Role of Mucosal Immunity in Herpes Simplex Virus Infection

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Role of Mucosal Immunity in Herpes Simplex Virus Infection

Nelly A. Kuklin, Massoud Daheshia, Sangjun Chun, and Barry T. Rouse

This study evaluates whether the vaginal mucosal surface of immunized mice can prevent invasion by herpes simplex virus (HSV) and aims to identify immune components that affect immunity after challenge at the vaginal mucosa. Despite the induction of both IgA and IgG vaginal Ab following immunization with recombinant vaccinia virus vectors expressing either glycoproteins B or D, viral infection occurred in most animals even after minimal viral dose challenge. Challenged immune animals, including those genetically unable to generate anti-HSV Ab, survived and showed few if any clinical signs of infection. Experiments with T cell subtype knockout animals and depletion with T cell subset-specific MAb indicated that immunity following vaginal challenge was principally dependent on the function of CD4+ T cells. Our results indicate that anti-HSV vaccines may not provide barrier immunity at the vaginal mucosal site but may be adequate to minimize clinical expression of disease. The Journal of Immunology, 1998, 160: 5998–6003.

Mucosal surfaces are vulnerable sites for pathogen invasion but if effectively protected by immunization could achieve immune exclusion. This represents an attractive prospect with agents such as herpes simplex virus (HSV)3 and HIV where successful infection sets the stage for later disease. For example, genital infection with HSV is not only a major cause of recurrent venereal disease but is also responsible for significant mortality in infants born to infected mothers (1). Currently, there is no effective HSV vaccine, although many have been advocated for use over the last two decades (2). Past vaccines against HSV were all administered systemically, and it is possible that they failed to prevent infection because of inadequate mucosal immune induction. In this report, we have addressed the issue of mucosal immunity against HSV in a mouse model system, paying particular attention to the effectiveness of barrier immunity. Our results demonstrate that in spite of high levels of vaginal Ab induced by mucosal vaccines, this is rarely sufficient to prevent viral infection. Our report adds further evidence to the concept that immunity to HSV is seldom if ever adequate to prevent viral invasion. In consequence, anti-HSV vaccines should be designed to minimize or prevent the expression of disease. As is also indicated in this report, immunity to disease following vaginal infection appears to be mainly a function of CD4+ T cell-mediated immunity.

Materials and Methods

Mice

Female BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). B cell-deficient mice (μMT−/−) made by targeted disruption of the membrane exon of the Ig μ-chain gene were provided by Dr. Werner Muller (Institute for Genetics, University of Cologne, Cologne, Germany) (3). Lack of IgM− B cells in μMT−/− mice was confirmed by FACS analysis. β2−Microglobulin (β2m) knockout mice made by Dr. Oliver Smithies (University of North Carolina) were purchased from The Jackson Laboratory (Bar Harbor, ME) (4). CD4 knockout mice were made by Dr. Niel Killen (5) (University of California, San Francisco, CA). β2m and CD4 knockouts were on B6/129 stock background and were backcrossed to 129 mice for four to five generations. The lack of CD4+ or CD8+ T cells in these mice was confirmed by FACS analysis.

During experimental procedures, the investigators adhered to guidelines proposed by the Committee of the Care of Laboratory Animal Resources, Commission of Life Sciences, National Research Council.

Virus

HSV strain KOS or McKrae cells were grown on monolayers of Vero cells (ATCC CCL81) and were titrated as described previously (6). Vaccinia virus expressing glycoprotein D (VgD) (7) or glycoprotein B (VgB) (8) and parent vaccinia virus vkr− (VV) were grown on monolayers of CV1 cells as described previously (7, 8). All viruses were stored in aliquots at −80°C until used.

Immunization of mice

Four- or five-week-old female mice were immunized intranasally (i/anas) with 105 pfu of VV expressing glycoprotein B or D of HSV (VgB, VgD) or VV in 20 μl volume. Some animals were given 106 pfu of HSV-1 KOS live or UV inactivated (titrated before inactivation). In some experiments, the immunization was repeated twice.

Virus challenge

To synchronize the estrous cycle at the progesterone-dominated stage as described by Parr et al. (9), the mice were injected s.c. with DepoProvera (DP) (Upjohn, Kalamazoo, MI) at a concentration of 2 mg/mouse in 50 μl of distilled H2O. Five days following DP administration, the animals were challenged with 103, 104, or 105 pfu of HSV-1 McKrae in 20 μl. The mice were examined daily for vaginal inflammation, neurologic illness, and death. The severity of disease was scored 1 to 5 (0, no change; 1, mild inflammation; 2, moderate swelling; 3, severe inflammation; 4, paralysis, death) (10).

Monoclonal antibodies

For T cell depletion, anti-CD4 (G.K. 1.5 ascites fluid; ATCC TIB 20F) or anti-CD8 (2.43 ascites fluid; ATCC TIB 210) mAbs were used. The concentration of the Abs was determined by ELISA. PharMingen (San Diego, CA) reagents, namely, rat anti-mouse CD4 FITC (catalog no. 10164D), rat anti-mouse CD8-phycocerythrin (catalog no. 10145B) and as isotype controls rat IgG2a-FITC (catalog no. 11024C) and rat IgG2a-phycocerythrin (catalog no. 11025A) were used for flow cytometry analysis. Additionally, for detection of IgM+ cells, biotinylated affinity pure goat anti-mouse IgM (catalog no. 115-065-075) (Jackson ImmunoResearch, West Grove, PA) and streptavidin-FITC (catalog no. 13024D) (PharMingen) were used.
Humoral immune response to immunization with recombinant VV expressing gB or gD of HSV (VgB, VgD)∗

<table>
<thead>
<tr>
<th>Immunization</th>
<th>VgB</th>
<th>VgD</th>
<th>VV</th>
<th>HSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum IgG Anti- (ng/ml)</td>
<td>14,225 ± 937</td>
<td>25 ± 15</td>
<td>&lt;20</td>
<td>22,893 ± 4,227</td>
</tr>
<tr>
<td>Vaginal IgA Anti- (ng/ml)</td>
<td>15 ± 10</td>
<td>3,226 ± 1,628</td>
<td>&lt;20</td>
<td>12,720 ± 970</td>
</tr>
<tr>
<td>Vaginal IgG Anti-gB (ng/ml)</td>
<td>687 ± 292</td>
<td>ND</td>
<td>&lt;10</td>
<td>526 ± 70</td>
</tr>
</tbody>
</table>

∗Twenty-four female BALB/c mice were immunized i/na with VgB and 14 were immunized with VgD. Additionally vtk− (VV) or live HSV-immunized mice were used as negative and positive controls, respectively. Twenty days after the immunization, serum and vaginal washings were collected. The levels of anti-gB or anti-gD responses were determined by ELISA.

In vivo T cell depletion

μMT−/− or BALB/c mice were given 10^3 pfu of HSV-1 KOS i/na. One group of mice received PBS and was used as negative control. Thirty days after the immunization, the mice were injected s.c. with 2 mg/mouse DP. At day 36, all animals were challenged intranasally with 5 × 10^3 pfu of HSV-1 McKrae (100 LD50). Depleting anti-CD4 or anti-CD8 mAbs (0.5 mg/mouse) were administered i.v. on days 34, 38, and 40 (before challenge and 2 and 4 days after challenge). Ten days after the third depletion, the mice were killed, spleens and vaginal draining lymph nodes were removed, and cells were pooled together and analyzed by two-color flow cytometry for CD4, CD8, and B220 markers (Consort-30 FACScan flow cytometer; Becton Dickinson, Mountain View, CA).

Antibody assays

Serum was collected by retroorbital bleeding. Vaginal washings were collected with a micropipette by introducing 100 μl of PBS into the vaginal cavity, and 50 to 70 μl were recovered per animal. The vaginal lavage sediments were subsequently removed by centrifugation. Fecal samples were weighed and suspended in PBS, 0.1% sodium azide at a concentration of 100 ng/ml. All samples were stored at −70°C until used. Ab detection was performed as previously described (6). Briefly, ELISA plates were coated overnight at 4°C with gB or gD protein, 2 μg/ml (kindly provided by Dr. Rae Lyn Burke, Chiron, Emeryville, CA) or anti-mouse IgG or IgA (Southern Biotechnology Associates (SBA). Birmingham, AL) in bicarbonate buffer. After blocking and washing the ELISA plates, standard mouse IgG or mouse IgA (SBA) was added at a concentration of 100 ng/ml to the anti-IgG- or anti-IgA-coated wells. Serum fecal or vaginal samples were added to gB- or gD-coated wells and were subsequently diluted twofold into the ELISA plates. After 2 h of incubation at 37°C and subsequent washing, goat anti-mouse IgG- or IgA-conjugated horseradish peroxidase (SBA) was added, and plates were incubated for 1 h at 37°C. Substrate 2,2′-azino-bis(ethylendiamine)-b-sulfonic acid was used for color development. The quantity of Ab bound was computed from a standard curve run simultaneously (6).

Statistics

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

Results

Generation of vaginal Ab after intranasal immunization with recombinant vaccine virus expressing gB and gD

Previous reports showed that i/na exposure to wild-type HSV or to recombinant viral vectors expressing HSV proteins leads to the generation of Ab responses at both systemic and at distal mucosal sites such as the vaginal tract (11, 12). In the present report, we have used two recombinant vaccinia virus vectors expressing, respectively, gB and gD. As is evident in Table I, immunization with both vectors led to the production of high titers of glycoprotein-specific IgA and IgG Ab in vaginal washings. The Ab levels observed were of the same magnitude as occurs in vaginal washings of mice immunized i/na with infectious virus.

Potency of protection against viral invasion

Immunized mice were given progesterone to standardize their susceptibility to HSV challenge as described by Parr et al. (9). This procedure actually changes the ratio of HSV-specific IgG to IgA in animals (13). In fact, in two experiments, we found ratios to change from an average of 0.6 to 4.5. Synchronized immune and control mice were challenged with different doses of infectious HSV. Animals were followed for signs of disease, and vaginal washings were collected for both virus titration and Ab measurement. It was reasoned that if levels of vaginal Ab were sufficient to neutralize virus and prevent viral invasion, then virus should be rapidly removed from vaginal tissues and animals should show no evidence of a secondary systemic immune responses to HSV. However, as indicated in Table II, which compares the results of such infection in VgB and control vaccinia (vtk−) virus-immune animals, the vaginal immunity was insufficient to prevent viral invasion in the majority of animals. Thus, all animals challenged with 10^7 pfu (200 LD50) of virus, and even one-third of those exposed to 10^6 pfu, developed Ab responses to gD. In a separate experiment, VgB-immunized mice were challenged with 10^5 pfu (before inactivation) of UV-inactivated virus. None of the animals developed gD-specific Ab (see Table III, Footnote h). Consequently, we assume the presence of gB Ab in the vaginal tract does not prevent viral infection of mucosal cells and the induction of an immune response. However, immunity following vaccination was sufficient to control the expression of clinical disease. Thus, even challenge of VgB vaccinees with 10^7 pfu of HSV failed to cause symptoms. In contrast, VV-immune animals all succumbed to 10^7 pfu challenge, and 20% of animals succumbed to as little as 10^6 pfu challenge.

In the case the failure to preclude viral invasion was unique to gB-specific Ab, experiments of a similar design were done with VgD immunization. In this instance, the generation of serum gB-specific Ab in challenged recipients was taken as evidence that viral invasion must have occurred. The results in Table III demonstrate that this was indeed the case. Thus, virus were recoverable from vaginal washings for up to 5 days at least in mice challenged with the 10^7 dose of virus, and the mice developed gB-specific Ab. In addition, 70% of animals challenged with 10^6 pfu of virus, a dose 10 times lower than that found to be nonimmunogenic with inactivated virus, developed anti-gB-specific Ab responses. As with the VgB-immune animals, protection against clinical disease was evident in all mice immunized i/na with VgD.

What protects mucosally immunized mice from clinical disease?

Since vaginal Ab was insufficient to prevent viral invasion, yet immunized animals were protected from clinical disease and in fact cleared virus more rapidly than control mice, the question arose as to possible immunologic mechanisms involved in the control of vaginal infection. Two types of experiments indicated a major function for CD4+ T lymphocytes. In the first series of experiments, mice were immunized i/na with HSV and all were shown to respond systemically and vaginally with specific Ab production. Animals were then hormonally synchronized, depleted of

Table I. Humoral immune response to immunization with recombinant VV expressing gB or gD of HSV (VgB, VgD)∗
either CD4+ or CD8+ T cells by mAb treatment, and then challenged intravaginally with 5 × 10^6 pfu HSV (100 LD_{50}). Vaginal wash samples were subsequently collected for the measurement of viral titers. The results in Figure 1 show that the CD4- and CD8-depleted animals had viral titers 2.5 logs higher than the CD8-depleted ones. In HSV-immune nondepleted mice, only one of five vaginal samples tested at day 2 postchallenge had detectable virus. These results indicate that CD4+ T cells are more important for vaginal immunity than are CD8+ T cells.

In the second approach, the susceptibility to vaginal challenge was compared in β2m knockout and CD4 knockout mice that had been immunized i/nas with HSV. Both HSV-immune and naive mice were hormonally synchronized before vaginal challenge with a high dose of virus (2 × 10^7 pfu). The results presented in Table IV demonstrate that CD4+ knockout mice were more susceptible to viral challenge than were to β2m knockout animals. In support of this conclusion, CD8+ T cells were added to the CD4+ knockout mice, and the results presented in Table III indicate that CD8+ T cells are not essential for protection against vaginal HSV challenge. Furthermore, a high dose of virus (2 × 10^7 pfu) was used in the challenge, and results presented in Table II indicate that CD8+ T cells are not essential for protection against vaginal HSV challenge.

### Table II. Clinical and Immunologic Responses in VgD-Immunized Mice Challenged Vaginally with HSV

<table>
<thead>
<tr>
<th>Challenge Dose</th>
<th>Immunization</th>
<th>Vaginal Virus Titer (pfu/ml)</th>
<th>72 h Virus Titer (pfu/ml)</th>
<th>Clinical Score Day 7</th>
<th>Anti-gD Serum IgG (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10^3 UV</td>
<td>VgD</td>
<td>0.2 ± 0.5</td>
<td>0</td>
<td>0</td>
<td>4/13</td>
</tr>
<tr>
<td>1 × 10^4 UV</td>
<td>VgD</td>
<td>1.1 ± 0.8</td>
<td>0</td>
<td>0</td>
<td>9/13</td>
</tr>
<tr>
<td>1 × 10^5 UV</td>
<td>VgD</td>
<td>3.2 ± 1.4</td>
<td>2.1 ± 1.7</td>
<td>0</td>
<td>6/6</td>
</tr>
<tr>
<td>1 × 10^6 UV</td>
<td>VgD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/4</td>
</tr>
<tr>
<td>1 × 10^7 UV</td>
<td>VgD</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/4</td>
</tr>
</tbody>
</table>

### Table III. Clinical and Immunologic Responses in VgD-Immunized Mice after Vaginal Challenge with HSV

<table>
<thead>
<tr>
<th>Challenge Dose</th>
<th>Immunization</th>
<th>Vaginal Virus Titer (pfu/ml)</th>
<th>72 h Virus Titer (pfu/ml)</th>
<th>Clinical Score Day 7</th>
<th>Anti-gB Serum IgG (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10^3 UV</td>
<td>VgD</td>
<td>0.4 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>4/7</td>
</tr>
<tr>
<td>1 × 10^4 UV</td>
<td>VgD</td>
<td>1.7 ± 0.9</td>
<td>0</td>
<td>0</td>
<td>5/7</td>
</tr>
<tr>
<td>1 × 10^5 UV</td>
<td>VV</td>
<td>2.3 ± 0.5</td>
<td>0</td>
<td>0</td>
<td>4/4</td>
</tr>
<tr>
<td>1 × 10^6 UV</td>
<td>VV</td>
<td>3.4 ± 0.7</td>
<td>1.7 ± 0.8</td>
<td>0</td>
<td>4/4</td>
</tr>
</tbody>
</table>

### Table IV. Challenge and Immunologic Responses in VgB-Immunized Mice Challenged Vaginally with HSV

<table>
<thead>
<tr>
<th>Challenge Dose</th>
<th>Immunization</th>
<th>Vaginal Virus Titer (pfu/ml)</th>
<th>72 h Virus Titer (pfu/ml)</th>
<th>Clinical Score Day 7</th>
<th>Anti-gD Serum IgG (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10^3 UV</td>
<td>VgB</td>
<td>0.2 ± 0.5</td>
<td>0</td>
<td>0</td>
<td>4/13</td>
</tr>
<tr>
<td>1 × 10^4 UV</td>
<td>VgB</td>
<td>1.1 ± 0.8</td>
<td>0</td>
<td>0</td>
<td>9/13</td>
</tr>
<tr>
<td>1 × 10^5 UV</td>
<td>VgB</td>
<td>3.2 ± 1.4</td>
<td>2.1 ± 1.7</td>
<td>0</td>
<td>6/6</td>
</tr>
<tr>
<td>1 × 10^6 UV</td>
<td>VgB</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/4</td>
</tr>
<tr>
<td>1 × 10^7 UV</td>
<td>VgB</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0/4</td>
</tr>
</tbody>
</table>

*Female BALB/c mice (4 to 5 wk old) were immunized i/nas with 1 × 10^7 PFU of VgB or VV. Twenty days after immunization, the mice were injected s.c. with 2 μg/mouse DP. Five days after immunization, the animals were challenged intravaginally with 1 × 10^3, 1 × 10^4, and 1 × 10^5 pfu of HSV-1 McKrae. Forty-eight hours postchallenge, vaginal washings were collected, and the virus titer was determined for each individual mouse by plaque assay. Twenty days after challenge, serum was collected and the titer of anti-gD was determined by ELISA. Data in parentheses are number of vaginal washings positive or virus total number of vaginal samples tested.*

*Viral titers were additionally measured at days 5 and 7 postintravaginal infection. Virus was recovered from vaginal washings up to day 5 of immune mice challenged with 1 × 10^7 pfu of HSV.*

*Four VgB-immune mice were given 1 × 10^7 pfu of UV HSV (titrated before inactivation) intravaginally.*

*ND, not done (since the VV immunized mice died at 7 to 9 days postchallenge).
against high dose intravaginal HSV challenge, unimmunized mice succumbed to viral challenge. We interpret m

is B cell function necessary for HSV immunity?

Taken together, the above experiments indicate that protection against vaginal viral challenge depends on the activity of T cell immunity, particularly CD4⁺ T cells. To evaluate whether B cell-mediated resistance plays an additional role in defense, the outcome of vaginal viral challenge was compared in BALB/c and immunocompromised μMT⁻/⁻ mice of the same genetic background. The data presented in Table V indicate that the failure to generate anti-HSV Ab responses did not impair the ability to resist infection. After intravaginal challenge with a low dose of HSV (10⁵ pfu), VgB-immunized μMT⁻/⁻ had marginally higher vaginal virus titers, but when animals were challenged with a high dose of HSV (10⁶ pfu), titers were approximately the same. Additionally, the time of viral clearance was similar in immune μMT⁻/⁻ and BALB/c mice. Furthermore, both immune B cell-immunocompromised and control mice survived low and high dose intravaginal challenge, whereas all naive animals succumbed to infection (Table V).

To further evaluate the role of CD4⁺ and CD8⁺ T cells in μMT⁻/⁻-immune mice, such animals were depleted of either CD4⁺ or CD8⁺ T cells before vaginal challenge with a high dose of HSV (200 LD₅₀). As is evident in Table VI, depletion of CD4⁺ T cells was more detrimental to such mice than was CD8⁺ T cell depletion. Thus, whereas both CD4⁺ and CD8⁺ T cell-depleted animals survived challenge, vaginal virus titers were higher and persisted for longer in the CD4⁺ T cell-depleted animals.

Discussion

This report evaluates mechanisms of immunity that confer protection against infection and disease in immunized mice following vaginal challenge with HSV. The intent was to assess whether barrier immunity at the mucosal site could prevent invasion and to determine which aspect of immunity protected against clinical disease. Our results show that in spite of robust vaginal Ab responses at the time of challenge, these were often insufficient to preclude viral invasion even by a modest dose of virus. However, immunized mice, including those genetically incapable of producing Ab, were immune to disease expression. Protection from disease appeared to be mediated principally by CD4⁺ T lymphocytes. Our results contrast with findings with typical mucosal viral agents such as influenza where Ab at the site of infection is effective to prevent invasion and viral clearance is conducted mainly by CD8⁺ T lymphocytes (14–17).

The conclusion that Ab at the site of mucosal infection was usually inadequate to prevent invasion came from experiments in which mice immunized i/nas with recombinant vaccinia expressing HSV glycoproteins were challenged vaginally with HSV. Despite high titers of both IgA and IgG, vaginal Ab against the immunizing glycoprotein,

<table>
<thead>
<tr>
<th>Mice</th>
<th>HSV Immunization</th>
<th>Log Virus Titer</th>
<th>Vaginal Inflammation</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
<td>Day 7</td>
<td>Day 5</td>
</tr>
<tr>
<td>CD4⁻/⁻</td>
<td>–</td>
<td>3.6 ± 0.4</td>
<td>3.5 ± 0.4</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>CD4⁺/⁺</td>
<td>+</td>
<td>3.0 ± 0.4</td>
<td>2.4 ± 1.7</td>
<td>1.8 ± 0.9</td>
</tr>
<tr>
<td>β₂₅⁻/⁻</td>
<td>–</td>
<td>4.0 ± 0.3</td>
<td>3.3 ± 0.5</td>
<td>2.0 ± 0.7</td>
</tr>
<tr>
<td>β₂₅⁺/⁺</td>
<td>+</td>
<td>1.7 ± 1.7</td>
<td>0</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>4.2 ± 0.2</td>
<td>3.3 ± 0.6</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>1.6 ± 0.8</td>
<td>0</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

*CD4⁻/⁻, β₂₅⁻/⁻ or control mice were immunized twice with i/nas with live HSV. Fifteen days after the last immunization, all groups of naive or HSV-immune mice were injected s.c. with 2 mg/mouse DP. Five days after hormone administration, the mice were challenged intravaginally with 2 × 10⁵ pfu of HSV-1 McKrae. Vaginal washings were collected at days 5 and 7 postchallenge, and virus titers were determined using plaque assay.

Table V. **Protective responses in VgB-immune B cell-deficient mice**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Immunization</th>
<th>Challenge Dose</th>
<th>Vaginal Virus Titer</th>
<th>No./Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 2</td>
<td>Day 5</td>
</tr>
<tr>
<td>μMT⁻/⁻</td>
<td>VgB</td>
<td>Low dose</td>
<td>3.1 ± 1.6 (6/6)¹</td>
<td>0.6 ± 1.6 (1/6)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>VgB</td>
<td></td>
<td>2.2 ± 1.0 (5/6)¹</td>
<td>0.0 (0/6)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>VV</td>
<td></td>
<td>5.4 ± 0.4 (7/7)¹</td>
<td>3.5 ± 0.4 (7/7)</td>
</tr>
<tr>
<td>μMT⁻/⁻</td>
<td>VgB</td>
<td>High dose</td>
<td>5.1 ± 0.1 (4/4)¹</td>
<td>1.2 ± 1.1 (3/4)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>VgB</td>
<td></td>
<td>4.7 ± 0.6 (4/4)¹</td>
<td>1.1 ± 1.2 (3/4)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>VV</td>
<td></td>
<td>6.0 ± 0.6 (4/4)¹</td>
<td>5.6 ± 0.6 (4/4)</td>
</tr>
</tbody>
</table>

¹ Groups of 6 female μMT⁻/⁻ B cell-deficient mice were challenged i/nas (2 × 10⁵ pfu of VgB and BALB/c mice with i/nas with 1 × 10⁵ pfu of VgB. Additionally, a group of BALB/c mice was given VV i/nas and used as controls. Fifteen days after immunization, all mice were injected with 2 mg/mouse DP. Ten days later, the mice were challenged intravaginally with 1 × 10⁶ or 1 × 10⁵ pfu of HSV-1 McKrae (200 LD₅₀). Vaginal washings were collected at days 2 and 5 after challenge, and the titers were determined by plaque assay. BALB/c and μMT⁻/⁻ mice were bled at day 30 after immunization, and the serum anti-HSV IgG titers were determined by ELISA. On average, immune BALB/c mice had titers of 20.305 ng/ml, whereas immune μMT⁻/⁻ mice had titers of <355 ng/ml.

2 Low dose, 10⁵ pfu; high dose, 2 × 10⁷ pfu.

3 Number of surviving mice/number of mice used.

4 Number of mice positive for virus/number of mice tested.
Table VI. Influence of CD4+ and CD8+ T cell depletion on viral clearance in immune μMT−/− mice

<table>
<thead>
<tr>
<th>μMT−/−</th>
<th>Vagal Virus Titer (pfu/ml)</th>
<th>Group</th>
<th>Day 2</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-immune CD4-depleted μMT−/−</td>
<td>b</td>
<td>4.9 ± 0.2</td>
<td>1.5 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>HSV-immune CD8-depleted μMT−/−</td>
<td>c</td>
<td>3.0 ± 0.12</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Naive undepleted μMT−/−</td>
<td>d</td>
<td>5.6 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

*Group of eight 4- to 5-wk-old female μMT−/− mice were immunized i/nas with 1 × 10^6 pfu of HSV-1 KOS. Additionally, a group of 3 μMT−/− mice were given PBS and used as negative controls. At day 30, the mice were injected s.c. with DP (2 mg/mouse). At day 36, all animals were challenged with 1 × 10^6 pfu of HSV-1 McKee. Depletion of CD4+ and CD8+ T cells was performed by i.v. administration of 0.5 mg/mouse anti-CD4 (1.5) or anti-CD8 (2.43) Abs on days 34, 38, and 40 (2 days before challenge, 2 and 4 days after challenge). Vaginal wash samples were collected at days 2 and 5 postchallenge, and the virus titer was determined by plaque assay. The experiments were performed simultaneously with the experiments presented in Figure 1. Groups b and c are statistically significant; p < 0.01 using Student’s t test. Group b, 1.6% CD4+ T cells and 31% CD8+ T cells; group c, 47% CD4+ T cells and 0.09% CD8+ T cells; group d, 23.8% CD4+ T cells and 10.04% CD8+ T cells. All groups b, c, and d had <0.09% IgM+ cells, whereas BALB/c control mice had 40% IgM+ B cells.

infection following vaginal challenge was assumed to occur in many animals. Thus, Ab responses against other glycoproteins were induced, and in addition challenged animals developed secondary Ab responses to the immunizing glycoprotein. This pattern of events was evident even in some immune animals challenged with a minimal dose (10^3 pfu) of virus. Our results indicate either that Ab, at least against gB or gD, the current favored candidates for subunit vaccines, fails to neutralize HSV in the vagina or that virus-Ab complexes remain infectious to FcR-bearing cells in the vaginal mucosa. Our observations do stand in contrast with some previous reports (18–20). For example Eis-Hubinger et al. (18) observed vaginal protection in mice following the i.v. administration of monoclonal, although not polyclonal, Ab. However, these workers failed to exclude whether or not viral invasion occurred. Thus we also routinely observed protection from disease in animals that did succumb to infection. In addition, disease following ocular infection may not become evident in mice previously given high concentrations of mAb (21). Nevertheless, such animals still establish latent infection.

In humans, some observations do indicate a role for Abs in protection from infection. For example, Brown et al. (22) observed that Ab may protect against invasion of the human infant. They observed that some infants born to infected mothers suffering recurrent disease were protected from infection during birth by their maternal Ab. However, not all infants born to immune mothers were protected from infection (22, 23). Other studies have indicated that the presence of cervical IgA in humans may diminish the duration of viral shedding in women (24, 25).

Whether or not Ab can fully protect against invasion is an important issue in HSV pathogenesis. Thus, if immunity is not sterile, then viral invasion can establish latency and thus set the scene for eventual recurrent disease. It is sobering to realize that no prophylactic vaccine against HSV has yet been deemed satisfactory by independent evaluation (2). Recently, one major company abandoned their prophylactic vaccine trial for thus far unknown reasons, and the results of a second trial using a subunit vaccine approach are still being evaluated.

We also readdressed the issue of which immune defenses are involved in protection against disease following mucosal challenge. This topic was addressed previously by others using animal models usually in a primary infection mode. For instance, McDermott et al. (26) demonstrated transfer of protection against intra-vaginal challenge by T cells from the genital lymph nodes draining the vaginal mucosa, but not by Ab-producing B cells. More recently, Milligan and Bernstein (27, 28) have assessed T cell role in vaginal protection against HSV-1 in naive mice and concluded that CD4+ cells are the principal means of defense and that the cells likely function by IFN-γ production. IFN-γ has been advocated as the mediator of systemic immunity conducted by both CD4+ and CD8+ T cells (29), although animals genetically unable to produce IFN-γ can still be protected from disease (30). More recently, one group have advocated that CD8+ T cells act as the major for mucosal defense entities against HSV infection (31).

The present study using immune animals makes a strong case that T cells, mainly CD4+, act as the principal mediators of mucosal defense and that Ab likely plays little or no role. Firstly, we observed that immunized μM knockout mice, lacking B cells and incapable of producing anti-HSV Ab, were equally as well protected against HSV challenge as were immunocompetent animals. Additionally, such μM knockout mice immunized with live HSV and subsequently depleted of CD4+ or CD8+ T cells were able to survive lethal intravaginal HSV challenge. However, significantly higher virus titers were recovered from the vaginal tract of the CD4+ than that of CD8+ T cell-depleted μM knockout mice. These results indicate that although CD8+ T cells may have some protective role, CD4+ T cells act as the principal mediators of vaginal immunity. Moreover, in the recombinant vaccinia experiments, the constructions used that provided protection were not able to induce CD8+ CTL responses in BALB/c mice (32, 33). Also arguing for a principal role for CD4+ T cells in mucosal immunity was the observation that whereas immunized βM knockout (low CD8+) were well protected from HSV vaginal challenge, immune CD4+ knockout animals were more susceptible. Thus, our studies add up to the conclusion that mucosal immunity to HSV, at least in the mouse, is principally the domain of CD4+ T cells. How such cells control infection in the vaginal tract still must be established, but we favor the idea that it involves multiple cytokines elaborated during the course of an inflammatory response orchestrated principally by CD4+ T cells. Whether vaccines can be designed that effectively enhance CD4+ T cell immunity so that infection, exogenous or reactivated, remains subclinical must be evaluated.

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References


