Long-Term Immunity and Protection against Herpes Simplex Virus Type 2 in the Murine Female Genital Tract after Mucosal but Not Systemic Immunization

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The degree and duration of immunity against herpes simplex virus type 2 (HSV-2) infection of the female genital tract were assessed after intranasal (inl) or intraperitoneal (ip) immunization with a recombinant adenovirus vector expressing HSV glycoprotein B (AdgB8). After intravaginal HSV-2 challenge, control mice rapidly developed disease and displayed high virus titers in vaginal washes. In contrast, virus titers decreased significantly and at similar rates in inl and ip immunized mice and by day 7 were undetectable in vaginal wash samples. Assessment of genital pathology and survival showed that only inl immunization provided long-term protection. Examination of antibody-secreting cells (ASCs) during the decline in vaginal virus titers revealed that gB-specific IgA ASCs were only observed in the genital tissues of inl immunized mice. These results indicate that mucosal immunization provides a high and long-lasting level of immunity from sexually transmitted infections of the female genital tract.

Protecting the female genital tract against viral sexually transmitted diseases (STDs), such as those caused by human immunodeficiency virus (HIV) and herpes simplex virus (HSV), will likely depend on the presence of both humoral and cellular immune functions [1, 2]. More specifically, the unique immune functions associated with the mucosal immune system can provide optimal protection against STDs [1, 2]. Secretory IgA (sIgA), which is considered the hallmark of mucosal immunity, can mediate protection of mucosal tissues through inactivation or neutralization of virus at the exterior surface of the mucosal epithelium and intracellularly during transcytosis [3–7]. Furthermore, the level of antibodies in external secretions correlates with protection against diseases of the respiratory and gastrointestinal tracts [8, 9]. As confirmation of the importance of mucosal IgA, anti-IgA, but not anti-IgG or IgM antiserum, instilled intranasally (inl) can abrogate immunity against influenza virus infection [10].

In the female genital tract, IgG antibodies are an important component of protection, because IgG antibodies specific for HSV can protect mice against intravaginal HSV type 2 (HSV-2) infection [11, 12]. Our studies indicate that the levels of antigen-specific IgA and IgG antibodies in vaginal wash samples of mice are inversely related and dependent on the stage of the estrous cycle [13]. These results suggest that the induction of both IgA and IgG will be required to maintain a blanket of humoral immunity in the female genital tract over the course of the estrous cycle. In addition to humoral immunity, T cells are involved in protecting mucosal tissues as shown by the role of mucosally derived T cells in protecting the female genital tract against HSV-2 infection [14].

The immune system has separate compartments in which the development of an immune response can be initiated. The systemic immune compartment consists of the bone marrow, spleen, and peripheral lymph nodes. The mucosal compartment consists of lymphoid aggregates in mucosae, external secretory glands, and the lymph nodes that drain these tissues [1, 15, 16]. The consequence of this compartmentalization is that antigen exposure and immune induction within one immune compartment results in the predominant expression of subsequent immune functions within the tissues associated with that compartment [1, 13, 15–17]. As a result, there is an increasing emphasis on the development of novel vaccines designed to induce mucosal-specific immune functions and provide protection against STDs [1].

Several groups pursuing this goal have shown that oral or inl inoculation with live viral or bacterial vectors [14, 18, 19] or conjugates of antigen with cholera toxin [20] successfully induces mucosal-specific immunity in the female genital tract. Alone or in conjunction with other routes, inl immunization successfully protects against STDs [21–23]. Our studies utilized an adenovirus vector expressing glycoprotein B (gB), AdgB8, of HSV-1 to induce immunity in the female genital tract. Our results showed that inl administration of AdgB8 results in gB-specific IgA and IgG antibodies and long-term cytotoxic T lymphocyte (CTL) memory responses in the female genital tract [17, 24]. Inl immunized mice were also highly
resistant to intravaginal HSV-2 infection [13]. In contrast, systemic administration of this vector resulted in only IgG antibodies and short-term CTL memory responses in the reproductive tract [17, 14]. Because of these results and the recent emphasis on mucosal immunization, it is imperative to compare systemic with mucosal routes of immunization with respect to protection from STDs. In the study presented here, mice immunized i.n. or intraperitoneally (i.p.) with AdgB8 were challenged intravaginally with HSV-2 and evaluated for the development of humoral immunity, clearance of virus, and genital pathology. 

Materials and Methods

Animals and cell cultures. Female C57Bl/6 (H-2b) mice (Constant, Quebec) were 6–8 weeks of age during primary immunization. Vero and 293 cells were grown in a-MEM (Gibco Laboratories, Burlington, Canada) supplemented with 10% fetal calf serum (Gibco), 1% penicillin-streptomycin, and L-glutamine (Gibco). 293-N2S cells, a nonadherent cell line derived from 293 cells, were grown in spinner flasks with Joklik’s medium supplemented as above.

Virus strains and inoculations. The construction of the replication-competent recombinant adeno-virus type 5 vector, AdgB8, has been described [24]. In brief, AdgB8 contains the gB gene from HSV-1 coupled to the SV40 promoter and inserted into the E3 region of human adenovirus type 5. Recombinant adenoviruses were grown in 293-N2S cells, purified twice on CsCl gradients, and titered on 293 cells. HSV-2 strain 333 was propagated and titered on Vero cells.

For AdgB8 immunization, mice were anesthetized with halothane and inoculated i.n. or i.p. with 10^5 pfu of AdgB8 as described [13, 24]. For intravaginal HSV-2 challenge, mice were first injected subcutaneously with 2 mg of progesterone/mouse (Depo-Provera; Upjohn, Don Mills, Canada). Five days later, while under halothane anesthesia, mice were swabbed vaginally with a cotton applicator, placed on their backs, and infected intravaginally for 1 h with 10 μL of HSV-2.

Viral replication and pathology in the reproductive tract. After intravaginal HSV-2 inoculation, samples were obtained daily by pipetting 30 μL of PBS into and out of mouse vaginas, which were then swabbed with a cotton applicator. Both the wash and the applicator were combined with 0.97 mL of PBS and frozen at −70°C. Virus titers were determined by plaque assay on Vero cell monolayers. The dilution of each vaginal wash supernatant was considered to be 10^−2.

Genital pathology was monitored daily after HSV-2 challenge and scoring was performed blinded. Pathology was scored on a 5-point scale: 0, no apparent infection; 1, slight redness of external vagina; 2, redness and swelling of external vagina; 3, severe redness and swelling of external vaginal and surrounding tissue; 4, genital ulceration with severe redness and swelling and hair loss of genital and surrounding tissue; and 5, severe genital ulceration extending to surrounding tissue. Mice were sacrificed upon reaching stage 5. The severity of pathology was measured as the area under the lesion score–day curve for the first 6 days following infection [13].

Antibody-secreting cell (ASC) enumeration by ELISPOT assay. Single cell suspensions from iliac lymph nodes and genital tissues were prepared as described [13]. Ninety-six well filtration plates were coated with nitrocellulose membrane (Millipore, Bedford, MA) with 10 μg/mL recombinant HSV-2 gB (provided by R. L. Burke, Chiron, Emeryville, CA) or 1/500 dilution of goat anti-mouse IgG or IgA (Southern Biotechnology Associates, Birmingham, AL) in PBS, and kept overnight at 4°C. Plates were blocked and lymphocytes were plated at 37°C for 16 h. The number of plasma cells in each preparation secreting total or HSV gB-specific IgA or IgG antibody was determined by the addition of biotinylated goat anti-mouse IgA or IgG (Southern Biotechnology Associates), followed by avidin-peroxidase. Spots representing individual ASC were visualized by developing with peroxidase substrate containing H2O2 and 3-amino-9-ethylcarboazole in acetic buffer. Spots were enumerated by digitized image analysis, and discrimination from background was based first on gray density and then by size (program written by L. Arsenault, Microscopy Group, McMaster University). Results are expressed as the mean number of ASC per 10^6 mononuclear cells for each tissue.

Statistical analysis. Data were analyzed by the InStat program (GraphPAD Software, San Diego). For comparisons between two groups, data were analyzed by Student’s t test or Fisher’s exact test as appropriate. Comparison among the means of multiple groups was done by analysis of variance.

Results

Virus titers in vaginal wash samples of mice infected intravaginally with HSV-2. Vaginal wash specimens from unimmunized mice and from mice immunized i.n. or i.p. with AdgB8 6 weeks earlier were analyzed daily for virus following intravaginal challenge with 2 × 10^4 pfu of HSV-2. Virus was detected in the samples of all 3 groups of mice over the first 4 days. However, i.n. immunized mice contained lower levels (P < .05) of detectable virus relative to unimmunized mice over the first 2 days (figure 1). By day 5, the virus titers in the vaginal wash samples of both i.n. and i.p. immunized mice had decreased dramatically (>2 logs; P < .001). This trend continued, and the number of immunized mice with detectable virus also decreased substantially. By day 7, virus was undetectable in vaginal wash samples of any of the immunized mice. In contrast, virus was present in the samples from all unimmunized control mice and remained at constant levels for 6 days after challenge.

Survival from intravaginal HSV-2 challenge in mice immunized with AdgB8. Six weeks after AdgB8 immunization, mice were monitored for survival following a lethal intravaginal HSV-2 (2 × 10^4 pfu) infection [13]. None of the unimmunized control mice survived past day 6 due to the rapid onset of pathology that consisted of severe genital ulceration extending to the surrounding tissue (figure 2). Similarly, 8 of 10 mice immunized i.p. with AdgB8 developed genital pathology, and 7 of these succumbed to disease by day 9; the 8th survived as did 2 others that did not display any overt symptoms (figure 2). In contrast, a significant number of mice immunized i.n.
with AdgB8 survived (8/10) the lethal HSV-2 challenge compared with controls (P ≤ .005). Of these mice, 60% were completely protected from any overt genital pathology (P ≤ .01 vs. unimmunized mice). Of interest, 1 of the ip and 2 of the i.n. immunized mice developed genital swelling and redness (genital pathology score ≥2); however, none of the 3 developed severe symptoms. Further, all mice that survived primary infection were free of overt genital pathology and virus in vaginal wash samples for 3 months after challenge.

Long- and short-term resistance to intravaginal HSV-2 infection in mice immunized with AdgB8. Resistance to low- and high-dose intravaginal HSV-2 challenge was examined at short (4–6 weeks) and long (9–10 months) periods after AdgB8 immunization and evaluated by presence and severity of genital pathology (table 1). Naive mice that served as controls were unimmunized and age-matched. The severity scores clearly showed that in low-dose challenge experiments both i.n. and ip immunized mice (at 4–6 weeks) had significantly less genital pathology. Of these mice, 60% were completely protected from any overt genital pathology (P ≤ .01 vs. unimmunized mice). Of interest, 1 of the ip and 2 of the i.n. immunized mice developed genital swelling and redness (genital pathology score ≥2); however, none of the 3 developed severe symptoms. Further, all mice that survived primary infection were free of overt genital pathology and virus in vaginal wash samples for 3 months after challenge.

Figure 1. Viral replication in vaginal wash samples of mice immunized 6 weeks earlier with AdgB8 after intravaginal HSV-2 infection (>100% lethal dose; 2 × 10^4 pfu). Daily vaginal wash samples were analyzed for virus by plaque formation on Vero cell monolayers. * Naive mice with overt genital pathology were sacrificed on day 7. Comparisons among means of groups were performed by analysis of variance. in, intranasal; ip, intraperitoneal.

Figure 2. Survival of mice immunized with AdgB8 6 weeks before intravaginal HSV-2 infection (2 × 10^4 pfu). Mice were monitored daily for survival and signs of disease. Those with genital pathology scores of 5 were sacrificed. For comparisons between 2 groups, data were analyzed by Fisher’s exact test. in, intranasal; ip, intraperitoneal.
pathology than naive mice ($P \leq .001$). However, consistent with the survival data (figure 2), more inl immunized mice remained disease-free with significantly lower overall pathology severity scores (0.6 vs. 3.7; $P \leq .05$) than ip immunized mice. Furthermore, when mice immunized 9–10 months earlier were challenged with a low dose of HSV-2, only inl immunized mice had significant resistance on the basis of severity of genital pathology ($P \leq .05$; table 1). Indeed, only 1 inl immunized mouse had any genital pathology, whereas most of the ip immunized mice developed overt symptoms of infection (4 of 5; table 1). The lower pathology severity scores in naive mice at the later challenge time (9–10 months) compared with the scores 4–6 weeks after challenge are likely due to slower development of genital pathology in aged mice. This increased “natural” resistance likely accounts for the decreased pathology scores observed in aged inl immunized mice (0.6 vs. 0.1).

The development of genital pathology after a higher challenge dose of HSV-2 was also assessed. At the higher dose, both inl and ip immunized mice still displayed significantly less genital pathology than did naive mice ($P \leq .001$) 4–6 weeks after AdgB8 immunization. In addition, mice immunized inl but not ip were significantly protected 9–10 months after AdgB8 immunization ($P \leq .001$). However, the number of inl immunized mice displaying overt pathology increased, as did the overall severity of infection, over that observed at low challenge dose. The severity of infection in ip immunized mice did not appear to change from the low challenge dose. As a result, there was no significant difference in severity of pathology between inl and ip immunized mice at the higher challenge dose at 4–6 weeks or 9–10 months after AdgB8 immunization. However, only inl immunized mice maintained a significant level of protection when compared with naive mice.

**ASC cells specific for HSV gB in the genital tissues of AdgB8 immunized mice 6 days after intravaginal HSV-2 infection.** Specific antibodies in the genital tracts of mice after AdgB8 immunization should neutralize and assist in clearance of virus during an infection. Therefore, the presence of gB-specific ASCs was assessed in the iliac lymph nodes (ILNs) that drain the genital tissues and in genital tissues (uterus, vagina, and fallopian tubes) of AdgB8 immunized and unimmunized mice 6 days after a lethal intravaginal HSV-2 infection. At short (3 weeks) and long (9 months) times after AdgB8 immunization, mice immunized inl and ip contained IgG and IgA gB-specific ASCs in the ILNs (table 2). The gB-specific ASCs in the ILNs represented a large percentage of total ASCs for both IgG and IgA memory responses, especially in mice immunized 3 weeks before HSV-2 challenge. In addition, the ILNs of mice immunized inl (compared with those immunized ip) contained far more IgA ASCs specific for gB both at 3 weeks and 9 months after immunization. In contrast, the ILNs of naive mice undergoing a primary response to HSV-2 infection lacked any IgA ASCs and contained only a few IgG ASCs (1% of total) specific for gB of HSV. Of note, only after inl immunization were gB-specific IgA ASCs found in genital tissues. Assessment of IgG ASCs demonstrated that both inl and ip immunized mice were capable of recruiting gB-specific IgG ASCs to the genital tissues after HSV-2 infection (table 2). However, at long periods after ip immunization (9 months), specific IgG ASCs were not always detectable in genital tissues.

**Discussion**

Many different formulations and routes of antigen exposure have been explored for the induction of immunity in the female genital tract against HSV and other sexually transmitted pathogens [1, 2, 6, 7, 15, 16]. Several studies have used the inl route of immunization to successfully provide humoral immunity in the female genital tract with both IgA and IgG specificities [18–20, 22, 25]. Our previous results using inl immunization with AdgB8 also demonstrated the presence of gB-specific IgG and IgA antibodies in mouse sera and vaginal wash specimens [24, 26]. In contrast, systemic immunization resulted only in IgG antibodies in vaginal fluids. We also observed that although both inl and ip AdgB8 immunization induced short-term CTL responses, only inl immunized mice maintained long-lived CTL responses in genital-associated lymphoid tissues [17]. This appears to be a relevant finding since McDermott et al. [14] in adoptive transfer studies demonstrated that immune T cells from the genital lymph nodes of mice infected intravaginally with an attenuated strain of HSV-2 could mediate protection against a subsequent intravaginal HSV-2 challenge.

<table>
<thead>
<tr>
<th>Table 1. Genital pathology after intravaginal HSV-2 infection of AdgB8-immunized mice.</th>
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<tr>
<td><strong>Time to challenge, group</strong></td>
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<tr>
<td><strong>4–6 weeks after immunization</strong></td>
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<td>Naive</td>
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<td><strong>9–10 months after immunization</strong></td>
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* Mice were immunized intranasally (inl) or intraperitoneally (ip) with AdgB8 and challenged intravaginally 4–6 weeks or 9–10 months later with $2 \times 10^4$ pfu (low dose) or $1 \times 10^5$ (high dose of HSV-2).

1 No. of mice that demonstrated overt genital pathology/total (i.e., score $\geq 1$).

2 Mean $\pm$ SD; measured as area under lesion score–day curve for first 6 days.

3 $P < .05$ (AdgB8 immunized vs. unimmunized mice).

4 $P < .001$ (AdgB8 immunized vs. unimmunized mice).

5 $P < .05$ (ip vs. ip immunized mice).
Table 2. HSV gB-specific antibody secreting cells (ASC) in genital tissues of AdgB8-immunized and naive mice 6 days after intravaginal HSV-2 infection.

<table>
<thead>
<tr>
<th>Tissue, immunization*</th>
<th>Time after immunization</th>
<th>Mean HSVgB-specific ASC/10⁶ mononuclear cells²</th>
<th>IgA</th>
<th>% of total</th>
<th>IgG</th>
<th>% of total</th>
</tr>
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<tr>
<td>Iliac lymph nodes</td>
<td>3 weeks</td>
<td>94 ± 11</td>
<td>70</td>
<td>1700 ± 131</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 months</td>
<td>29 ± 7</td>
<td>30</td>
<td>595 ± 76</td>
<td>17</td>
<td></td>
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<tr>
<td></td>
<td>ip</td>
<td>18 ± 4</td>
<td>26</td>
<td>1540 ± 177</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 months</td>
<td>9 ± 2</td>
<td>14</td>
<td>2025 ± 237</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Naive Ð</td>
<td>—</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>65 ± 41</td>
<td>1</td>
<td></td>
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<tr>
<td>Genital tract</td>
<td>3 weeks</td>
<td>17 ± 3</td>
<td>11</td>
<td>30 ± 26</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 months</td>
<td>7 ± 3</td>
<td>4</td>
<td>10 ± 20</td>
<td>4</td>
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<tr>
<td></td>
<td>ip</td>
<td>&lt;2</td>
<td>&lt;1</td>
<td>15 ± 10</td>
<td>5</td>
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<td></td>
<td>9 months</td>
<td>&lt;2</td>
<td>&lt;1</td>
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* Naive or AdgB8-immunized mice were challenged intravaginally with 10⁵ pfu of HSV-2, and 6 days later iliac lymph nodes and genital tracts were isolated from groups of 5 mice. Mononuclear cells were analyzed by ELISPOT for HSV gB-specific antibody-secreting cells.

² Results are expressed as mean ± SD of triplicate wells and as % of total ASC observed.

Here we compared the level of protection after inl or ip immunization at short and long periods against an intravaginal HSV-2 infection. Although no unimmunized mice survived a lethal HSV-2 intravaginal challenge, we observed a large degree of protection shortly after AdgB8 immunization, especially in mice immunized inl. Moreover, long-term protection was primarily maintained in mice immunized inl. It is conceivable that the mice immunized inl received a larger immune dose, perhaps due to viral replication in the respiratory tract. However, our previous studies indicated that with the same vaccine dose, the initial levels of systemic and mucosal antibodies and CTL [17, 24, 26] (except for IgA) were similar with either route of AdgB8 immunization. Thus, the superior and long-lasting protection observed in inl immunized mice against an intravaginal HSV-2 infection is likely attributable to other factors, such as the presence of sIgA or long-lived CTL responses [13, 17, 24, 26]. The fact that ip immunized mice are significantly protected for short periods after immunization is in agreement with the proposal [17] that shortly after immunization there is a period during which immune lymphocytes are present in all immune compartments, regardless of the immunization route. Conversely, at longer periods following immunization, memory lymphocytes tend to segregate to tissues related to those in which they were first induced [17].

The doses of challenge virus that we used likely exceed those that would occur naturally, and perhaps as a consequence of this, we observed a dose dependency on severity of infection in inl immunized mice. At a high HSV-2 challenge dose, there was an increase in both the severity of infection and the number of mice with overt genital pathology. These symptoms were similar to those observed in ip immunized mice, suggesting that mucosal-specific immune functions may become overwhelmed, whereas systemic immune functions can still maintain some level of protection during higher challenge doses. These results are in agreement with our earlier observations [13] and those of others [21, 23] that showed intranasal immunization is capable of mediating protection against infections of the female genital tract. We believe these observations to be the first to indicate that inl immunization provides long-lasting and superior resistance to intravaginal infection with a sexually transmitted virus compared with ip immunization at the same dose. Consistent with these observations, Marx et al. [22] showed that intramuscular plus intratracheal or oral, but not intramuscular plus intramuscular, vaccination protected macaques against intravaginal simian immunodeficiency virus infection [22]. The results of McLean et al. [27] also suggest that mucosal immunization provides optimal mucosal protection. In their studies, intravaginal immunization of guinea pigs with a genetically disabled HSV-1 virus better protected the animals against intravaginal infection with wild type virus than did ip immunization.

sIgA, which is considered to be of primary importance in mucosal immunity, can prevent infection of mucosal tissues [1, 2, 6, 7, 15, 16]. The main activity of IgA has been generally regarded as its ability to neutralize or prevent attachment of pathogens at the apical surface of mucosal tissues [1, 2, 6, 7, 15, 16]. However, in vitro [4, 5] and in vivo [3] models have shown that sIgA can form complexes with viruses intracellularly, inhibiting viral replication and subsequently preventing primary or resolving chronic infections. Of interest, in our studies of immunized mice infected intravaginally with HSV-2, viral replication continued in the genital tract for the first 4 days. Thus, AdgB8 immunization did not result in sterile immu-
nity in the genital tract. This may have been due to the large challenge doses or because to successfully infect mice intravaginally with HSV-2, it is necessary to induce them into a diestrous-like state. This is achieved following progesterone administration and, as we have observed [13], this treatment results in increased levels of IgG antibodies and conversely in decreased levels of IgA antibodies in genital fluids. Nevertheless, by day 5, the levels of virus in vaginal wash samples had significantly decreased, and by day 7, none of the immunized mice had any free virus. Of particular importance was the observation that virus became undetectable, even in the vaginal samples of mice that developed severe genital pathology. We therefore assessed the ASCs present in the genital-associated lymphoid tissues and in the genital tract during this period of virus resolution to determine whether local antibody production may be responsible for neutralization of virus in vaginal samples late in infection.

The genital tissues of mice immunized inl or ip with AdgB8, but not unimmunized mice, contained gB-specific IgG ASCs. Significantly, only inl immunized mice had IgA ASCs in the genital tissues, even in the progesterone-dominated environment. Furthermore, while both inl and ip immunized mice developed high numbers of gB-specific IgG ASCs in the ILNs draining the genital tissues, inl immunized mice generated much stronger IgA ASC responses than did ip immunized mice. These results suggest that gB-specific IgA antibodies in inl immunized mice are present locally during virus clearance and are likely responsible for the neutralization of virus. The superior protection afforded inl immunized mice may thus reflect the mucosal specific functions of local IgA antibodies in addition to local cellular responses [17].

In terms of the development of successful vaccine strategies for STDs, numerous routes and antigen formulations have been examined [1, 2, 6, 7, 15, 16]. We previously showed that inl immunization with an adenovirus vector expressing a single HSV glycoprotein can induce long-lived mucosal specific IgA and IgG antibodies as well as CTL [13, 17, 24, 26]. In the present study, when we compared the inl and ip routes of immunization, we found that mice immunized inl with AdgB8 maintained higher levels of protection against a lethal intravaginal HSV-2 challenge. Protection occurred even though the mice were initially infected. The enhanced survival in inl immunized mice was consistent with the presence of a long-lived herpes-specific IgA ASC response in the genital tissues and not with vaginal wash virus titers.

Acknowledgments

We thank Rae Lyn Burke for the recombinant gB used in the ELISPOT assays, Frank L. Graham and David C. Johnson for the recombinant adenovirus vector, AdgB8, and L. Arsenault and the Electron Microscopy Group, McMaster University, for writing the computer program and for providing equipment for analysis of ELISPOT data.

References


