  | 1.2 (1/5)           |
| i.n. vector <sup>j</sup>         | 0/4   | 7.8 $\pm$ 0.7                     | 3.0 $\pm$ 0.7                          | 3.7 $\pm$ 1.2 | 4.5 $\pm$ 1.0 | 5.0 $\pm$ 0   | 4.4 $\pm$ 0.1 (4/4)  | 3.4 $\pm$ 0.5 (4/4) |

<sup>a</sup> Mice were immunized as described for Table 5. Thirty-five days following the last immunization the mice were infected intravaginally with 10<sup>4</sup> PFU of HSV McKrae. Vaginal viral titers were determined at days 2 and 6 following infection. The mice were observed every day for vaginal inflammation. Clinical severity was graded as follows: 0, no inflammation; 1, mild inflammation; 2, moderate swelling and redness; 3, severe inflammation; 4, paralysis; and 5, death.

<sup>b</sup> *d*, *g*, *h*, and *i* versus *j* at day 2 postchallenge,  $P < 0.05$ ; *d*, *g*, and *i* versus *j* at day 6 postchallenge,  $P < 0.05$ .

<sup>c</sup> Data in parentheses are number of vaginal washings positive for virus/total number of vaginal samples tested.

<sup>e</sup> No virus was recovered from the vaginal washings of HSV-immunized mice at 24 and 48 h following infections.

<sup>f</sup> ND, no death was observed.

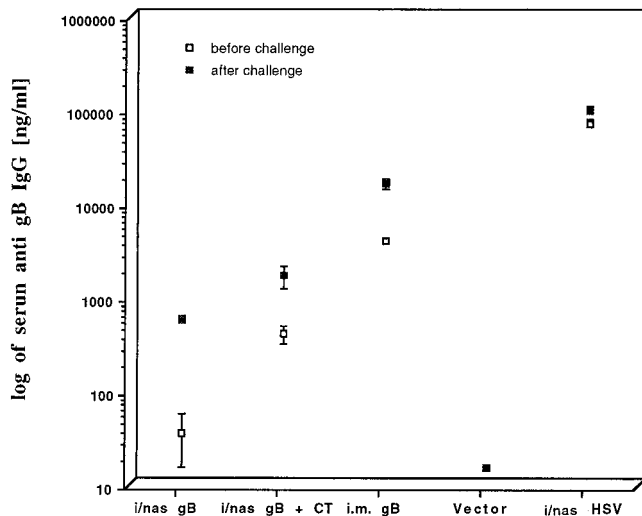


FIG. 3. Serum IgG titer change 5 days postchallenge with HSV-1 McKrae. BALB/c mice were immunized i.n. with 100  $\mu$ g of DNA or  $10^5$  PFU of live HSV. The immunization was repeated three times over a 3-week period. Thirty days following the last immunization serum was collected by retroorbital bleeding, and the mice were subcutaneously given 2 mg of DP. Five days after the hormone administration the mice were challenged intravaginally with  $10^4$  PFU of HSV-1 McKrae. Five days following the challenge serum was collected and both prechallenge and postchallenge serum samples were tested for anti-gB IgG antibodies by ELISA. Not shown is the fact that mice which responded to  $10^4$  PFU of inactivated virus failed to show secondary rises in antibody titers. i/nas, intranasal.

than on humoral antibodies functioning in the vaginal canal. This conclusion was based on a comparison of the effectiveness of adoptive transfer with T versus B cells (14). On the other hand, some reports of studies using a passive antibody transfer approach do indicate that clearance of the virus in the genital mucosa can be mediated by humoral immunity (2, 28). Clearly, more investigations are required to resolve the respective roles of cellular and humoral defenses against HSV in the genital tract.

In our studies, animals immunized i.m. with DNA, and lacking significant IgA in vaginal washings, were as resistant to vaginal challenge as were animals with IgA as a consequence of i.n. immunization with DNA plus CT. Accordingly, we are led to the conclusion that although mucosal immunization with DNA, especially when adjuvanted with CT, is an effective means of inducing mucosal immunity, this may not result in complete protection against HSV. Indeed, protection against this agent, which if not total will result in the establishment of latency (25), is more a property of CD4<sup>+</sup> and CD8<sup>+</sup> T cells which generate type 1 cytokines (10, 11). We are currently evaluating the mucosal DNA immunity approach for its effectiveness in protecting against other agents as well as exploring means of enhancing the effectiveness of delivery systems which may incur barrier immunity. The use of other DNA constructs delivered simultaneously with antigen constructs may allow modification of the immune response. Indeed, coadministration of cytokine constructs has proven useful in directing the immune response following systemic immunization with plasmid DNA (IL-2, IL-4, transforming growth factor  $\beta$ , granulocyte-macrophage colony-stimulating factor, and IL-12) (20, 31). In addition to mucosal adjuvants such as CT, the use of recent technologies may also enhance the efficiency of mucosally delivered DNA constructs. Recent developments have provided novel mucosal delivery systems, such as the use of

cochleates (7), which may provide a needed optimization of mucosal DNA delivery.

Finally, it is worth commenting that DNA administered by the noninvasive mucosal route appears to be an effective means of obtaining protein expression in the lung. In our studies using a plasmid encoding  $\beta$ -Gal, many cells in the alveolar epithelium, as well as in other sites in the lung, were shown to express protein. It might well be that the mucosally administered naked plasmid DNA delivery approach will prove to be a useful means of effecting gene therapy (26, 29). We are currently evaluating the use of this approach to influence the lung environment with various cytokines prior to mucosal immunization with HSV vaccines.

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