Cervical Antibody Responses to a Herpes Simplex Virus Type 2 Glycoprotein Subunit Vaccine

Rhoda L. Ashley, Flor-Mari Crisostomo, Michael Doss, Rose E. Sekulovich, Rae Lyn Burke, Mary Shaughnessy, Lawrence Corey, Nayak L. Polissar, and Andria G. M. Langenberg

Effective vaccines against genital herpes simplex virus type 2 (HSV-2) may need to induce genital tract immune responses. To determine local antibody responses to HSV-2 glycoproteins gB2 and gD2 in an intramuscular subunit vaccine, cervical secretions from HSV-seronegative women and HSV-1-seropositive women were tested for IgG and IgA to gB2 and gD2 by enhanced chemiluminescence Western blot. Most (94%) of the seronegative subjects developed cervical IgG to gB2, IgG to gD2, and IgA to gB2; 72% developed IgA to gD2. All HSV-1-seropositive subjects had cervical IgG responses to vaccine gB2 and gD2, 85% had IgA responses to gB2, and 50% had IgA responses to gD2. Responses were more rapid and titers more consistently sustained in the HSV-1-seropositive women. Further, vaccination resulted in cervical IgG and IgA titers comparable to those to HSV-2 gB2 and gD2 in response to recurrent HSV-2 genital infection.

Genital herpes continues to spread, partly because of the high proportion of subclinical or unrecognized cases and the frequency of viral shedding in the absence of symptoms [1]. That prior immunity to herpes simplex virus (HSV) gives at least partial protection against infection is inferred from observations that genital infection with >1 strain of HSV-2 is rare [2], that acquisition of genital herpes is lower in subjects with prior HSV-1 antibodies than in HSV-naïve patients [3], and that vertical transmission of genital HSV-2 to the newborn is infrequent from mothers with established infection [4]. These findings suggest that vaccine-induced immunity could be an effective means of slowing the spread of genital herpes. Because HSV infections begin at mucosal sites, induction of a consistent local antibody response is a desirable characteristic of any candidate vaccine for HSV.

Previously we characterized cervical antibody responses to first episodes of genital herpes in women. These studies showed a predominant IgG response to viral protein antigens similar to those of serum antibodies. However, HSV-specific IgA responses differed qualitatively from cognate serum IgA responses [5]. We also demonstrated local anamnestic antibody responses in the cervix when HSV-1-seropositive women acquired genital HSV-2 infections [6].

Here we report the isotype and kinetics of cervical antibody development after each of three intramuscular doses of an HSV-2 subunit vaccine. Eighteen HSV-seronegative women and 20 women with prior HSV-1 immunity completed a protocol to receive three doses of vaccine. Their responses were compared to determine the effects of prior mucosal priming (in the subjects with prior HSV-1) on local antibody development after parenteral boosting.

Materials and Methods

Subjects. Women aged 18–55 years were recruited, enrolled, and followed up at the University of Washington Virology Research Clinic. Women were not pregnant at entry, agreed to use reliable birth control methods throughout the study, and were human immunodeficiency virus-negative. Subjects were tested for the presence of HSV-1 and HSV-2 serum antibodies by Western blot [7].

Twenty HSV-seronegative women and 20 HSV-1-seropositive women were enrolled. Further serologic testing by a more sensitive technique [8] confirmed that 19 women lacked antibodies to HSV-1 and HSV-2, providing serologic evidence that they had not been infected with either HSV subtype. This additional testing revealed that 1 subject who was HSV-seronegative by standard screening assay had a low titer of antibodies to HSV-1. This person, therefore, was included in the HSV-1-seropositive group, giving 19 HSV-seronegative and 21 HSV-1-seropositive subjects. None of the subjects had histories of genital herpes; 4 of the HSV-1-seropositive subjects had a history of oral herpes.

Eighteen HSV-seronegative and 20 HSV-1-seropositive subjects completed the vaccine schedule of intramuscular doses at days 0, 28, and 180. Fourteen seronegative and 16 HSV-1-seropositive subjects consented to a fourth dose given ~1 year after entry. The vaccine comprised 30 µg each of HSV-2 recombinant glycoproteins gB2 and gD2 in MF59 adjuvant (Chiron Vaccines, Emeryville, CA [9]). Sera drawn at the end of the trial were tested...
by Western blot in parallel with sera drawn at the start of the trial to detect serologic evidence of HSV-2 infection in all subjects and of HSV-1 infection in the HSV-seronegative subjects during the trial. None of the subjects was found to have seroconverted to HSV-1 or HSV-2.

Cervical secretions from 11 subjects who were seronegative for HSV by enhanced chemiluminescence Western blot (ECL-WB) [8] were pooled to use as background control samples in the ECL-WB test. An additional 6 control subjects were HSV-2–seropositive with histories of culture-documented recurrent genital herpes lesions. Cervical and vulvar samples from these HSV-2–seropositive control subjects were further characterized by HSV polymerase chain reaction as being negative for HSV shedding at the time of cervical sampling.

**Sampling.** Cervical samples were collected on days 0, 28, 42, 90, 180, 194, 270, and 360 and 14 days after the fourth dose. As described in detail [5], two sequential pairs of tear flow indicator strips were placed in the cervical os until secretions had soaked up to the strips’ shoulders. After trimming, the four saturated stubs were placed in a vial with 0.5 mL of 0.01% sodium azide in PBS and frozen at −20°C. Serum was obtained at each clinic visit and stored at −20°C.

**Local antibody testing.** Samples were processed as described previously [5]; none was positive for blood (Hemoccult; Smith-Kline Diagnostics, San Jose, CA).

Samples were tested by ECL-WB using recombinant gB2 and gD2 as the antigens [8]. Because the recombinant proteins contained several species with similar electrophoretic mobilities, gB2 and gD2 were run on separate gels. Twenty microliters of each cervical sample was diluted 1:50 in Blotto and incubated with blots overnight at room temperature. Blots were washed, and bound antibodies were detected with goat anti-human IgA (α chain) or goat anti-human IgG (γ chain) and ECL detection reagents (Amer sham Life Sciences, Oakville, Canada) [5].

Samples from days 0 to 270 (8 samples) from a single subject were run in the same immunostaining run on blots derived from a single gel containing gB2 and a second gel containing gD2. A second set of blots was used for days 360 and 374 to assess responses to the fourth dose. Blots were exposed to film for periods from 10 s to 4 min. Film profiles were selected for scoring, using the maximal film exposure time that gave discrete banding in the lane developed with a cervical secretion control pool from unvaccinated HSV-2–seropositive subjects. Most profiles were selected for scoring from 1-min exposures (IgA) or 30-s exposures (IgG).

Profiles were scored as positive for vaccine-related response in seronegative subjects if vaccine bands were detectable 28 days after the first vaccine dose and 14 days after subsequent doses compared with the profile of cervical secretions from the same subject just before that immunization. Profiles were scored as positive for vaccine-related responses in HSV-1–seropositive subjects if vaccine bands appeared de novo or increased in intensity in blots of secretions taken 28 days after the first vaccine dose compared with the day 0 sample and 14 days after subsequent doses of vaccine compared with profiles of secretions collected immediately before that immunization.

Of 17 HSV-1–seropositive subjects with evaluable specimens for cervical IgA at day 0, 14 (82%) had IgA to gB2 and 4 (24%) had IgA to gD2. The 18th subject’s day 0 specimen resulted in test artifacts that prevented accurate IgA assessment. Of 18 day 0 specimens from subjects that were tested for cervical IgG, 14 (78%) were positive for gD2 and 12 (67%) for gD2. As shown previously, HSV-1 infection results in detectable cervical antibodies to gB2 and gD2 because of the extensive cross-reactivity of these proteins between HSV-1 and HSV-2 [6].

**Titer determination by strip immunoblot assay.** Cervical secretions were serially diluted 4-fold in 4% goat serum in PBS starting at a dilution of 1:20 for IgA and 1:80 for IgG. Custom nitrocellulose strips containing bands of non-denatured recombinant gB2 and gD2 were provided by Chiron Vaccines. Strips were incubated for 1 h in 0.5 Tween 20 in PBS, and then used as described [5]. Strips were exposed to film for 30 s and then for periods ranging from 2 s to 2 min. End-point titers were taken as the reciprocal of the highest dilution showing a clear gB2 or gD2 band at the selected exposure time, as shown for cervical IgG (figure 1A) and cervical IgA (figure 1B) from 1 HSV-1–seropositive subject. The test was repeated if titer determinations of two readers were not in agreement. By comparing end-point titers with those generated by humanized monoclonal antibodies of known concentration (see below), we estimate that a cervical IgG titer of 1:80 represents ~78 ng/mL IgG to gB and 313 ng/mL IgG to gD.

Humanized IgG monoclonal antibodies against gB2 and gD2 (provided by R. Whitley, University of Alabama, Birmingham) were used to select films for end-point determination. Monoclonal antibodies were serially diluted 4-fold in 4% goat serum in PBS starting at 1:4000 and reacted with assay strips as described above. A monoclonal antibody test was run with each subject’s secretions. The exposure time for the film to be used for end-point determination was determined by comparing the monoclonal antibody band intensity at each dilution with that of an external reference standard run. Films were selected to be as close as possible in intensity of these control bands to minimize reading artifact (figure 1C).

**Statistical methods.** The proportions of seronegative and HSV-1–seropositive subjects having an IgG and IgA response to the vaccine glycoproteins were compared by cross-tabulation, with statistical significance based on χ² or Fisher’s exact test.

IgA values <1:20 and IgG values <1:80 were assigned values of 1:10 and titers were log₁₀ transformed for these analyses. Geometric mean titers are presented along with their SDs. Because of the presence of outliers or extreme values for some samples, the nonparametric Wilcoxon–Mann-Whitney test was used to calculate statistical significance of differences between groups (e.g., seronegative vs. seropositive.) The Wilcoxon signed ranks test was used to compare paired values within a group (e.g., titers before and after a dose).

The Spearman correlation coefficient was used to determine if serum and cervical IgG responses tended to change together over time. For each subject, the Spearman correlation coefficient between paired values (serum, cervical) was calculated for all observation times. The correlation ranged between 0.0 and ±1.0, representing no correlation and perfect correlation (positive or negative), respectively. The Wilcoxon signed ranks test was used to test the null hypothesis that the mean correlation is zero, that is, that serum and cervical responses are unrelated.

**Results**

**Cervical IgG and IgA responses to vaccine in seronegative subjects.** Paired Western blots of pre- and postvaccine secre-
Figure 1. Strip immunoblot end-point titer determination for IgG (A) and IgA (B) to gB2 and gD2 in HSV-1-seropositive subject receiving HSV-2 gD2-gB2 subunit vaccine. Cervical secretions from day 0, 14, 28, 42, and 90 were serially diluted 4-fold from 1:80 (IgG) or from 1:20 (IgA) and reacted with immunoblots containing gD2 and gB2. C shows results of IgG humanized monoclonal antibody (HuMAb) against gD2 and gB2 4-fold diluted from 1:4000 to 1:1,024,000. Set of blots at left is standard against which films were compared from test runs; set on the right was generated in this run. Film exposure times showing end-point titer of 1:64,000 for gB2 and 1:16,000 for gD2 were used for vaccine antibody titer determinations. Control bands on strips include, from top, high-titer human IgG, mouse monoclonal antibody to human IgM, and human IgG.

Cervical antibody responses to the vaccine proteins were observed in most subjects, but not necessarily to each dose. Of the 18 seronegative subjects who received three doses, 17 (94%) had IgG responses to gB2 in at least 1 pair of samples. For the other antibody responses, 17 pairs could be evaluated. Sixteen (94%) of 17 seronegative subjects developed IgG to gD2 and IgA to gB2 after at least one dose; 13 (72%) of 17 developed IgA responses to gD2 that were detected in at least 1 pair of samples. Responses to dose 4 in the 14 seronegative subjects were less prevalent than to earlier doses (table 1).

The responses to individual doses 2, 3, and 4 represented first-ever responses in some subjects, recovery of antibody to dose 1 that had been lost before subsequent doses in other subjects, or boosting of prior responses as noted by an increased band intensity over that of the prior postvaccine sample. Figure 2 illustrates a typical pattern of cervical IgG (figure 2A) and cervical IgA responses (figure 2B) in a seronegative subject. Comparing day 28 profiles to day 0 profiles revealed the presence of de novo IgG to gB2 and gD2 and de novo IgA to gB and gD. IgA to gD2 also was apparent after the third dose in this subject. Responses to dose 2 were scored by comparing day 42 profiles with those from day 28. Intensity of gB2 bands actually decreased slightly for both IgG and IgA; thus, no response was scored for the second dose. The IgG gD2 band increased in intensity at day 42 and was therefore scored as a response. Comparing day 194 and day 180 profiles resulted in scoring an IgG response to both gB2 and gD2 given in dose 3. This subject demonstrated decreased levels of IgG and IgA at day 270, suggesting a waning of titers 3 months after the third dose.

All of this subject’s post-dose profiles at day 28, 42, and 194 have antibody not detected at day 0, before immunization. However, with IgA in particular, intensity of gB2 bands did not change dramatically once a response was noted. Responses to the fourth dose were determined in paired day 360 and day 374 secretions analyzed at a later date with a second set of blots. IgG or IgA responses were not apparent to gB2 or gD2 bands.

Table 1. Prevalence of cervical IgG and IgA responses to HSV-2 gD2-gB2 subunit vaccine in seronegative subjects.

<table>
<thead>
<tr>
<th>Vaccine dose</th>
<th>No. of responses/total no. of evaluable specimen pairs (%)</th>
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<tbody>
<tr>
<td></td>
<td>IgG to gB2</td>
</tr>
<tr>
<td>1</td>
<td>6/16 (38)</td>
</tr>
<tr>
<td>2</td>
<td>11/17 (65)</td>
</tr>
<tr>
<td>3</td>
<td>13/17 (76)</td>
</tr>
<tr>
<td>4</td>
<td>5/14 (36)</td>
</tr>
</tbody>
</table>

NOTE. Paired specimens from days 0 and 28 (dose 1), from days 28 and 42 (dose 2), from days 180 and 194 (dose 3), and from days 360 and 374 (dose 4) were subjected to enhanced chemiluminescence Western blot. De novo appearance of gB2 or gD2 bands or increased intensity of gB2 or gD2 bands following vaccination was recorded as response.
mean titers than those observed after the second or third doses (figure 3A).

Mean end point titers for specimens taken at days 90 and 180 (between doses 2 and 3) showed progressive waning of antibody titers (figure 3A). Similarly, mean titers dropped by day 360 between the third and fourth doses (figure 3A). Thus, in seronegative subjects, the third and fourth doses did not boost antibody titers above those achieved with two doses but, rather, served to partially or fully restore titers that had waned.

Cervical IgG and IgA responses to vaccine in seropositive subjects. All of the 20 seropositive subjects receiving three doses had increased cervical IgG band intensities of gB2 and gD2 after one to three doses. Seventeen (85%) of 20 subjects had increased levels of cervical IgA to gB2 and 10 (50%) developed additional IgA to gD2 after at least one dose. A higher proportion of subjects responded to the first dose than to later doses (table 2).

Response patterns differed between seronegative and HSV-1−seropositive subjects in the following ways: A higher proportion of HSV-1−seropositive than seronegative subjects had responses to the first dose (P < .01 for IgG to gB2 and gD2). In contrast, a higher proportion of seronegative subjects had responses to the second dose (P < .05 for IgG to gD2; P < .01 for IgA to gB2 and gD2). Thus, it appears that seronegative subjects require more antigen presentation events to give evidence of vaccine response than do those with prior experience with HSV-1. Seronegative subjects were also more likely to respond to the third dose (P < .05 for IgG to gD2).

Magnitude and duration of cervical IgG and IgA responses in seropositive subjects. End-point titers established using strip immunoblots are illustrated for 1 HSV-1−seropositive subject for cervical IgG (figure 1A) and IgA (figure 1B) in specimens collected on days 0, 14, 28, 42, and 90. In this subject, IgG and IgA were present to both gB2 and gD2 before vaccination (day 0). Both IgG and IgA titers increased by day 14. IgA titers waned; day 42 titers did not recover to day 14 levels after a second dose. Similarly, IgG titers at day 42 did not reflect a response to the second dose given at day 28.

In the seropositive subjects, geometric mean IgG titers rose after dose 1 and remained quite constant thereafter (figure 3B). Mean titers of IgA in seropositive subjects rose after the first dose and remained relatively constant except for slight titer decreases before the third dose (day 180) and before the fourth dose (day 360). Thus, HSV-1−seropositive subjects had less dramatic fluctuations in titers over the course of the vaccination protocol than did seronegative subjects.

Titers of cervical antibodies to vaccine versus natural infection. Western blots from this study had gB2 and gD2 band intensities that were similar to or more intense than those produced by cervical specimens from patients with genital herpes, suggesting that vaccine responses were comparable in titer with those elicited by mucosal infection. To test this hypothesis, mean end-point titers after three doses (day 194) were compared with those obtained from women with culture-documented recurrent infection.
Figure 3. Geometric mean titers in HSV-seronegative (A) and HSV-1-seropositive subjects (B) receiving 3 or 4 doses of HSV-2 gD2-gB2 subunit vaccine. Titers of IgG to gB2, IgG to gD2, IgA to gB2, and IgA to gD2 were measured by end-point dilution. Geometric mean titers were plotted to demonstrate fluctuations in responses with time and with additional doses of vaccine. Bars denote 1 SE. No. of subjects varied from 6 to 11 per time point for seronegative and 11 to 19 for seropositive subjects, as noted at base of figure.

HSV-2 infections. The HSV-2-infected subjects were sampled for cervical antibodies and, at the same visit, by polymerase chain reaction testing to detect HSV DNA. Only samples taken at times when HSV DNA was not detected were used in this comparison to avoid bias introduced by local antigen-antibody complexes, which would not be detected by the strip immunoblot system. Titers from 8 seronegative vaccine recipients, from 18 HSV-1-seropositive vaccine recipients, and from 9 naturally HSV-2-infected subjects were compared (table 3).

Mean titers of IgG and IgA to gB2 and gD2 were significantly higher in the HSV-1-seropositive vaccine recipients than in either the seronegative vaccine recipients (P < .01 for IgG to gB2 and P < .05 for IgG to gD2) or the HSV-2-infected subjects (P < .01 for IgG to gB2 and gD2). There was a trend toward higher IgA titers in HSV-1-seropositive vaccine recipients than in either seronegative or naturally infected subjects. IgG and IgA titers of the infected subjects were somewhat higher than those of seronegative vaccine subjects, but the differences were not statistically significant.

Comparison of cervical and serum IgG titers. This study offered the unique opportunity to explore the relationship between serum responses and the development of cervical anti-
We report here that intramuscular injections of an HSV-2 subunit vaccine resulted in cervical IgG and IgA to both of the vaccine glycoprotein components in virtually all human subjects tested. As we found with natural HSV-2 genital herpes [5, 6], IgG was the predominant cervical antibody isotype induced by the subunit vaccine. Total IgG levels in the female genital tract usually exceed those of IgA [10, 11]. Furthermore, IgG has been shown to be the major genital tract isotype in response to human immunodeficiency virus [12] and to intramuscular immunization with tetanus toxoid [13].

The source of cervical IgG may be, in part, local plasma cells [14, 15], or the IgG may be derived from serum pools, either by passive diffusion [16] or by an active transport mechanism [11, 13]. The finding that fluctuations in cervical IgG titers were significantly correlated with those of serum IgG is consistent with the notion that most of the cervical IgG may be derived from the serum compartment after immunization with this gD2-gB2-MF59 subunit vaccine. A similar correlation between serum and parotid or nasal IgG to a cytomegalovirus subunit vaccine in MF59 adjuvant has been reported [17], suggesting that this adjuvant may affect the induction and delivery mechanisms for IgG in oral and respiratory as well as genital secretions. A more direct method to infer serum antibody transudation has been described but was not applied in these studies [18].

Passive immunization with serum IgG has been shown to be protective against vaginal challenge with HSV in mice [19]. It has been suggested that in humans, threshold serum IgG levels induced by vaccines against mucosal pathogens are most closely correlated with protection [20]. Additional study will be needed to determine the function of the IgG identified in this study.

Cervical IgA to the subunit vaccine was less prevalent than IgG and lower in titer than cervical IgG. The methods used for this study did not identify different molecular forms of IgA (dimeric versus monomeric), nor were we able to determine if the IgA detected contained secretory component, a covalently bound moiety obtained via active transport and maturation of dimeric IgA into the mucusosal lumen. Because the serum EIA did not measure IgA, we could not determine if the fluctuations in IgA titers reflected serum IgA fluctuations. Thus, while IgA appears to constitute a substantial minority of the immunoglob-

### Table 2. Prevalence of cervical IgG and IgA responses to HSV-2 gB-gD subunit vaccine in HSV-seropositive subjects.

<table>
<thead>
<tr>
<th>Vaccine dose</th>
<th>No. of responses/total no. of evaluable specimen pairs (%)</th>
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<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>IgG to gB</td>
<td>IgG to gD</td>
<td>IgA to gB</td>
<td>IgA to gD</td>
</tr>
<tr>
<td>1</td>
<td>20/20* (100)</td>
<td>17/20* (85)</td>
<td>12/19 (63)</td>
<td>10/19 (53)</td>
</tr>
<tr>
<td>2</td>
<td>6/19 (32)</td>
<td>8/19* (42)</td>
<td>3/19* (16)</td>
<td>1/19* (5)</td>
</tr>
<tr>
<td>3</td>
<td>10/20 (50)</td>
<td>9/20* (45)</td>
<td>6/19 (32)</td>
<td>3/19 (16)</td>
</tr>
<tr>
<td>4</td>
<td>9/16 (56)</td>
<td>6/16 (38)</td>
<td>9/16 (56)</td>
<td>1/16 (6)</td>
</tr>
</tbody>
</table>

NOTE. Paired specimens from days 0 and 28 (dose 1), from days 28 and 42 (dose 2), from days 180 and 194 (dose 3), and from days 360 and 374 (dose 4) were subjected to enhanced chemiluminescence Western blot. Increased intensity of gB2 or gD2 band was recorded as response.

* P < .01 vs. seronegative subjects.

### Table 3. Comparison of cervical IgG and IgA geometric mean titers in subjects who received 3 doses of vaccine with those from subjects with genital HSV-2 infection.

<table>
<thead>
<tr>
<th>Group</th>
<th>IgG</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gB2</td>
<td>gD2</td>
</tr>
<tr>
<td>Seronegative vaccine recipients</td>
<td>2.7 ± 1.2*</td>
<td>2.9 ± 1.4*</td>
</tr>
<tr>
<td>Seropositive vaccine recipients</td>
<td>3.9 ± 0.4*</td>
<td>3.9 ± 0.4*</td>
</tr>
<tr>
<td>Subjects with HSV-2 infection</td>
<td>3.2 ± 0.6*</td>
<td>3.3 ± 0.6*</td>
</tr>
</tbody>
</table>

NOTE. Data are geometric mean cervical log_{10} end-point titers (± SD).

* P < .01 between seronegative and seropositive groups.

* P < .05 between seronegative and seropositive groups.

* P < .01 between seropositive and infected groups.
Cervical Antibodies to HSV-2 Vaccine

ulins that arise in response to the HSV subunit vaccine, its characteristics and possible source are unclear.

Previous studies with this HSV-2 vaccine revealed equivalent or higher serum antibody titers to vaccine than to natural HSV-2 infection [9]. Assuming local antibody functions either protect against HSV-2 superinfection or are markers of other protective local factors, cervical antibody titers in natural infections provide a benchmark for potentially protective levels of antibodies induced by vaccine. Mean cervical antibody titers after three doses of vaccine in the seropositive group approximated (IgA) or significantly exceeded (IgG) those seen with natural HSV-2 infection. The seronegative group had lower mean antibody titers than did either the seropositive vaccine group or the group with natural HSV-2 infection.

Seropositive subjects responded to the first dose with titers that remained high over the course of the study. Seronegative subjects required two doses for maximal titers and had sharper declines in titer between doses. These differences in kinetics can most logically be ascribed to prior mucosal stimulation with antigen via oral HSV-1 infection in the group with prior HSV-1 antibodies. Cervical IgG and IgA to HSV are present in varying levels in subjects with prior HSV-1 infection [6]. The relatively flat mean titer curve of the seropositive subjects over four doses of vaccine and the fact that many did not increase their titers after doses 3 or 4 suggest that an upper limit of responsiveness was reached beyond which further antigen presentation failed to elicit a heightened response.

The functional utility of the genital tract antibodies that we have identified to a systematically delivered subunit HSV vaccine is not known. An efficacious vaccine for prevention of genital HSV-2 is likely to be associated with meaningful elicitation of genital tract immune responses.

Acknowledgments

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