Immunoglobulin Concentrations and Antigen-Specific Antibody Levels in Cervicovaginal Lavages of Rhesus Macaques Are Influenced by the Stage of the Menstrual Cycle

F. XUSHENG LÜ,† 1,2 ZHONGMIN MA, TH. TRACY ROURKE,† SEEMA SRINIVASAN,† MICHAEL MCCHESEY,† AND CHRISTOPHER J. MILLER† 1,2,3*

California Regional Primate Research Center,† 1 Center for Comparative Medicine, 2 and Department of Pathology, Microbiology and Immunology,†3 School of Veterinary Medicine, University of California Davis, Davis, California 95616

Received 14 June 1999/Returned for modification 27 August 1999/Accepted 1 September 1999

The levels of antigen-specific antibodies (Abs) and immunoglobulins in the cervical mucus of women vary with the menstrual cycle; the highest levels occur during menses, and the lowest occur during the periovulatory period. The rhesus macaque is a widely used animal model of female genital tract immunity. We sought to determine whether rhesus macaques have a cyclical pattern of changing cervicovaginal Ab and immunoglobulin levels that is similar to that of the human female. This study examined the relationship of the stages of the menstrual cycle to genital mucosal and systemic immunoglobulin concentrations and Ab levels in rhesus macaques. In all seven rhesus macaques studied, the immunoglobulins G and A and some antibodies in cervicovaginal lavages varied with the stages of the menstrual cycle, and in many cases this variation reached the level of statistical significance. In a pattern similar to that of women, the highest levels of Abs and immunoglobulins occurred during menses, and the lowest levels occurred around the time of ovulation. However, the Ab and immunoglobulin levels in serum and rectal lavages did not change with the menstrual cycle stage. The results of this study are consistent with the hypothesis that the ovarian hormones that drive the menstrual cycle influence genital tract immunity in female primates.

Mucous membranes comprise a large surface area (ca. 400 m² in adult humans) and include the intestinal, respiratory, and genital tracts. These mucosal surfaces are the first line of defense against many pathogenic organisms (15). Immune responses are elicited and independently regulated in mucosal and systemic immune compartments (16). Secretory immunoglobulin A (S-IgA) characterizes mucosal immune responses, whereas, systemic humoral immunity is dominated by IgG. The induction of protective immunity at the mucosal membranes is being considered with increasing emphasis in the development of vaccines against pathogens (3, 11, 14, 17). An understanding of genital and rectal mucosal immunity and the role of the ovarian hormone cycle or menstrual cycle in the regulation of immunity in the genital tract is necessary to develop vaccines against sexually transmitted diseases, including AIDS.

The menstrual cycle is regulated by the cyclic production of the ovarian sex steroid hormones progesterone and estrogen. Sex steroid hormones influence immune function in the genital tract. In rats, the stage of the estrous cycle influences the accumulation of IgA and IgG in uterine secretions (27, 28). In mice immunized intranasally with a recombinant adenovirus vector expressing herpes simplex virus glycoprotein B, specific IgA antibody titers in vaginal washes are higher during estrus than during diestrus or proestrus (5). Estrous-cycle-dependent changes have been documented in the immune cell populations of the rat uterus and vagina (8). Schumacher and Yang demonstrated, in studies of healthy women, that IgG and IgA levels in cervical secretions are lowest 1 day before ovulation and on the day of ovulation (22). Similarly, Kutteh et al. reported that IgA levels in human cervical secretions drop to the lowest level at ovulation (10). Jalanti and Isliker reported that more cervicovaginal lavage (CVL) IgA than CVL IgG is present at midcycle (7). Other investigators reported that levels of IgA and IgG in cervical secretions remain constant throughout the cycle (1). Expression of the polymeric immunoglobulin receptor by cervical and uterine epithelial cells varies with the stage of the menstrual cycle; this may be a reason that the S-IgA levels in cervical mucus of women vary with the menstrual cycle (2). Other potential mechanisms by which estrogen and other sex hormones affect immunity in the female genital tract remain to be determined.

In a study of intravaginally immunized macaques, the levels of antibodies (Abs) in the cervical mucosal were lowest at midcycle (29). However, it is not known if total immunoglobulin levels in the genital tract secretions of normal rhesus macaques vary with the menstrual cycle. The effect of sex steroid hormone levels on systemic immunoglobulin levels or immunoglobulin levels in other mucosal secretions has not been reported. Because macaques are becoming widely used for studies of genital tract vaccine development, the purpose of this study was to confirm the relationship between the menstrual cycle and immunoglobulin or Ab levels in CVL of female rhesus macaques and to determine whether this relationship extended to other mucosal or systemic immune compartments. In all macaques studied, the concentrations of IgG and IgA in the CVL were highest during menstruation and lowest in the periovulatory period. However, the menstrual cycle had no effect on immunoglobulin concentrations in rectal lavages (RL) or serum. These data demonstrate that the ovarian hormones, which control the menstrual cycle, influence immunoglobulin concentrations and specific Ab levels in the CVL of the female macaque.
EU definition. The levels of antigen-specific IgG and IgA antibodies in serum, CVL, and RL are expressed as ELISA units (EU). The EU value of a sample was determined by comparison with pooled, hyperimmune sera. A standard curve was generated for each ELISA plate and run in quadruplicate. The EU value was calculated based on the following equation: EU = ([sample OD – minimum OD standard curve] / maximum OD standard curve – minimum OD standard curve) × 100. OD is defined as the difference between the mean OD values of duplicate antigen-coated wells and the control wells. The maximum OD standard curve (ODsc) and minimum ODsc were defined as the OD values at the opposite endpoints of the linear portion of the standard curve. According to the equation, the maximum EU is 100 and the minimum EU is 0. Differences of all samples were tested.

Antibody ELISAs. Antibody levels are presented as EU as described above. The levels of TT-, KLH-, and CT-specific antibodies were determined by ELISAs performed in flat-bottom plates (Nunc Immunoplate II Maxisorb; Applied Scientific, San Francisco, Calif.). Plates were coated with TT (50 μg/ml), KLH (2.5 μg/ml), or CT (2 μg/ml) in Na2CO3-NaHCO3 coating buffer (pH 9.6) and then incubated overnight at 4°C. After plates were blocked with 4% skim milk, serial dilutions of standard hyperimmune sera and one dilution of each sample were added to the wells. The IgG antibody was detected with peroxidase-conjugated goat anti-monkey IgG secondary antibody (Nordic Laboratories). To measure antigen-specific serum IgG responses, different dilutions of serum samples were used for each antigen: 1:30 for CT, 1:20,000 for KLH, and 1:4,000 for TT. To measure antigen-specific serum IgA responses, different dilutions of serum samples were used for each antigen: 1:30 for CT and 1:10 for KLH and TT. All antibody responses in RL were tested on undiluted samples. To measure antigen-specific CVL IgG responses, different dilutions of CVL samples were used for each antigen: 1:60, 1:30, 1:15, and 1:7.5 for CT and CT and 1:60, 1:30, 1:15, and 1:7.5 for TT. To measure antigen-specific CVL IgA responses, different dilutions of CVL samples were used for each antigen: 1:2 for CT and KLH and 1:4 for TT. All antibody responses in RL were tested on undiluted samples. Preliminary experiments determined the dilution of each particular sample type that was most likely to give a OD value in the linear portion of the standard curve. The remaining steps were the same as those described for the measurement of IgA in serum (see above).

Measurement of urine prostate specific and estradiol levels. In order to evaluate ovarian hormone levels, daily urine estrone conjugates (E1C) and pregnanediol-3-glucuronide (Hy-PdG) of seven monkeys were measured by enzyme immunoassay (EIA) (6, 23). Flat-bottom microtiter plates (Nunc Maxisorb; Applied Scientific) were coated with 50 μl of rabbit anti-E1C antiserum (1:2,000) and 50 μl of monoclonal antibody to Hy-PdG (1:10,000) in bicarbonate coating buffer (pH 9.6) and then incubated at 4°C for a minimum of 12 h. The E1C antisera and the Hy-PdG antibody were provided by Bill Lasley (University of California, Davis). After incubation, plates were washed twice with a solution of 1.5 M NaCl and 0.05% Tween 20 (Sigma) and blocked for at least 30 min with 30 μl of PBS (0.1 M, pH 7) containing 0.1% of bovine serum albumin. Samples and standards (estrone β-glucuronide and β-pregnane-3,20α-diol-glucuronide; Sigma) were then added to the plates. Aliquots (40 μl) of urine and standard were added to the wells and incubated at 4°C overnight. After the samples and standards were incubated, they were washed, and 50 μl of distilled water was added to all wells, and plates were read on a spectrophotometer at 490 nm with a reference filter of 650 nm. Concentrations of IgA in samples were interpolated from the standard curve and corrected by multiplying by the dilution factor using the SOFTmax program (Molecular Devices Co., Sunnyvale, Calif.). Concentrations of IgA in sera were presented as milligrams per milliliter.

Statistical analysis. The immunoglobulin concentrations (Table 1) in serum, CVL, and RL were analyzed using Student's t-test as described above. The results were expressed as mean ± standard error (SE). The IgG and IgA levels were compared using a one-way ANOVA followed by a Student's t-test. The significance level was set at p < 0.05.
TABLE 1. Immunoglobulin concentrations in mucosal secretions and serum of normal female rhesus macaques

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>Conc in CVL (μg/ml [range])a</th>
<th>Conc in serum (mg/ml [range])b</th>
<th>Conc in RL (μg/ml [range])b</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>9.01 ± 3.73 (3.2–18.87)</td>
<td>1.74 ± 0.27 (0.63–3.63)</td>
<td>24.56 ± 3.90 (3.42–46.95)</td>
</tr>
<tr>
<td>IgG</td>
<td>14.18 ± 4.27 (3.53–55.00)</td>
<td>21.21 ± 1.1 (15.6–28.5)</td>
<td>0.98 ± 0.55 (0.03–1.27)</td>
</tr>
</tbody>
</table>

a Results presented are the mean immunoglobulin concentration ± the SE of the CVL samples collected from the animals during a single menstrual cycle. Only samples taken during the menstrual cycles used to produce Fig. 1 (see the text) were used.

b Results presented are the mean ± the SE of the samples from animals on the initial day of the study. *P < 0.0001 versus IgA in serum; **P < 0.0001 versus IgG in rectal secretions.

approach was used to compare mean immunoglobulin or Ab levels in CVL at ovulation versus 2 to 4 days before ovulation (Fig. 1).  

RESULTS

Accurate assessment of the relationship of the menstrual cycle to cervicovaginal immunoglobulin and Ab depends on close approximation of the day of ovulation. In this study, ovulation was estimated by measuring estrogen and progesterone levels in daily urine samples and by daily observation for menstrual bleeding (Fig. 1 to 3). The stage of the menstrual cycle at the time of immunization was variable among the animals, since no attempt was made to standardize the timing of the immunizations.

Immunoglobulin concentrations in serum. The mean concentrations of IgG and IgA in serum of the animals on the initial day of the study are shown in Table 1. The mean serum IgG concentration of 21.21 mg/ml was more than 10 times higher than the mean serum IgA concentration of 1.74 mg/ml (P < 0.0001). The results of the serum IgG analysis were consistent with our previous data (13). The booster immunization increased the serum IgG concentration of individual animals by 10 to 35 mg/ml over the 90-day observation period. However, cyclic variations in serum IgG or IgA concentrations were not present during the course of the menstrual cycle in any of the animals studied.

Immunoglobulin concentrations in rectal secretions. The mean concentrations of IgG and IgA in RL on the initial day of the study (day 0) are presented in Table 1. In contrast to serum, the mean RL IgA concentration was 20 times higher than the mean RL IgG concentration (P < 0.001). However, cyclic variation did not occur in the concentrations of immunoglobulins in the RL of individual animals during the course of the menstrual cycle.

Immunoglobulin concentrations in cervicovaginal secretions. The mean IgG and IgA concentrations of all CVL samples collected from the seven animals over the course of one menstrual cycle are presented in Table 1. All seven macaques studied had a higher concentration of IgG than IgA in CVL. The mean immunoglobulin levels in the CVL of the seven animals at specific time points in the menstrual cycle are presented in Fig. 1. The cycles of the different animals were aligned to one another by using the estimated day of ovulation as day 0. The highest IgA concentration in CVL of all animals occurred late in menses (in samples contaminated with menstrual blood); the IgA concentration remained elevated throughout the follicular phase (Fig. 1C). IgA levels dropped to the lowest concentration around ovulation and rose again through the luteal phase. Although the trend of high IgA levels during menses and low IgA levels at ovulation was clear, the difference in IgA concentration at these time points did not rise to the level of statistical significance. The IgG concentration in CVL was highest at menses (in samples contaminated with menstrual blood) and declined slightly but remained elevated through the follicular phase. The IgG concentration dropped to the lowest levels after the preovulatory estrogen peak and then rose again through the luteal phase to a peak again at the next menses. The difference in IgG concentration at menstruation versus ovulation was statistically significant (P < 0.05, day –1 to 1 versus day –14) (Fig. 1B). Variations from the pattern above of CVL immunoglobulin concentration among individual animals were often, but not always, coincident with unusual ovarian hormone profiles. When the observation of menstrual cycle period was narrowed to 4 days before and 3 days after ovulation, a clear trend was evident, with IgG and IgA concentrations highest 2 to 4 days before ovulation (Fig. 1B and C, inset). However, no statistically significant difference in immunoglobulin concentration existed between the periovulatory period (day –1 to day 1) and 2 to 4 days before ovulation.

Anti-TT and KLH Ab levels in serum after systemic immunization. Serum anti-TT IgG levels were 4000- to 8000-fold higher than serum anti-TT IgA levels. The serum anti-TT IgG levels increased from immunization day 0 (mean EU, 2.3 ± 2) to day 6 (mean EU, 15 ± 5), maintained high levels (mean EU, 47 ± 10) from day 12, and dropped at day 33 (mean EU, 23 ± 4). Anti-TT IgG increased to a mean EU value of 48 ± 7 within 7 days of the second immunization, and these elevated levels were maintained for the remainder of the study period (mean EU, 60 ± 6). Although the strength of the anti-KLH IgG and IgA response was much lower (note the dilutions used), the pattern of response was similar to that of the serum anti-TT Abs. Ab levels in serum did not vary cyclically during the course of the menstrual cycle (data not shown).

Anti-TT and KLH Ab levels in rectal secretions after systemic immunization. Anti-TT IgG and IgA in RL were detected intermittently but did not vary cyclically with menstrual cycles. Anti-TT IgG and IgA appeared 12 days after immunization (mean EU, 15 ± 6), increased 15 days after the second immunization (mean EU, 20 ± 10) and decreased to lower levels (mean EU, 5 ± 2) by day 65. The level of anti-KLH Abs in RL was low (mean EU, 10 ± 5 at the highest level). The anti-KLH IgG and IgA profiles in RL were similar to those of the anti-TT Abs (data not shown).

Anti-TT and KLH Ab levels in cervicovaginal secretions after systemic immunization. The anti-TT IgG and IgA levels in CVL were highest (EU IgG, 75 to 100; EU IgA, 40 to 50) during menstruation, when samples were contaminated with...
menstrual blood (Fig. 2B and 3B). After menstruation, anti-TT IgA decreased to below detectable levels (Fig. 3B). On the day of ovulation and 2 to 4 days after ovulation, the anti-TT IgG decreased to lowest levels (mean EU, 10) but remained detectable in some monkeys (EU from 20 to 40) (Fig. 2B). The difference in mean anti-TT IgG and IgA levels in CVL between day 21 and the periovulatory period (day 2 to day 1) was statistically significant ($P < 0.0001$). The menstrual cycle-related variation in KLH Ab levels was similar to the pattern seen with anti-TT Abs (Fig. 2C and 3C). The trend in changing IgG anti-KLH levels in CVL was statistically significant ($P < 0.01$). However, the variation of IgA anti-KLH levels in CVL did not reach a statistically significant level. When the observation of menstrual cycle period was narrowed to 4 days before and 3 days after ovulation, anti-TT IgG and IgA was highest 2 to 4 days before ovulation (Fig. 2B and 3B). However, a statistically significant difference between the Ab concentration during the periovulatory period versus that 2 to 4 days before ovulation was reached only for anti-TT IgG ($P < 0.05$).

**Anti-CT Ab levels in serum after oral immunization.** Serum anti-CT IgG was first detected (mean EU, 3.2 ± 1.1) 15 days after oral CT immunization. The second oral CT immunization boosted anti-CT IgG levels (EU, from 28 ± 9 on day 33 to 51 ± 11 on day 45) in all animals, and these elevated levels (mean EU, 40 ± 10) were maintained for the period of observation. Serum anti-CT IgA was first detected (mean EU, 2.2 ± 0.8) 27 days after immunization, increased by 7 days after the second immunization (mean EU, 15 ± 6.7), and was undetectable by day 54. Longitudinal analysis indicated that serum anti-CT Ab levels were not influenced by the menstrual cycle (data not shown).

**Anti-CT Ab levels in RL after oral immunization.** RL anti-CT IgA was intermittently detected (mean EU, 8 ± 4) beginning 9 days after oral immunization, increased 7 days after
the second immunization (mean EU, 15 ± 6), and dropped to the lowest level (mean EU, 5 ± 3) by day 65. No RL anti-CT IgG was detected. There was no correlation between anti-CT IgA levels in RL and anti-CT Abs in serum. Longitudinal analysis indicated that the Ab levels in RL did not change cyclically during the course of the menstrual cycle (data not shown).

**Anti-CT Ab levels in CVL after oral immunization.** The highest levels of anti-CT IgG (mean EU, 20 ± 5) in CVL occurred during menstruation when samples were contaminated with menstrual blood (Fig. 2D). Anti-CT IgA was for the most part undetectable, except for low levels (mean EU, 5 ± 2) detected during menstruation (Fig. 3D). The highest anti-CT IgG level in CVL occurred during menstruation, and the lowest Ab level occurred at ovulation. However, this trend in changing anti-CT Ab levels did not reach the level of statistical significance. No statistical correlation was found between anti-CT IgG levels in CVL and serum.

**DISCUSSION**

In this study, we examined the relationship of the menstrual cycle to mucosal and systemic immunity in rhesus macaques. We determined that the levels of IgG and IgA and the levels of antigen-specific Abs in the CVL vary at different stages of the menstrual cycle. The highest levels of immunoglobulins and specific Abs were present during menstruation, when samples were contaminated with menstrual blood, and in the early follicular phase, while the lowest levels occurred around the time of ovulation. However, the variations in immunoglobulin levels reached statistically significant levels only for CVL IgG. It is likely that a larger group of animals would be required to...
demonstrate that the clear trend in changing IgA levels in CVL was statistically significant. We also demonstrated that, as in women (12, 25), IgG is the predominant immunoglobulin in the genital secretions of rhesus macaques. The IgG and IgA concentrations in RL and serum were not influenced by the menstrual cycle. These data demonstrate that IgG and IgA antibody levels in CVL, induced by either systemic or oral immunization, are influenced by the menstrual cycle. The pattern of low immunoglobulin and Ab levels around the time of ovulation, reported here for rhesus macaque CVL, is consistent with previous reports on Ab levels in the cervical mucus of rhesus macaques (29) and on the IgG and IgA levels in the cervical mucus and vaginal fluid of women (22, 26). Further confirmation of the similarity between women and female rhesus macaques was provided in a recent report by Kutteh et al. In that report, analysis was limited to human cervical mucus samples collected from 5 days before ovulation to 3 days after ovulation. Within this narrow window of the menstrual cycle, IgA levels were highest 2 to 3 days before ovulation (10). However, this report did not demonstrate statistically significant differences in IgA levels at these time points. If the analysis of our results is limited to a similar window of the menstrual cycle, then the pattern in monkeys is very similar. The highest IgA levels in macaques CVL occurred 2 to 4 days before ovulation, and this was followed by decreasing IgA levels during and just after ovulation. However, despite a clear trend, these changes were not statistically significant. This pattern was more pronounced for the IgG in CVL but again did not rise to the level of statistical significance. This pattern extended to antibodies in CVL generated by both systemic and oral immunization and reached the level of statistical significance for anti-TT IgG ($P < 0.05$) (Fig. 2B and D).
One objective of our laboratory is to characterize the antiviral immune defenses of the female genital tract. Because many sexually transmitted viruses (human papilloma virus, simian immunodeficiency virus, human immunodeficiency virus, and herpes simplex virus type 2) infect both the vagina and the cervix, we chose to study the mixture of CVL. CVL best reflects the Abs present and capable of blocking infection by a pathogen in the lower genital tract. These studies now have analyzed the changes in immunoglobulin levels in the CVL over the entire menstrual cycle, including menses when samples were contaminated with menstrual blood. Any discrepancies in the data from women and monkeys may relate to differences in sample collection (CVL compared to cervical mucus) or alignment of the macaque menstrual cycles without using the luteinizing-hormone peak which indicates ovulation. However, taken together, the data from women and female rhesus monkeys indicate that immunoglobulin and Ab levels in genital tract secretions are lowest around the time of ovulation. In women, the drop in cervical mucus immunoglobulin concentration and Ab levels around the time of ovulation coincides with a large increase in the volume of cervical mucus (4, 10). Thus, the apparent decline in immunoglobulin and Ab concentration in the genital secretions of female menstrual primates around ovulation is likely to be an effect of dilution.

The results of the oral CT immunizations in the current study are consistent with our previous studies of oral immunization in the rhesus macaque (9). Oral CT immunization induced an anti-CT IgA response in RL but no anti-CT IgA response in CVL (9). The combined results of these studies suggest that oral immunization may not generate strong genital mucosal immune responses.

The source of the IgA and IgG in CVL is not known. Plasma-derived immunoglobulin in CVL may play an important role in humoral immune defenses of the female genital tract, especially during menstruation, when CVL is contaminated with menstrual blood. Numerous serum proteins, including immunoglobulins, complement, and albumin, are found in the CVL of women (21), while other plasma serum proteins, including α2-macroglobulin and high-molecular-weight lipoproteins cannot be detected in human CVL (21). This suggests that some plasma proteins may be secreted selectively into CVL. The concentration of the plasma proteins in CVL of women is lowest at midcycle (22). Thus, in women, the concentration of immunoglobulins in CVL parallels the concentration of other plasma proteins in CVL, suggesting that at least some immunoglobulin in CVL is plasma derived. The presence of serum antibodies in menstrual blood likely explains why the IgG and IgA concentrations in CVL are highest during menstruation.

The mean concentration of the serum IgG in the seven rhesus macaques in this report was determined to be 21 mg/ml (Table 1). This value is higher than was reported in another study (18) that used an ELISA system and human IgG as the standard. In the present study, the serum IgG concentration was determined by RID assay with rhesus macaque purified IgG as the standard. We previously demonstrated that, due to a much smaller chance of pipetting error and the use of a rhesus macaque IgG standard, the RID assay is more reliable than ELISAs for measuring IgG in rhesus macaque sera (13).

The results presented here demonstrate that the variation in immunoglobulin concentration in genital tract secretions during the menstrual cycle is similar in female rhesus monkeys and women. In addition, we have extended to macaques the observation previously reported in women that Ab levels as well as immunoglobulin concentrations in CVL vary with the stage of the menstrual cycle. Finally, the data presented here strengthen the growing body of evidence that the ovarian hormones that drive the menstrual cycle influence genital tract immunity but do not affect systemic immunoglobulin or Ab levels.

ACKNOWLEDGMENTS

We thank Jiri Mestecky for critical review of the manuscript and Judy Torten, Steve Joyce, Kristen Bost, Lisa Laughlin, David Bennett, and Linda Hirst for technical assistance.

This work was supported by National Institutes of Health grants NICHD ROI 33169, NCRR P51 AG00169, and NICHD U54 29125.

REFERENCES


