Effects of the Estrous Cycle on Local Humoral Immune Responses and Protection of Intranasally Immunized Female Mice against Herpes Simplex Virus Type 2 Infection in the Genital Tract

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This study demonstrates that the levels of gB-specific IgG and IgA in vaginal washes of mice immunized intranasally (i.n.) with a recombinant adenovirus vector expressing herpes simplex virus (HSV) glycoprotein B (AdgB8) vary inversely with each other and are dependent on the stage of the estrous cycle. Anti-gB IgA titers in vaginal washes were significantly higher during estrus than diestrus or proestrus, whereas specific IgG titers were significantly higher during diestrus than estrus. This was further demonstrated in hormone-treated mice, where progesterone administration induced a diestrus-like state that resulted in elevated specific IgG-to-IgA ratios. Interestingly, unimmunized mice were only susceptible to intravaginal (ivag) infection with HSV-2 during diestrus. Mice immunized i.n. with AdgB8 and given progesterone were protected from a lethal intravaginal HSV-2 challenge, despite the fact that virus replication was present for 4 days postchallenge. Further, high numbers of gB-specific IgA and IgG antibody-secreting cells were present in both the genital tracts and the draining iliac lymph nodes of i.n.-immunized, but not unimmunized, mice 6 days following ivag HSV-2 challenge. These results demonstrate that the levels of specific antibodies in the female genital tract are dependent on the stage of the estrous cycle. Furthermore, i.n. AdgB8 immunization provided a significant level of protection and specific IgA and IgG antibody-secreting cells in the genital tissues during resolution of an ivag infection with HSV-2.

INTRODUCTION

Antibodies in the mucosal secretions of the genital tract represent the first specific immune barrier against the penetration of sexually transmitted pathogens (Brandtzæg et al., 1994; Mestecky, 1987; Mestecky et al., 1994; Mogens and Russell, 1994). Antibodies of both IgG and IgA isotypes are present in genital secretions and both have been shown to be important in this protection (Brunham et al., 1983; Eis-Hubinger et al., 1993; Merriman et al., 1984; Mogens and Russell, 1994; Whaley et al., 1994). Secretory IgA is particularly well suited for mucosal surfaces due to its greater avidity and resistance to proteolytic cleavage (Magnusson and Stjernstrom, 1982; Mogens and Russell, 1994). Although the primary function of IgA has been regarded as the ability to neutralize pathogens at the mucosal surface (Mogens and Russell, 1994), more recently, in vitro (Mazanec et al., 1992, 1995) and in vivo (Burns et al., 1996) models have demonstrated that secretory IgA is capable of forming intracellular complexes with viruses and inhibiting virus replication and subsequently preventing primary or resolving chronic infections.

The presence of secretory IgA at the mucosal surface of the murine genital tract is mainly a result of active transport by secretory component (SC) through the uterine epithelium, whereas IgG found in cervicovaginal secretions is generally due to transudation through the vaginal epithelium (Brandtzæg et al., 1994; Mestecky, 1987; Mestecky et al., 1994; Parr and Parr, 1990, 1994a; Underdown and Mestecky, 1994). The levels of SC and total immunoglobulins in the female genital tract have been reported to change over the course of the estrous cycle (Sullivan and Wira, 1983; Wira and Sandoe, 1977, 1980; Wira et al., 1994). In rodents, SC is present at its highest levels in uterine fluids during proestrus and estrus (Sullivan and Wira, 1983). Since SC is responsible for delivering IgA to the mucosal surface it is not surprising that total IgA levels in rat uterine secretions were significantly higher during estrus and proestrus when compared to diestrus (Wira and Sandoe, 1977, 1980; Wira et al., 1994). The levels of total IgG have also been shown to change with the estrous cycle with the highest levels occurring during proestrus (Wira and Sandoe, 1977, 1980; Wira et al., 1994). The migration of lymphocytes into the genital tract is also influenced by the estrous cycle since increased numbers of IgA plasma cells are observed in genital tissues during proestrus and estrus (Canning and Billington, 1983; McDermott et al., 1980; Rachman et al., 1983). These cyclic changes in SC and total antibody levels are also observed in humans (Schumacher, 1980;
Sullivan et al., 1984; Suzuki et al., 1984; Usala et al., 1989), suggesting that the hormones that control the reproductive cycle are intimately involved in these changes. Indeed, progesterone and estrogen have been shown to be directly responsible for influencing the levels of SC and antibodies, as well as the numbers of B cells in the genital tract (Parr and Parr, 1994a; Sullivan et al., 1983; Wang et al., 1996; Wira and Sandoe, 1977, 1980; Wira et al., 1994).

As mentioned, the induction of specific antibodies in genital secretions is considered necessary for protection against infection with sexually transmitted pathogens such as herpes simplex virus (HSV) or HIV (Brandtzæg et al., 1994; Mestecky, 1987; Mestecky et al., 1994; Mogens and Russell, 1994). In examining the induction of humoral immunity in the female genital tract, immunization by systemic routes (sc, im, and ip) has been shown to induce specific antibodies in several species including humans (Bouvet et al., 1994; Gallichan and Rosenthal, 1995; Miller et al., 1992; Nakao et al., 1994; Ogra and Ogra, 1973; Parr and Parr, 1990; Thapar et al., 1990a,b). Although high titers of specific IgG antibodies were induced in these studies, the levels of specific IgA were generally low or not reported. As a mucosal route of immunization, intravaginal (ivag) or intrauterine inoculation has been shown by some groups to induce both specific IgG and IgA antibodies in the genital tract (Milligan and Bernstein, 1995; Ogra and Ogra, 1973; Wira et al., 1994). However, in many other studies the titers were low or consisted mainly of IgG antibodies (Gallichan and Rosenthal, 1995; Lehner et al., 1992; McDermott et al., 1990; Miller et al., 1992; Parr and Parr, 1994; Parr et al., 1988; Thapar et al., 1990a,b), perhaps attributable to the generalized lack of secondary lymphoid nodules in the genital tract (Parr and Parr, 1994). In contrast, immunization at other mucosal sites (ig or i.n.) has been successful in generating specific IgG and IgA antibodies in the genital tract (Gallichan and Rosenthal, 1995; Lubeck et al., 1994; Muster et al., 1995; Wu and Russell, 1993). Few of the studies concerned with specific antibodies in the genital tract took into account the stage of the reproductive cycle during sampling and evaluation of antibody titers.

Herpes simplex virus type 2 is a sexually transmitted agent that attaches, penetrates, and undergoes infectious cycles of replication in the epithelium of the genital tract. Most studies concerned with protection against genital HSV-2 infection have focused on systemic immunization (Bernstein et al., 1990; Burke et al., 1994; Byars et al., 1994; Heineman et al., 1995; Nakao et al., 1994; Straus et al., 1994), despite the evidence supporting mucosal immunization as the optimum route for the induction of both IgA and IgG antibodies in the genital tract. The exception to this involves ivag immunization with attenuated strains of HSV-2 that protected mice against ivag HSV-2 challenge (McDermott et al., 1987; McLean et al., 1994; Milligan and Bernstein, 1995; Parr et al., 1994). Recently we showed that intranasal (i.n.) immunization with a recombinant adenovirus capable of expressing gB of HSV-1 (AdgB8) induced both gB-specific IgA and IgG antibodies in vaginal washes of mice (Gallichan and Rosenthal, 1995). In this study we examined the influence of the estrous cycle on the levels of HSV gB-specific IgA and IgG antibodies in the female genital tract following i.n. immunization with AdgB8. In addition, mice immunized intranasally with AdgB8 were challenged intravaginally with HSV-2 and the subsequent pathology, viral replication, and B cell responses in the genital tissues were evaluated.

**MATERIALS AND METHODS**

**Animals and cell cultures**

Female C57Bl/6 (H-2b) mice used in immunization and challenge studies and in the evaluation of vaginal wash antibody levels were 6 to 8 weeks of age during primary immunization and were obtained from Charles River Laboratories (Constant, Quebec, Canada). Mouse colonies were maintained on a 12-hr light/dark cycle. Vero and 293 cells were grown in α-MEM (GIBCO Laboratories, Burlington, Canada), supplemented with 10% fetal calf serum (FCS; GIBCO) and 1% penicillin–streptomycin and L-glutamine (GIBCO). 293-N2S cells are a nonadherent cell line derived from 293 cells and were grown in spinner flasks with Joklik’s media supplemented as above.

**Virus strains and inoculations**

The construction of the replication-competent recombinant adenovirus type 5 vector, AdgB8, was reported elsewhere (Hutchinson et al., 1993). Briefly, 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 titrated on 293 cells. HSV-2 strain 333 was propagated and titrated on Vero cells.

For AdgB8 immunization, mice were ether anesthetized, inverted, and inoculated i.n. with 10^{6} PFU of AdgB8 by introducing virus in 10 μl of phosphate-buffered saline (PBS, pH 7.4) directly into the nares by means of a micropipet. For ivag HSV-2 challenge, mice were first injected subcutaneously with 2 mg of progesterone/mouse (Depo-Provera; Upjohn, Don Mills, Ontario), and 5 days later mice were anesthetized using halothane, swabbed ivag with a cotton applicator, placed on their backs, and infected intravaginally for 1 hr with 10 μl of HSV-2 while being maintained under anesthetic.

**Collection of fluids and estrus staging**

Vaginal fluid for estrus staging and antibody determinations was collected by pipetting 30 μl of PBS into and
out of the vagina several times. The staging of the estrous cycle for each mouse was based on a smear from these washings (Allen, 1922). Smears were stained with Diff-Quik (Baxter Scientific Products, Miami, FL). By examining the cells present in the smears we were able to determine whether the animal was in estrus, metestrus, diestrus, or proestrus. The vaginal washings were then centrifuged to remove particulate matter and the supernatants were stored at −20°C for subsequent antibody determination.

Viral replication and pathology in the reproductive tract

Following ivag HSV-2 inoculation, mice were sampled daily for 6 days by pipetting 30 μl of PBS into and out of the vagina followed by a swabbing with a cotton applicator. Both the wash and the applicator were combined with 0.97 ml of PBS and frozen at −70°C. Viral 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 following 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, swelling and hair loss of genital and surrounding tissue; 5, severe genital ulceration extending to surrounding tissue. Mice were sacrificed upon reaching stage 5.

Antibody determination

Total and HSV gB-specific antibody titers were determined by ELISAs performed in flat-bottomed microtiter plates (Costar, Cambridge, MA). Plates were precoated with 2.5 μg/ml recombinant HSV-2 gB (kindly provided by R. L. Burke, Chiron, Emeryville, CA) for gB-specific antibody titers, and 1/1000 dilution of goat anti-mouse IgG or IgA (Southern Biotechnology Associates, Birmingham, AL) for total antibody titers, in borate-buffered saline, pH 8.5, and kept overnight at 4°C. A Tris-buffered saline solution containing 10 mg/ml bovine serum albumin, pH 7.4, was used to block any nitrocellulose not precoated with antibody or gB protein. Serially diluted single cell suspensions of iliac lymph nodes or genital tract digests plus supplemented RPMI medium (10% FCS) were plated at 37°C for 16 hr. The number of plasma cells in each preparation secreting total or HSV gB-specific IgA or IgG antibody was determined by ELISPOT assay.

Antibody-secreting cell (ASC) enumeration by ELISPOT assay

Ninety-six-well filtration plates backed with nitrocellulose membrane (Millipore Corp., Bedford, MA) were coated with 10 μg/ml recombinant HSV-2 gB (provided by R. L. Burke) or 1/500 dilution of goat anti-mouse IgG or IgA (Southern Biotechnology Associates) in PBS, and kept overnight at 4°C. A PBS solution containing 10 mg/ml bovine serum albumin, pH 7.4, was used to block any nitrocellulose not precoated with antibody or gB protein. Serially diluted single cell suspensions of iliac lymph nodes or genital tract digests plus supplemented RPMI media (10% FCS) were plated at 37°C for 16 hr. The number of plasma cells in each preparation secreting total or HSV gB-specific IgA or IgG antibody was determined by washing each well with PBS - Tween 20 to remove cells and developing the plate by the addition of biotinylated goat anti-mouse IgA or anti-IgG (Southern Biotechnology Associates), followed by avidin - peroxidase. Spots representing individual antibody-secreting cells were visualized by developing with peroxidase substrate containing H2O2 and 3-amino-9-ethylcarbazole in acetate buffer. Spots were enumerated by digitized image analysis and discrimination from background was based on gray density and then size (program written by Dr. L. Arsenault, Microscopy Group, McMaster University, Hamilton, Ontario). Counts were visually confirmed in case of overlapping spots. Results are expressed as the mean number of ASC per million mononuclear cells for each tissue.
Statistical analysis

Data were analyzed using the GraphPAD InStat program (GraphPAD Software, San Diego, CA). 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 carried out using analysis of variance.

RESULTS

Variation in specific vaginal wash antibody levels during the estrous cycle

In our previous studies we observed the presence of specific antibodies to gB of HSV-1 in the vaginal washes of mice immunized i.n. with AdgB8 (Gallichan and Rosenthal, 1995). The levels of these antibodies tended to vary greatly between mice and within individual mice over time. Since immunoglobulin levels are influenced by the estrous cycle, we determined the titers of HSV gB-specific IgG and IgA in vaginal washes on a daily basis and correlated them with the stage of the estrous cycle. Figure 1A shows data from a representative mouse immunized 6 weeks previously with AdgB8 and sampled daily over two estrous cycles. The absolute titers of gB-specific antibodies in this mouse demonstrated that, indeed, there was a large variation from day to day. Moreover, there appeared to be a cyclic fluctuation in the titers of gB-specific IgG and IgA that varied with the stage of the estrous cycle. Anti-gB IgA titers were generally highest during estrus and, conversely, anti-gB IgG titers tended to be lowest during estrus and higher during other periods of the cycle. To examine these fluctuations in a group of mice, titers from the vaginal washes of four mice immunized i.n. with AdgB8 were pooled over two estrous cycles and expressed as percentages of maximums to accommodate the varying magnitudes of specific antibodies observed. In Fig. 2A it is clear that the levels of anti-gB IgA and IgG fluctuated with the estrous cycle. In fact, the levels of anti-gB IgA during estrus were significantly higher than those observed during diestrus or proestrus ($P \leq 0.05$). In contrast, the levels of anti-gB IgG observed during diestrus were significantly higher than during estrus ($P \leq 0.001$) (Fig. 2A). This inverse relationship between isotype expression indicates that over the course of the estrous cycle specific IgA levels are highest during periods of estrus and lowest during diestrus, whereas specific IgG levels are highest during periods of diestrus and lowest during estrus. The levels of specific IgA and IgG during metestrus and proestrus lie in between.

Ratios of specific IgG-to-IgA antibodies in vaginal washes

The relationship between the levels of specific IgG relative to IgA was examined at each stage of the estrous cycle by determining the ratios of gB-specific IgG to IgA.

**FIG. 1.** The effect of the estrous cycle on gB-specific antibody levels in the vaginal washes of a mouse immunized intranasally with AdgB8. The stage of the estrous cycle was determined daily by cytology from a smear of the vaginal washes of a representative mouse over a 16-day period: estrus (E), metestrus (M), diestrus (D), proestrus (P). (A) The levels of HSV gB-specific IgA (hatched bars) and IgG (solid bars) were determined daily using a gB-specific ELISA. (B) The ratios of HSV gB-specific IgG to IgA over the 16 days for the same mouse in A.

Figure 1B demonstrates that the gB-specific IgG-to-IgA ratios from the vaginal washes of the mouse in Fig. 1A followed a cyclic pattern and were highest during diestrus and lowest during estrus. In addition, there was as much as a 93-fold difference in ratios between estrus and diestrus. These cyclic trends were similar in all mice that we examined. To examine a group of mice, the ratios of anti-gB IgG to IgA from four mice were pooled (over two cycles each) from each of the four estrous stages by first normalizing the ratios in each mouse to a scale of 1.0. The ratios in Fig. 2B demonstrate that the relative levels of gB-specific IgG compared to IgA were significantly higher during diestrus compared to all other stages ($P \leq 0.001$). In addition, the ratio at diestrus was 15 times greater than during estrus, and conversely, during estrus the levels of specific IgA were relatively much higher than IgG. The inverse relationship between levels of gB-specific IgG and IgA at each stage of estrus was
observed in the vaginal washes of mice infected during diestrus or late metestrus. There was no virus replication observed in mice infected at estrus. The results presented in Table 1 are representative of several experiments examining a number of animals at each stage. Interestingly, the dose of HSV-2 used for infection represents more than a 1000-fold lethal dose (unpublished data). Thus mice appeared to be highly resistant to intravaginal HSV-2 infection at estrus. The stages of the estrous cycle occurring on days subsequent to infection did not seem to influence the amount of virus recovered in vaginal washes (data not shown). By Day 6 the mice that were infected during diestrus or late metestrus had developed moderate to severe pathology of the exterior genital tissues. In contrast, mice infected during estrus displayed no genital pathology.

To examine the site and extent of tissues infected, the uterus, vagina (including the cervix), and nerves innervating the genital tissues were isolated. Only the tissues from mice infected during diestrus or late metestrus contained virus (Table 1). The level of infection in vaginal tissues was severalfold higher than in the uterus of all mice we examined, indicating that the primary site of HSV-2 infection following intravaginal inoculation is in the vagina. Virus was also observed in the nerves of severely infected animals. These results indicate that mice are primarily susceptible to HSV-2 infection during early to late periods of diestrus and that the primary site of infection is in the vagina, with further infection of the uterus and ultimately the nerves occurring in severely infected animals.

Influence of progesterone on specific antibody ratios in vaginal washes

Administration of progesterone induces mice into a diestrus-like state and is known to make mice highly susceptible to intravaginal infection with HSV (Parr et al., 1994). To determine the influence of this treatment on specific antibody levels in the genital tract we examined the ratios of vaginal wash anti-gB IgG to IgA in six mice that were immunized intranasally with AdgB8. By Day 3 the ratios had increased dramatically and were significantly greater than the preceding days (P < 0.05) (Fig. 3). In addition, by Day 3 post-hormone treatment all six mice had entered a diestrus-like state which was maintained for more than a week. During this diestrus-like state the titers of gB-specific IgA antibodies were extremely low or undetectable.

Intranasal immunization protects against intravaginal HSV-2 infection

Next we determined whether mice immunized intranasally with AdgB8 were protected from ivag HSV-2 infection during a progesterone-induced diestrus-like state. Five days following progesterone treatment, control (un-
TABLE 1

Effects of the Estrous Cycle on HSV-2 Replication in the Genital Tract Following Intravaginal Infection

<table>
<thead>
<tr>
<th>Estrous stage</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>HSV-2 titer (10^7 PFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage</td>
<td>Titer</td>
<td>Stage</td>
<td>Titer</td>
</tr>
<tr>
<td>Estrous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>M</td>
<td>0</td>
<td>M</td>
<td>0</td>
</tr>
<tr>
<td>M</td>
<td>D</td>
<td>1</td>
<td>D</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>D</td>
<td>11</td>
<td>D</td>
<td>740</td>
</tr>
<tr>
<td>P</td>
<td>E</td>
<td>1</td>
<td>E</td>
<td>0</td>
</tr>
</tbody>
</table>

a Mice were inoculated intravaginally with 2 x 10^7 PFU HSV-2 and vaginal washes were taken daily for determination of virus titers and the estrus stage.

b On the 6th day following infection tissues were isolated and homogenized, and viral titers were determined.

c Estrous staging was determined histologically: estrus (E), metestrus (M), diestrus (D), proestrus (P).

immunized) and immunized mice were inoculated ivag with a lethal dose of HSV-2 (Table 2). Subsequent daily evaluation of genital pathology, survival, and virus replication in vaginal washes of control and immunized animals was determined in a blinded fashion. All of the control animals were infected with virus and demonstrated genital pathology by Day 4, eventually succumbing to infection on Day 6 (having reached a genital pathology score of 5). In contrast, mice immunized i.n. with AdgB8 demonstrated a significant level of protection against HSV-2 infection. Although virus replication was observed in the genital tracts of all the immunized mice for the first 4 days postinfection, by Day 5, virus was isolated from only 8 of the immunized mice (P < 0.01). In addition, by Day 5 there was a significant decrease in viral replication in the 6 remaining immunized mice (P < 0.0001). The severity of infection or genital pathology was also significantly reduced in the i.n.-immunized mice over the 6 days (P < 0.0001). Of the 14 AdgB8-immunized mice, 5 were completely protected from any discernable genital pathology. Interestingly, these 5 mice were among the mice that had cleared any signs of viral replication by Day 5. The remaining 9 mice all developed genital pathology; however, 4 of these mice displayed only minor symptoms (pathology score ≤ 3), and subsequently cleared the infection by Day 7 and any signs of pathology by Day 12. Therefore, a significant number of immunized mice (9 of the 14) survived past acute infection (P < 0.005).

Detection of specific antibody-secreting cells in the genital tract

The presence of ASC specific for gB of HSV-2 was examined in the genital tracts and iliac lymph nodes (ILN) of immunized and control mice following an ivag HSV-2 challenge. High numbers of gB-specific IgA and IgG ASC were present in both the genital tracts and the draining ILN of i.n.-immunized mice 6 days following ivag HSV-2 challenge (Table 3). The gB-specific ASC observed in the ILN represented 65 and 35% of the total IgA and IgG ASC present, respectively. In the genital tracts they represented 8 and 92% of the total IgA and IgG ASC present, respectively. This is in contrast to control animals in which no gB-specific ASC were observed in the genital tissues and only a small number of IgG ASC were observed in the ILN. The IgG ASC present in the ILN of control animals likely represents the development of a primary response and consisted of only 2% of the total IgG ASC present (and was 19 times less than that observed in the ILN of immunized mice). These results were confirmed in two separate experiments.

DISCUSSION

The capability of the female genital tract to maintain a high level of sterility and at the same time provide an
Protection and Virus Replication in the Genital Tracts of Mice Immunized Intranasally with AdgB8 and Challenged Intravaginally with HSV-2

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus isolation</th>
<th>Vaginal viral replication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days 1-4</td>
<td>Day 5</td>
</tr>
<tr>
<td>Unimmunized</td>
<td>14/14</td>
<td>14/14</td>
</tr>
<tr>
<td>Immunized</td>
<td>14/14</td>
<td>8/14***</td>
</tr>
</tbody>
</table>

Note. Significance between AdgB8-immunized and unimmunized mice: *P < 0.0001; **P < 0.005; ***P < 0.01; ****P < 0.025.

Mice were intravaginally challenged with 2 x 10^4 PFU of HSV-2; immunized mice were inoculated with AdgB8 3 weeks previously.

Number of mice in which virus was detected/total.

Mean ± SD log₁₀ PFU; calculated using only animals that experienced viral replication.

Mean ± SD; measured as area under the lesion score-day curve for first 6 days.

Number of mice with that demonstrated no overt genital pathology/total (i.e., score of 0).

Number of mice surviving infection/total.

This inverse relationship in specific antibody levels likely reflects the changes that occur in the female reproductive tract during the course of the estrous cycle. During estrus, or the time of mating, the female genital tract is subjected to numerous pathogens (Parr and Parr, 1994; Profet, 1993; Tristram and Ogra, 1994). In fact, sperm has been shown to be a vector for bacteria, whereby the bacteria attach to the tails of sperm as they move up the reproductive tract (Profet, 1993). In terms of protection at mucosal surfaces secretory IgA is an important component (Brandzaeg et al., 1994; Magnusson and Stjernstrom, 1982; Mestecky, 1987; Mestecky et al., 1994; Mogens and Russell, 1994; Underdown and Mestecky, 1994) and therefore, it follows that there be high levels of IgA present during this period to deal with the increased

TABLE 3

HSVgB-Specific Antibody-Secreting Cells in Genital Tissues of Intranasally Immunized Mice 6 Days Following Intravaginal HSV-2 Challenge

<table>
<thead>
<tr>
<th>Immunization</th>
<th>IgA</th>
<th>% of total</th>
<th>IgG</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILN</td>
<td>66.8 ± 6.3</td>
<td>65</td>
<td>1810 ± 151</td>
<td>34.5</td>
</tr>
<tr>
<td>i.n. AdgB</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>95.0 ± 56.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Unimmunized</td>
<td>68 ± 4.0</td>
<td>8.1</td>
<td>1275 ± 170</td>
<td>91.6</td>
</tr>
<tr>
<td>Genital tract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.n. AdgB</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Unimmunized</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Mice were unimmunized or immunized intranasally with AdgB8 3 weeks prior to ivag HSV-2 challenge. Six days following challenge the ILN and genital tracts were isolated from groups of five mice and the mononuclear cells were isolated and analyzed by ELISPOT for HSVgB-specific antibody-secreting cells.

Results are expressed as the mean ± SD of triplicate wells and as a percentage of total antibody-secreting mononuclear cells observed.

environment suitable for conception requires the coordination of a large number of specific and nonspecific factors. The main specific factors that contribute to protection in the genital tract include the effector functions of humoral- and cell-mediated immunity (Brandzaeg et al., 1994; Mestecky et al., 1994; Parr and Parr, 1994; Tristram and Ogra, 1994; Underdown and Mestecky, 1994). Indeed, both arms of the immune system have been shown to be important for resistance to various pathogens that infect the female genital tract (Brunham et al., 1983; Eishubinger et al., 1993; McDermott et al., 1987, 1989, 1990; Merriman et al., 1984; Parr and Parr, 1994; Whaley et al., 1994). Arguably, humoral immunity or antibodies are the first specific barrier encountered by sexually transmitted pathogens (Brandzaeg et al., 1994; Mestecky, 1987; Mestecky et al., 1994; Underdown and Mestecky, 1994). Both IgA and IgG are present in secretions from the murine genital tract and, anatomically, IgA originates mainly from the uterus/endocervix and IgG from the vagina (Mestecky, 1987; Parr and Parr, 1990, 1994; Wira et al., 1994). In our previous investigations, we demonstrated that i.n. immunization with a recombinant adenovirus vector capable of expressing gB of HSV-1 (AdgB8) induced both IgA and IgG specific for gB in the genital tract (Gallichan and Rosenthal, 1995). The current study demonstrates that the levels of specific IgG and IgA antibodies in the genital tract of intranasally immunized mice vary inversely with each other and are dependent on the stage of the estrous cycle. Indeed, specific IgA titers were higher during estrus than diestrus and specific IgG titers were higher during diestrus than estrus. This was further demonstrated in hormone-treated mice where progesterone administration induced a diestrus-like state that resulted in elevated specific IgG-to-IgA ratios. In fact, while specific IgG titers reached their highest levels, specific IgA titers were extremely low to undetectable. The fluctuations in levels of specific antibodies that we detected are similar to those reported for total (Parr, 1994; Sullivan et al., 1984; Wang et al., 1996; Wira et al., 1994) and specific (Wira and Sandoe, 1980) antibodies during the various stages of the estrous cycle or under the influence of sex hormones.
pathogen load associated with mating. Indeed, antibodies can be found in the uterine lumen bound to bacteria shortly after mating (Parr and Parr, 1985). There may be two hormonally controlled factors that contribute to increased IgA levels during estrus. First, there is an increased migration of plasma cells to the genital tract resulting in a greater number of IgA plasma cells during proestrus and estrus (McDermott et al., 1980; Rachman et al., 1983). Second, there is an increase in production of SC in the uterine epithelium that fluctuates with the estrous cycle and is associated with estradiol administration (Sullivan et al., 1983; Wira et al., 1994). Interestingly, exogenous estradiol and progesterone influence the levels of total IgA or IgG in the genital tract as well as both of the above phenomena, suggesting that the increased IgA levels observed during estrus are directly a result of the fluctuating hormone levels that occur over the course of the estrous cycle (Wang et al., 1996; Wira et al., 1994; Wira and Sandoe, 1980).

The factors contributing to the relative decrease in IgG during estrus are likely due in part to architecture changes in the epithelium of the vagina. IgG originates mainly from serum transudation through the vagina and the vaginal epithelium is known to undergo changes from a thin (3 to 7 layers) stratified epithelium at diestrus to a thick (12 to 13 layers) keratinized epithelium with a clearly defined basement membrane at estrus (Allen, 1922; Parr and Parr, 1994). These changes are also influenced by hormones with estrogen-dominated and progesterone-dominated vaginal epithelium being similar to that of estrus and diestrus, respectively (Parr et al., 1994). The physical structure of the vaginal epithelium, which is thinner during estrus, also explains the increase in specific IgG that we observed during this stage and which is likely due to increased serum transudation. In fact, proteins can penetrate the vaginal epithelium during diestrus and IgG has been localized in the intercellular spaces during this period (Parr and Parr, 1994). It would thus appear that the results presented here reflect the ability of the genital tract to compensate for the decrease in protective IgG during estrus by actively recruiting IgA plasma cells and transporting IgA via SC into the uterine lumen.

The physical changes that occur in the vagina during the transition from diestrus to estrus are likely in preparation for mating and the increased pathogen load associated with this activity. Our data clearly show that unimmunized mice were susceptible to intravaginal HSV-2 infection during diestrus but not during estrus. Indeed, even at 1000-fold lethal dose mice were resistant to vaginal infection during estrus. Thus our findings confirm those originally made by Tepee et al. (1990) and Parr et al. (1994). Following infection at diestrus, the stage of the estrous cycle did not affect the level of virus recovered in vaginal washes. In other words, once infection was established subsequent stages of the estrous cycle did not appear to influence virus replication. The difference in thickness and permeability of the vaginal epithelium, as well as availability of viral receptors, during these periods may be responsible (Allen, 1922; Parr and Parr, 1994).

To examine immunity in the genital tract within the environment of increased viral susceptibility (but decreased IgA levels) animals were induced into a diestrus-like state by progesterone treatment prior to intravaginal HSV-2 challenge. Our results demonstrate that while none of the control animals survived infection, a significant number of mice immunized intranasally with AdgB8 did survive (Table 2). Subsequent studies showed that intranasally immunized mice infected with a lower but still lethal dose of virus were completely protected from mortality as well as genital pathology (W. S. Gallichan and K. L. Rosenthal, manuscript in preparation). Of interest was the fact that although gB-specific antibodies (mainly IgG) were present at the time of infection in immunized animals, sterile immunity was not observed and the viral replication that occurred over the first 4 days postinfection was similar to that occurring in control mice. In addition, while some immunized mice developed genital pathology, several of these completely cleared the primary infection and lacked any genital morbidity by Day 12. These results suggest that the induction of specific anamnestic responses and not neutralization by mucosal IgG antibodies present during initial infection were responsible for subsequent protection. In examining the immune mechanisms present during the period of viral clearance, we observed that the draining ILN and the genital tissues of immunized but not control mice contained a large percentage of ASC specific for gB of HSV-2. Both IgG- and IgA-secreting plasma cells were present demonstrating that intranasal immunization induced memory lymphocytes of both isotypes that were capable of responding to infection and entering the genital tract. Interestingly, the gB-specific ASC in both the ILN and the genital tissues represented a large percentage of the total ASC present; however, the gB-specific IgA ASC in the genital tissues were not as highly represented (Table 3). This may be due to the progesterone-dominated environment since it has been observed that the circulation of IgA plasma cells into the genital tissues decreases during diestrus (McDermott et al., 1980). Nevertheless, gB-specific IgA ASC were present in the genital tissues during the resolution phase of infection and were more numerous than the HSV-2-specific ASC reported in similar experiments where mice were protected from a lethal HSV-2 challenge following intravaginal immunization with an attenuated HSV-2 virus (Milligan and Bernstein, 1995). Interestingly, Eis-Hubinger et al. (1993) demonstrated that administration of gB-specific monoclonal antibodies resulted in the rapid clearance of virus from the genital tracts of mice that had an established HSV-1 infection. Moreover, recent studies have revealed that in addition to neutralization at the mucosal surface, se-
cretery IgA is capable of resolving established viral infections in mucosal tissues through intracellular neutralization of virus. Therefore, although we have not demonstrated that specific antibodies were responsible for protection, our results clearly demonstrate that intranasal immunization with AdgB8 provided a memory B cell response in the genital tract consisting of both IgA- and IgG-specific antibody-secreting cells during the same period in which viral clearance was observed in protected animals. It is important to note that we recently observed that mice immunized intranasally with AdgB8 and challenged ivag with HSV-2 contained strong CTL recall responses in their ILNs (Gallichan and Rosenthal, 1996). Furthermore, in light of the work by McDermott et al. (1989) which demonstrated that HSV-2-specific T cells from the ILNs protected mice from ivag HSV-2 challenge, it is unclear whether cellular or humoral immunity or both are responsible for protection in our model.

The changes in specific antibody levels and susceptibility to HSV-2 infection that occur over the course of the normal estrous cycle have important implications for the development and analysis of mucosal vaccines designed against sexually transmitted pathogens. In evaluating humoral immunity, isotype expression and the stage of the estrous or menstrual cycle should be taken into account. Indeed, there is evidence in humans that a similar dependence exists between the presence of each isotype and the menstrual cycle (Schumacher, 1980; Sullivan et al., 1984; Suzuki et al., 1984; Usala et al., 1989). Furthermore, in light of our observations it is clear that the induction of specific antibodies of both IgG and IgA isotypes in mucosal secretions is a requirement that vaccines will have to meet in order to maintain a blanket of humoral immunity in the female genital tract over the course of the reproductive cycle, thus increasing the likelihood of sterile immunity. Alternatively, despite the narrow window of susceptibility of mice to ivag HSV-2 infection during diestrus, the period dominated by IgG, mice immunized intranasally displayed a rapid recall response of both IgG and IgA antibody-secreting cells and were protected. Finally, these results demonstrate for the first time that intranasal immunization provides a significant level of protection in the genital tract against an intravaginal HSV-2 infection and a B cell memory response capable of providing both IgA and IgG ASC in the genital tissues during resolution of a primary HSV-2 infection.

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REFERENCES


Magnusson, K., and Stjernstrom, I. (1982). Mucosal barrier mecha-
nisms: Interplay between secretory IgA (S-IgA), IgG and mucins on the surface properties and association of salmonellae with intestine and granulocytes. Immunology 45, 239–248.


