Specific Antibody Levels at the Cervix During the Menstrual Cycle of Women Vaccinated With Human Papillomavirus 16 Virus–Like Particles

Denise Nardelli-Haefliger, Daniel Wirthner, John T. Schiller, Douglas R. Lowy, Allan Hildesheim, Françoise Ponci, Pierre De Grandi

Background: **In early-phase trials, a human papillomavirus 16 (HPV16) virus–like particle (VLP) vaccine has been shown to be well tolerated, immunogenic, and protective against HPV16 in women, most of whom were taking oral contraceptives. Previous studies have not determined whether HPV immunization results in specific antibody levels in the human genital tract or whether these levels might vary during contraceptive or ovulatory cycles. Therefore, we determined the levels of total and specific antibodies in the cervical secretions of women who had been immunized with HPV16 VLPs and examined the influence of the menstrual cycle and oral contraceptive use on these levels.** ***Methods:*** **Two groups of women were immunized, seven who were taking oral contraceptives and 11 who were ovulating. After seroconversion, serum and cervical secretions were collected twice weekly for 5 weeks. Total immunoglobulins (IgG and IgA) and vaccine-specific IgGs were determined by enzyme-linked immunosorbent assay. Nonparametric statistical analyses were used to determine the statistical significance of differences in IgG levels between groups, and correlations between serum- and cervical-specific IgG levels were determined by the Spearman correlation coefficient.** ***Results:*** **All participants developed detectable titers of anti-HPV16 VLP IgGs in their cervical secretions after immunization. The cervical titers of specific IgG and total IgGs and IgAs among participants in the contraceptive group were relatively constant throughout the contraceptive cycle. In contrast, the cervical titers of specific IgG and total IgGs and IgAs among participants in the ovulatory group varied during the menstrual cycle, being highest during the proliferative phase, decreasing approximately ninefold around ovulation, and increasing approximately threefold during the luteal phase. Serum- and cervical-specific IgG levels were correlated (r = .86) in women in the contraceptive group but not in women in the ovulatory group (r = .27).** ***Conclusions:*** **The relatively high titer of anti-HPV16 antibodies at the cervix is promising in terms of vaccine efficacy; however, the decrease in antibody titer around ovulation raises the possibility that the HPV16 VLP vaccine might be less effective during the peri-ovulatory phase. [J Natl Cancer Inst 2003;95: 1128–37]**

Protective immunization requires the induction of an effective immune response at disease-relevant sites, where the presence of neutralizing antibodies is regarded as a key effector of many prophylactic vaccines *(1).* Most approved preventive vaccines are administered systemically, including some candidate vaccines against genital infection with herpes simplex viruses (HSVs) and human papillomaviruses (HPVs).

Although a mucosal route of immunization can induce the local production of polymeric secretory immunoglobulin A (sIgA) in the female genital tract *(2),* the predominant immunoglobulin in female genital mucosal secretions is monomeric immunoglobulin G (IgG), which is in contrast to the secretions of most mucosae, which are predominantly sIgA *(3–5).* The majority of IgG in the female genital tract probably results from the transudation of serum antibodies; however, it is possible that some IgG may be actively transported or locally produced in the mucosa *(6,7).* Immunization of rodents via a systemic route has been shown to lead to vaccine-specific IgG in the female genital tract *(8–10).* In addition, systemic immunization *(11,12)* or passive transfer of induced serum antibodies *(13)* can confer protection against HSV infection in animal models.

Much less is known about the immune response in the human female genital tract following systemic immunization, although specific antibody levels achieved at that site are likely to be related directly to vaccine efficacy. Parenteral immunization with tetanus toxoid vaccine has been shown to elicit vaccine-specific IgG in the female genital tract, but negative results were obtained with parenteral influenza vaccine *(14,15).* As is the case in rodents *(9,16–18),* the overall level of Igs in human female genital tract secretions is likely to be subject to considerable hormone-dependent variations during the menstrual cycle *(14,19–25).* However, no studies involving systemic immunization have examined the degree to which the menstrual cycle or the hormones present in oral contraceptives might affect vaccine-specific Igs in the genital tract.

PVs are epitheliotropic viruses whose sites of infection include the female genital tract *(26).* Although most genital HPV infections have a benign outcome, infection with a subset of HPV types, of which HPV16 is the most common, accounts for almost all cases of cervical cancer *(26),* which is the second most common cause of cancer deaths among women worldwide *(26,27).* Cervical cancer remains an important public health concern in industrialized countries despite well-established screening programs, and it occurs even more frequently in developing countries than it does in industrialized countries because of the lack of resources for population-wide screening.

Affiliations of authors: D. Nardelli-Haefliger, D. Wirthner, F. Ponci, P. De Grandi, Department of Gynecology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland; J. T. Schiller, D. R. Lowy (Laboratory of Cellular Oncology, Center for Cancer Research), A. Hildesheim (Environmental Epidemiology Branch, Division of Cancer Epidemiology and Genetics), National Cancer Institute, Bethesda, MD.

Correspondence to: Douglas R. Lowy, MD, Laboratory of Cellular Oncology, Bldg. 37, Rm. 4106, National Cancer Institute, Bethesda, MD 20892 (e-mail: drl@helix.nih.gov).

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The main public health goal of an HPV vaccine is to reduce the incidence of cervical cancer and precancerous lesions by preventing the establishment of persistent HPV infections that can lead to this malignancy. Candidate prophylactic HPV vaccines currently being tested in human trials are based on the observation that expression of the PV L1 major capsid protein leads to self-assembly of virus-like particles (VLPs) that resemble authentic virions and contain immunodominant neutralization epitopes [reviewed in *(28)*]. Animal models of PV infection have shown that parenteral immunization with VLP vaccines confers protection against challenge by the homologous virus in two oral mucosal PV models *(29,30)* and one cutaneous PV model *(31,32).* Studies involving passive transfer of immune IgG have shown that protection against cutaneous or oral animal PV infection is mediated largely by neutralizing IgGs. In early-phase human trials, systemic immunization with candidate L1 VLP vaccines involving HPV16 and other HPV types has been well tolerated, has induced high titers of neutralizing antibodies, and has been protective against HPV16 infection in women, most of whom were taking oral contraceptives *(33–36).* Although the level of specific antibodies in the human female genital tract is likely to be an important determinant of vaccine efficacy, previous studies *(33–36)* have not determined whether HPV immunization results in specific antibodies in the human female genital tract or whether these levels might vary during contraceptive or ovulatory cycles. Therefore, we followed healthy adult women who had received parenteral immunization with a candidate HPV L1 VLP vaccine to monitor vaccine-specific and total Ig levels in their genital tract during different phases of ovulatory and contraceptive cycles and examined the influence of menstrual cycle stage and oral contraceptive use on these levels.

Participants and Methods

Participants

Healthy adult women aged 18 to 45 years, with no history of positive Papanicolaou (Pap) smear, were recruited. Each participant's health was confirmed by medical history, serologic tests, and urinalysis for the presence of leukocytes. Participants underwent a routine gynecologic examination, which included Thinprep (Cytyc, Boxborough, MA) cervical sampling. Participants diagnosed with *Candida* (n = 1) or urinary tract infections (n = 2) were treated prior to immunization. Participants were excluded if they had an abnormal Pap smear (i.e., atypical squamous cytology of undetermined significance [ASCUS] or low-grade squamous intraepithelial lesions [LSILs]), human immunodeficiency virus seropositivity, HPV16 seropositivity, were taking chronic medication, were pregnant, or were taking oral contraceptives other than the estroprogestative Gynera or Minulet (0.03 mg of ethinylestradiol and 0.075 mg of gestodene).

For logistical reasons, two separate groups of women were recruited; the first group followed immunization schedule 1 and the second group followed immunization schedule 2 (*see* "Vaccine and Immunization Schedule" section below). Following the screening visit of the first group of participants (n = 14), 10 women were enrolled in the study. Four women were excluded from the study because they had HPV16 IgG titers higher than 50 (n = 2), or their Pap smears indicated the presence of ASCUS (n = 1) or LSILs (n = 1). One additional participant had to be excluded from the study after the first immunization because she needed chronic medication (i.e., Rocutan), and two other participants dropped out of the study after the second immunization for personal reasons, leaving seven participants in the first recruitment group.

A second round of recruitment accrued 14 participants. Following the screening visit of the second group of participants, 11 women were enrolled in the study. Three women were excluded from the study because they had HPV16 IgG titers higher than 50 (n = 1), their Pap smears indicated the presence of ASCUS (n = 1), or they used an oral contraceptive other than Gynera or Minulet (n = 1). None of the 11 participants in the second group were excluded or dropped out of the study after the start of the immunization schedule. Therefore, a total of 18 participants were included in this study.

The participants were then separated into two groups according to whether they were taking oral contraceptives (the contraceptive group, n = 7) or not (the ovulatory group, n = 11). The ovulatory group included participants who used condoms or intrauterine devices for contraception or who had been sterilized via tubal ligation. The clinical protocol for this study was approved by the local ethics committee, and written informed consent was obtained from each participant after the study had been explained to them in detail.

Vaccine and Immunization Schedule

Clinical lots of recombinant HPV16 L1 VLP vaccine, purified from insect cells and suspended in saline without adjuvant, were provided by Novavax (Columbia, MD) and were prepared according to good manufacturing practices guidelines, as previously described *(33).* Two immunization schedules were followed. For schedule 1, participants (three women from the contraceptive group and four women from the ovulatory group) received two 2-μg doses of HPV16 L1 VLP vaccine at weeks 0 and 4, followed by two 50-μg doses at weeks 12 and 15. For schedule 2, participants (four women from the contraceptive group and seven women from the ovulatory group) received two 50-μg doses of HPV16 L1 VLP vaccine at weeks 0 and 4. The stage of the menstrual or contraceptive cycle at the time of immunization varied among the participants because no attempt was made to standardize the timing of the immunizations.

Clinical Procedures

All participants had serum and cervical samples collected at 0 and 4 weeks after their last immunization and then twice per week for a period of 5 weeks. For technical reasons, cervical samples were not taken during the high-blood-flow period of menstruation (i.e., days 25 to 28 of contraceptive cycles and after day +13 of ovulatory cycles, with the day of ovulation being day 0). Otherwise, cervical samples were taken at any time during the cycles because they were considered to be physiologically relevant, even though some samples may have contained blood. Cervical samples were collected with Weck-cell sponges *(15,24)* as part of the gynecologic examination. This sampling method has been reported to be non-traumatic *(24),* but we cannot exclude the possibility that, in a few participants, bleeding may have been induced by the examination. To avoid any possible bias introduced by the presence of blood in the samples, all the samples containing blood (14 of 52 in the contraceptive cycles and 13 of 48 in the ovulatory cycles) were excluded from the statistical analysis of the ratios between cervical and serum

antibody titers in relation to the phases of the ovulatory cycle (*see* Fig. 3).

To collect the cervical samples, a speculum was inserted into the vagina and two sponges were placed sequentially in the endocervical canal for 1 minute each. Each sponge was then removed and placed in a 0.5-mL Eppendorf tube on ice, and 150 μL of phosphate-buffered saline (PBS) containing protease inhibitors (pepstatin at 10 μg/mL, leupeptin at 10 μg/mL, antipain at 10 μg/mL, and benzamidine at 50 μg/mL; all from Sigma Chemical Co., St. Louis, MO) was added. Each tube was then weighed, and the weight of each cervical secretion was calculated by subtracting the combined weight of an empty tube plus a dry sponge plus 150 μL of PBS. The tubes were pierced at the bottom with a needle, placed in large 2-mL Eppendorf tubes without lids, and centrifuged at 10 000*g* for 10 minutes at 4 °C. The liquid fraction (i.e., diluted cervical secretion) was then frozen at –70 °C until analysis. For each sample, a dilution factor was calculated by dividing the total volume of the diluted samples by the weight of the cervical secretion collected.

Determination of Menstrual Cycle Stage

To determine the ovulation day(s) for each participant in the ovulatory group for the 5-week sampling period, the number of days after (or before) menstruation was considered together with the serum levels of estrogen, luteinizing hormone (LH), and progesterone. Ovulation occurs approximately 36 hours after the onset of the pre-ovulatory surge of LH. As the LH peak is reached, estradiol levels decrease and progesterone levels continue to increase. The luteal phase begins with the expulsion of the oocyte shortly after ovulation. Progesterone secretion generally increases to a peak 6–8 days after the LH surge. If the oocyte is not fertilized, progesterone levels decrease until the onset of menses. LH, estradiol, and progesterone levels were measured in the sera of participants starting at least 9 days after their first day of menstruation. If a clear increase in LH level was not observed, hormone levels were determined again at the next visit (i.e., 3–4 days later). When a clear LH peak was observed, together with a decrease in estradiol level and an increase in progesterone level, the day of ovulation was estimated to have occurred 24 hours later. If the participant had a high progesterone level (i.e., >10 ng/mL), the date of ovulation was confirmed or determined as being 14 days before the first day of the participant's next menstruation.

Using these criteria, we were able to determine the ovulation day(s) for eight of the 11 participants in the ovulatory group, two of whom had ovulated twice during the sampling period. For the three participants for whom we were unable to determine the ovulation day(s), one did not appear to ovulate during the sampling period (i.e., the participant had no detectable increase in LH levels at visits occurring at days 15 and 18 of her first cycle and days 11, 15, 18, and 23 of a second consecutive cycle), and for the other two participants, the day of ovulation could not be determined with enough precision. One of these participants (who had a long cycle of 45 days) started her sampling visits 22 days after the start of menstruation and, although an intermediate increase in LH level was observed, the precise date of ovulation could not be determined. The second participant presented with relatively high levels of LH at day 16 of her cycle; however, menstruation occurred 21 days later. Relatively high levels of LH were again measured at day 11 of her next consecutive cycle; however, this time frame was the end of the sampling period for that participant. One of the eight participants who had her ovulation day determined and who had low HPV16 VLP serum IgG antibody titers (i.e., 800 before cycle sampling and 200 afterward) had undetectable vaccine-specific IgG titers in cervical secretions at some time points during her ovulatory cycle; therefore, her data were not included in the analysis of genital tract antibodies.

For the contraceptive group, cervical secretion samples were assigned to a particular stage of the contraceptive cycle according to which day the contraceptive pill was taken (i.e., day 1 = first day of the 21-day packet).

Determination of Ig Levels

Ig levels were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, for determination of HPV16 VLP–specific IgG and IgA levels, Greiner 96 microtiter plates (Greiner Bio-One, Poitiers, France) were coated with purified HPV16 VLPs (85 ng) in PBS in each well. After blocking with a solution of PBS, 0.1% Tween, and 1% dry powdered milk for 1 hour at 37 °C, vaccine-specific Ig in both cervical and serum samples was determined by adding biotinylated rabbit anti-human IgG (1 : 500; Dako, Kyoto, Japan) or goat anti-human IgA (1 : 1000; Chemicon, Temecula, CA) secondary antibodies followed by peroxidase-conjugated streptavidin (1 : 5000; Dako) and *o*-phenylenediamine (1 mg/mL; Sigma Chemical Co.) to each well. Neutralizing antibody titers were not determined because a good correlation between ELISA titers of HPV16 VLP–specific antibodies and HPV16 neutralizing titers has been previously reported for both serum *(33,37)* and genital secretions *(9)*. For determination of total IgG and IgA levels, Greiner 96 microtiter plates were coated with a rabbit anti-human IgG (1 : 3000; Dako) or a mouse monoclonal anti-human IgA (1 : 250; Zymed, San Franciso, CA), respectively. The Ig levels in the samples were determined as described above for the vaccine-specific Igs.

Each serum and cervical sample was tested in duplicate, with purified human serum IgA (standard curves with eight consecutive twofold dilutions starting at 102 ng/mL; Cappel, Organon Teknika, Durham, NC) or IgG (standard curves with eight consecutive twofold dilutions starting at 46 ng/mL; Cappel, Organon Teknika) included in the assays as reference standards. For determination of HPV16 VLP–specific Ig levels, twofold dilutions (in PBS, 0.1% Tween, and 1% dry powdered milk) of the serum and cervical secretion samples were tested, starting at a dilution of 1 : 50 for sera and 1 : 5 for cervical secretions. The 96-well plates were then read on an automatic plate reader (Dynatech Laboratories, Chantilly, VA) at a wavelength of 490 nm and a filter of 640 nm. The variability of the duplicate assays for each serum and cervical sample was less than 15%. When higher variabilities were observed, each sample was tested again in duplicate.

The titers for vaccine-specific Igs were determined as the reciprocal end-point dilution that yielded an optical density (OD) of more than 0.100, which corresponded to three times the mean pre-immune OD (0.0127) plus three standard deviations (SDs) (0.0066) for the serum dilution of 1 : 50 and two times the mean pre-immune OD (0.0132) plus three SDs (0.0129) for the cervical dilution of 1 : 5. These criteria have been used previously on a panel of serum samples from vaccinees *(33)*; similar titers were obtained in this study (data not shown). Participants were considered to be HPV16-seropositive at recruitment if their serum yielded an OD of more than 0.100 at a 1 : 50 dilution;

these participants were excluded from the study, as previously noted.

Statistical Analysis

All antibody levels are presented as geometric mean titers (GMTs) calculated as the arithmetic mean of the log titers. Nonparametric statistical analyses were used to determine the statistical significance of differences in Ig levels between groups. When two groups were compared, the Wilcoxon *t* test was used, and when more than two groups were compared, the Kruskal–Wallis test was used. When patterns over time within participants were evaluated (i.e., cervical-to-serum antibody ratio, total IgGs and IgAs, and weight of secretions), unpaired rather than paired tests were used to avoid the exclusion of results from participants with incomplete data. When evaluating the association of menstrual cycle stage with cervical and serum antibodies over time, the ovulatory cycle was divided, *a priori,* into the proliferative (days –11 to –6), peri-ovulatory (days –2 to +2), and luteal phases (days +8 to +12) and the contraceptive cycle was divided (again, *a priori*) into three time periods, days 4–7, 11–14, and 18–21. Mean titer levels were used whenever a participant had more than one sample taken during a specific period within her cycle. For example, if a participant had samples taken twice during the luteal phase of her cycle, the mean of the two samples was used in analyses. Correlations between antibody titers in serum and cervical secretions were determined by the Spearman correlation coefficient. All statistical tests were two-sided.

Results

Serum Antibody Responses to HPV Immunization

To assess the antibody response to HPV immunization, participants received intramuscular injections of HPV16 VLPs without adjuvant following two different immunization schedules: schedule 1 consisted of two 2-μg doses of HPV16 VLPs followed by two 50-μg doses, and schedule 2 consisted of two 50-μg doses only. All participants, regardless of immunization schedule, had seroconverted by 4 weeks after the last immunization; the serum anti-HPV16 VLP IgG GMTs were 4158 (95% confidence interval [CI] = 1872 to 9236) for the participants on schedule 1 and 1925 (95% CI = 1088 to 3406) for the participants on schedule 2. Because the serum anti-HPV16 VLP IgG GMTs of the two immunization groups were not statistically significantly different ($P = .23$), additional comparisons were not stratified by the number of vaccine doses received because the purpose of this study was to investigate the variation in specific antibody levels at the cervix of each participant over time independently of the serum HPV16 VLP titer reached by each vaccine. Therefore, the rest of the results focus on comparisons between the ovulatory and contraceptive groups and between different stages of the ovulatory and contraceptive cycles within each group.

Four weeks after the last immunization, the serum GMT of anti-HPV16 VLP IgG obtained in the contraceptive group (GMT = 3189, 95% CI = 1143 to 8898) was not statistically significantly different from the serum GMT obtained in the ovulatory group (GMT = 2278, 95% CI = 1398 to 3714; $P = .47$). Six weeks later, at the end of the sampling period, the GMTs for the anti-HPV16 VLP IgGs in the contraceptive and ovulatory groups had decreased, although not statistically significantly, to 2348 (95% CI = 849 to 6491; $P = .71$) and 1023 (95% CI = 580 to 1802; $P = .08$), respectively. A similar decrease in serum anti-HPV16 VLP IgG GMTs was also seen 2 months after the second 50-μg dose in an earlier group of participants *(33).*

During the sampling period, a maximal twofold variation in the HPV16 VLP serum IgG titers was observed between two consecutive samples (data not shown). The serum GMTs for HPV16 VLP–specific IgA were relatively low in both groups of participants (74 [95% CI = 25 to 223] for the contraceptive group versus 100 [95% CI = 58 to 173] for the ovulatory group), as expected from previous findings *(33).* Anti-HPV16 VLP–specific IgA cervical titers were also very low (maximum titer of 40) and were often undetectable (data not shown).

HPV16 VLP–Specific Antibody Responses in Cervical Secretions

In addition to determining the serum antibody responses to HPV immunization, we also assessed the levels of cervical antibodies in both the ovulatory and contraceptive groups. Regardless of immunization schedule, all participants developed cervical anti-HPV16 antibodies (Figs. 1 and 2). Therefore, we examined possible variations in vaccine-specific cervical antibody levels during ovulatory and contraceptive cycles. Anti-HPV16 VLP IgG titers during individual ovulatory cycles for seven participants (Fig. 1, A; day 0 is the day of ovulation) were examined, two of whom (participants E and G) had two ovulations during the sampling period. Each participant's cervical anti-HPV VLP IgG titers were highest during the proliferative phase, decreased an average of ninefold around ovulation, and increased (except for participant F, whose titers remained constant after ovulation), on average, threefold during the luteal phase.

For the two participants whose day of ovulation could not be precisely determined by serum hormone levels, the lowest cervical anti-HPV VLP IgG titers were observed around 14 days before menstruation, which was in agreement with their putative ovulation date (data not shown). The two participants who had cervical samples from two consecutive menstrual cycles (participants E and G; Fig. 1, A and B) had similar antibody profiles for both cycles. However, there was considerable variation in the individual cervical titer curves between participants, even when the cervical titers were normalized to each participant's corresponding serum antibody titers (Fig. 1, B).

The ratios between cervical and serum antibody titers were also analyzed in relation to the phases of the ovulatory cycle (Fig. 3, A). The proliferative phase had the highest ratio (median = 0.09, interquartile range [IQR] = 0.04 to 0.16), the luteal phase had an intermediate ratio (median = 0.03, IQR = 0.02 to 0.04), and the ovulation phase had the lowest ratio (median = 0.01, IQR = 0.01 to 0.02; $P = .05$, compared with the proliferative or luteal phases). We also examined whether there was a correlation between cervical and serum antibody titers and found that cervical antibody titers did not correlate with the serum antibody titers ($r = .27$; Spearman correlation coefficient), suggesting that there may not be a direct relationship between serum and cervical antibody titer among participants in the ovulatory group.

The HPV16 antibody profiles for the participants in the contraceptive group displayed some important differences from those of the participants in the ovulatory group (Figs. 2 and 3). First, the cervical anti-HPV VLP IgG titers were relatively con-

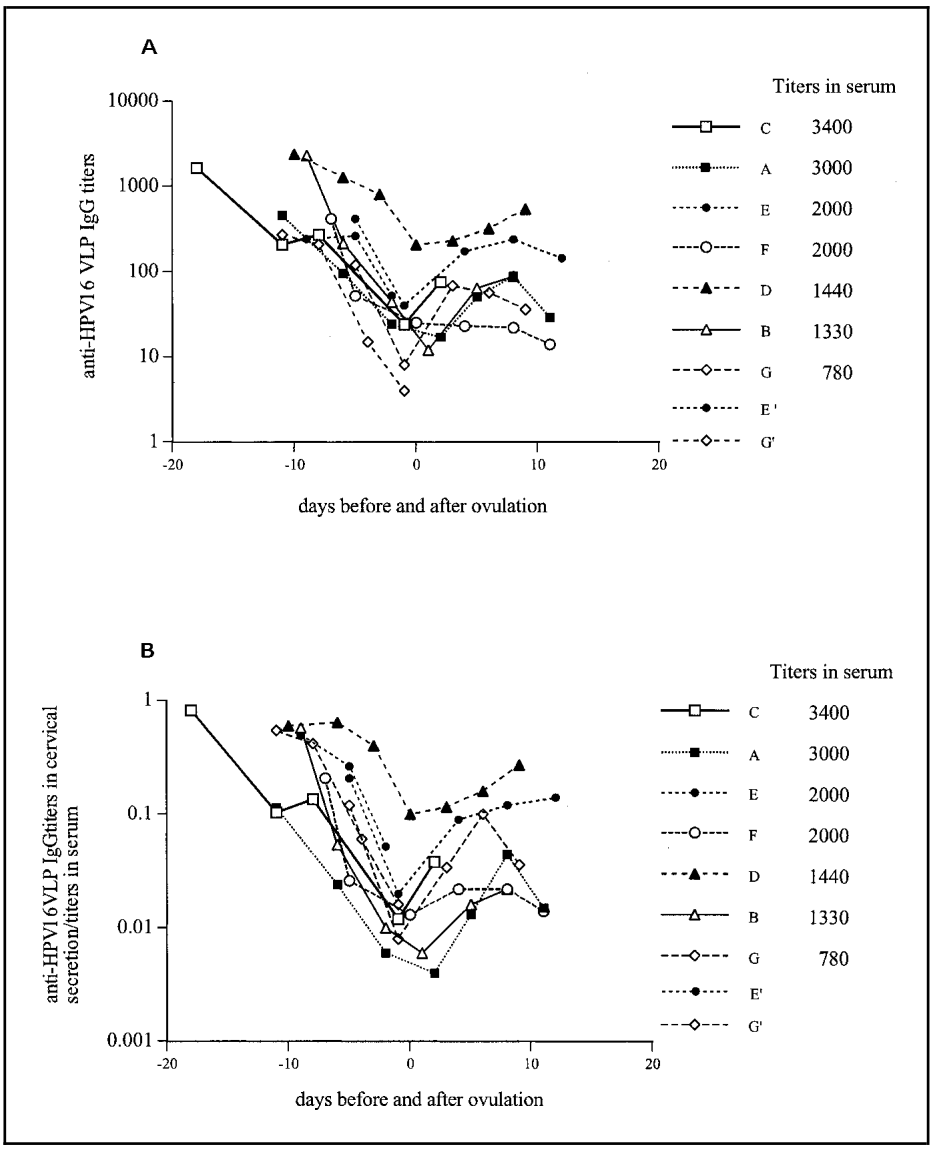

Fig. 1. Anti-human papillomavirus 16 (HPV16) virus-like particle (VLP) immunoglobulin G (IgG) titers in cervical secretions during ovulatory cycles. **A**) Vaccine-specific antibody titers in cervical secretions from women in the ovulatory group. Each **curve** represents the cervical antibody titers during one ovulatory cycle for each of seven participants (**A–G**). E′ and G′ represent the vaccine-specific antibody titers during a second ovulatory cycle for participants E and G, respectively. The mean serum anti-HPV16 VLP IgG titers during the sampling period are listed on the **right** of the figure adjacent to each participant's letter designation and symbol. **B**) The ratio of cervical anti-HPV16 VLP antibody titers at each time point in **panel A** to the serum anti-HPV16 VLP antibody titer at the same time point. Each **curve** represents the ratio during one ovulatory cycle for participants A–G. E′ and G′ represent the ratio during the second ovulatory cycle for participants E and G, respectively. The mean serum anti-HPV16 VLP IgG titers during the sampling period are listed on the **right** of the figure adjacent to each participant's letter designation.

stant throughout the contraceptive cycle—that is, there was no variability in anti-HPV16 VLP IgG titers throughout the cycle (Fig. 2, A and B) like there was in the ovulatory group. Interestingly, the cervical titers of the one participant in the ovulatory group who did not ovulate were also relatively constant throughout her sampling period (data not shown). Second, when the contraceptive cycle was divided into three phases (days 4–7, days 11–14, and days 18–21), the cervical-to-serum antibody ratios were not statistically significantly different, with median ratios of 0.06 (IQR = 0.05 to 0.08), 0.11 (IQR = 0.06 to 0.13), and 0.06 (IQR = 0.03 to 0.13) for the three phases, respectively (P = .71, Kruskal–Wallis test; Fig. 3, A). Third, the median cervical-to-serum antibody ratio during the contraceptive cycle (0.06, IQR = 0.04 to 0.10; days 1–24) was statistically significantly higher than it was in the peri-ovulatory phase (0.01, IQR = 0.01 to 0.02: days –2 to +2) (P<.001) of the ovulatory group. Finally, although the cervical antibody titers differed among the participants (Fig. 2), there was a correlation between the cervical antibody titer and the serum antibody titer in the contraceptive group (r = .86; Spearman correlation coefficient), whereas there was no such correlation in the ovulatory group, suggesting a direct relationship between cervical and serum antibodies among participants in the contraceptive but not the ovulatory group.

Total IgG and IgA Content in Cervical Secretions

To examine whether the observed relationship between serum and cervical anti-HPV16 antibody levels might also apply to overall antibody levels, total cervical and serum IgG and IgA levels were determined for each participant (Fig. 3, B and C, respectively). The median concentrations of total IgGs and IgAs in the cervical samples during contraceptive cycles were 530 μg/mL (IQR = 327 to 680 μg/mL) and 20.0 μg/mL (IQR = 13.4 to 34.0 μg/mL), respectively. The total IgG concentration was similar to that previously reported for cervical samples collected from women taking oral contraceptives (mean value ± standard error [SE] = 1169.2 ± 326.2 μg/mL) *(15)*; however, the total IgA concentration was lower than that previously reported (mean ± SE = 270.1 ± 57.8 μg/mL) *(15)*. We confirmed the low IgA concentration in our samples with a second standard human IgA, and we also verified that our detection of IgA was not hindered by the presence of high IgG levels by detecting known amounts of purified IgA that were added to the cervical secretions. Interestingly, vaginal secretions have been reported

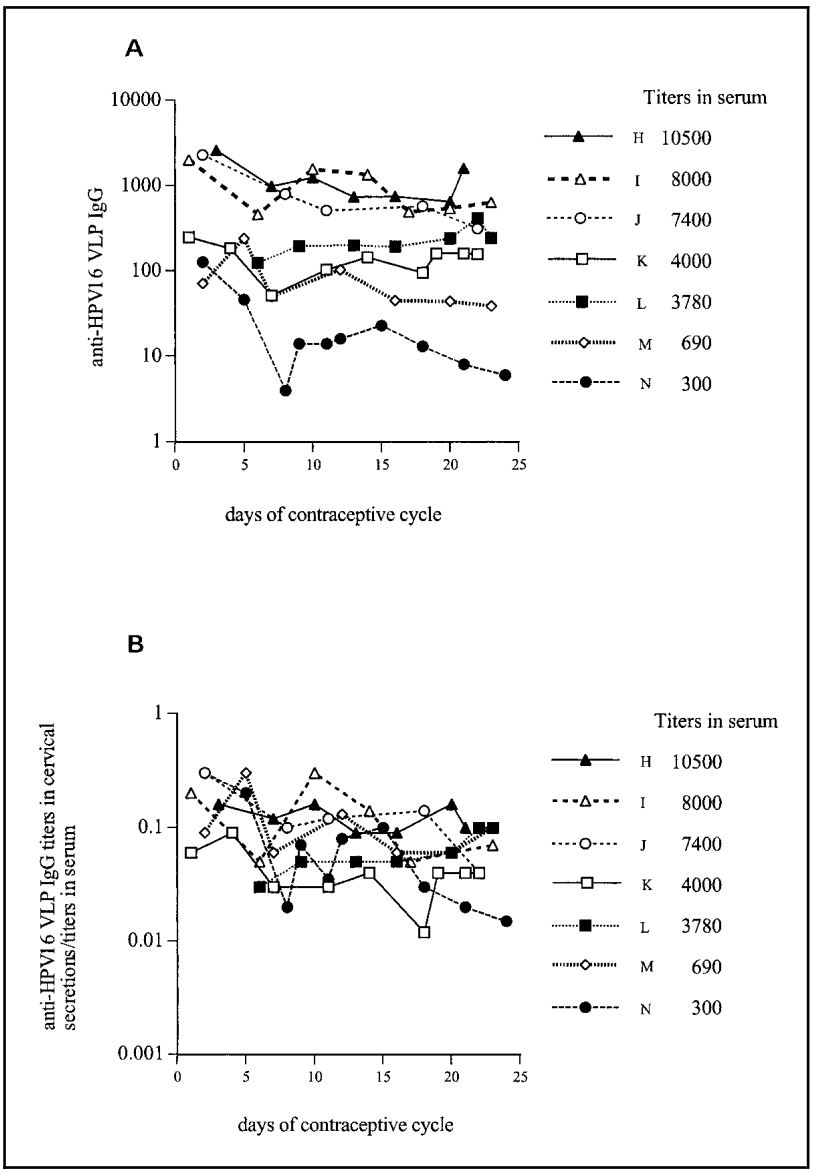

Fig. 2. Anti-human papillomavirus 16 (HPV16) virus-like particle (VLP) immunoglobulin G (IgG) titers in cervical secretions during contraceptive cycles. **A**) Vaccine-specific antibody titers in cervical secretions from women in the contraceptive group. Each **curve** represents the cervical antibody titers during one menstrual cycle for each of seven participants (**H–N**). The mean serum anti-HPV16 VLP IgG titers during the sampling period are listed on the **right** of the figure adjacent to each participant's letter designation. **B**) The ratio of cervical anti-HPV16 VLP antibody titers at each time point in **panel A** to the serum anti-HPV16 VLP antibody titer at the same time point. Each **curve** represents the ratio during one menstrual cycle for participants H–N. The mean serum anti-HPV16 VLP IgG titers during the sampling period are listed on the right of the figure adjacent to each participant's letter designation.

to have lower concentrations of IgA (mean ± SE = 43.6 ± 8.3 μg/mL) *(15)* than cervical secretions. Hence, although we placed our Weck-cell sponges in the cervical os and efforts were made to avoid touching the vaginal wall, we cannot exclude the possibility that some vaginal secretions that were loosely attached to the cervical mucus may have been included in our samples, thus potentially lowering the IgA concentration.

During the contraceptive cycles, both total IgG (Fig. 3, B) and total IgA (Fig. 3, C) concentrations were relatively constant ($P = .92$ and $P = .18$, respectively), with median values of 485 μg/mL (IQR = 370 to 605 μg/mL), 535 μg/mL (IQR = 421 to 1242 μg/mL), and 460 μg/mL (IQR = 439 to 567 μg/mL) for IgG and 13.4 μg/mL (IQR = 11.0 to 25.5 μg/mL), 32.8 μg/mL (IQR = 27.0 to 49.0 μg/mL), and 19.7 μg/mL (IQR = 15.0 to 28.5 μg/mL) for IgA during days 4–7, 11–14, and 18–21, respectively. During the ovulatory cycles, total IgG decreased statistically significantly between the proliferative phase and the peri-ovulatory phase (from 497 μg/mL, IQR = 306 to 793 μg/mL to 71 μg/mL, IQR = 49 to 114 μg/mL; $P = .04$) and rose again, although not statistically significantly, during the luteal phase (to 322 μg/mL, IQR = 175 to 551 μg/mL; $P = .11$).

The variation in total IgG concentration during the ovulatory cycles (Fig. 3, B) paralleled that observed for the vaccine-specific IgGs (Fig. 3, A). The decrease in median total IgG concentration from the proliferative phase to the peri-ovulatory phase was sevenfold, which was similar to the ninefold decrease in vaccine-specific IgG concentration. The same was also true for the fold increase from the peri-ovulatory phase to the luteal phase (fivefold in total IgG and threefold in specific IgG). These results suggest that variations in HPV16 VLP–specific IgG during ovulatory cycles are closely linked to the regulation of transudation of Igs in general and not to the vaccination procedure. In contrast, variation in the titers of total IgA during the ovulatory cycle (Fig. 3, C) was not statistically significant, but the number of participants in that group was small.

Effect of the Amount of Cervical Secretion on Antibody Levels

To determine whether changes in the amount of cervical secretion might account for the variation in antibody levels observed in the cervix, we analyzed the median weight of the cervical secretions obtained with the Weck-cell sponge during

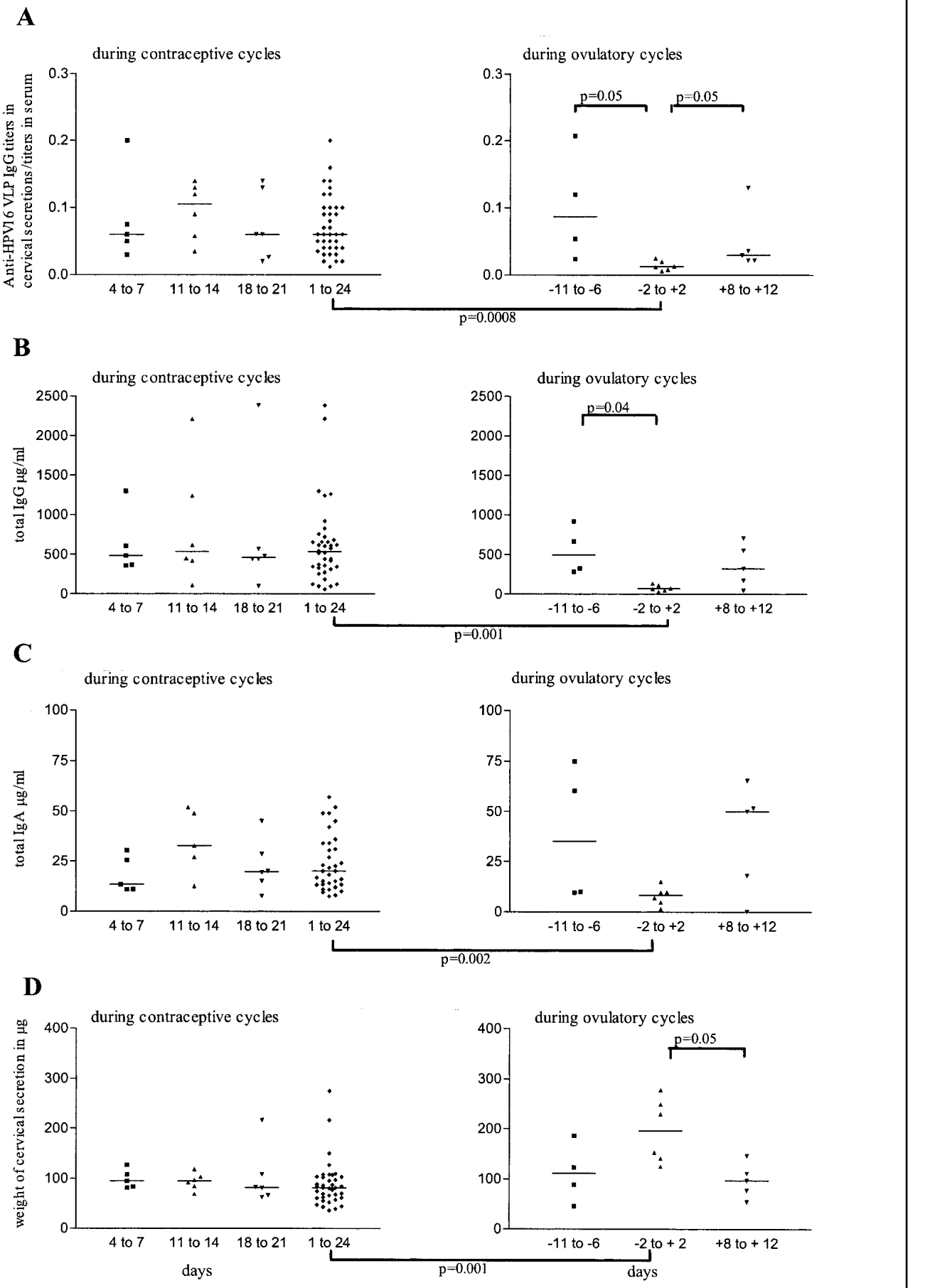
A
during contraceptive cycles
during ovulatory cycles
Anti-HPV16 VLP IgG titers in cervical secretions/titers in serum
0.3
0.2
0.1
0.0
p=0.05
p=0.05
4 to 7
11 to 14
18 to 21
1 to 24
-11 to -6
-2 to +2
+8 to +12
p=0.0008
B
during contraceptive cycles
during ovulatory cycles
total IgG µg/ml
2500
2000
1500
1000
500
0
p=0.04
p=0.001
C
during contraceptive cycles
during ovulatory cycles
total IgA µg/ml
100
75
50
25
0
p=0.002
D
during contraceptive cycles
during ovulatory cycles
weight of cervical secretion in µg
400
300
200
100
0
p=0.05
p=0.001
days
days

Fig. 3. Comparison of cervical antibody levels during ovulatory cycles versus oral contraceptive cycles. Comparisons were made by treating each data point as an independent observation in the contraceptive group (days 1–24) and as mean levels whenever a woman had more than one sample taken during a specific stage during her cycle. Only cervical samples that did not contain blood have been included in this analysis. **Horizontal bars** represent the median values of the indicated parameter at each phase of the contraceptive and ovulatory cycles. **A**) Level of anti-human papillomavirus 16 (HPV16) virus-like particle (VLP) immunoglobulin G (IgG) titers in cervical secretions. The **vertical axis** represents the ratio of cervical anti-HPV16 VLP antibody titers to the serum anti-HPV16 VLP antibody titer for each participant. The **horizontal axis** represents the data for the individual determinations shown in Fig. 1, B, for the ovulatory cycles and Fig. 2, B, for the contraceptive cycles. **B**) Level of total IgG in cervical secretions. **C**) Level of total IgA in cervical secretions. **D**) Weight of cervical secretions. For **panels A, B, C,** and **D,** the **horizontal axis** represents the data for the individuals shown in Fig. 1, B, for the ovulatory cycles and Fig. 2, B, for the contraceptive cycles during the *a priori* arbitrarily defined periods of the cycle indicated below each **horizontal axis.**

the peri-ovulatory phase and found that that phase yielded approximately two times more cervical secretions than the luteal phase (191.3 μg, IQR = 141 to 250 μg versus 97 μg, IQR = 77 to 111 μg, respectively; $P = .05$; Fig. 3, D). Results similar to those in the luteal phase were found in the proliferative phase (106.3 μg, IQR = 68 to 155 μg; $P = .91$). These modest changes in the amount of cervical secretions collected during the different phases of the ovulatory cycle cannot, however, account for the decrease in Ig concentration or in HPV16 VLP–specific IgG titers seen during the peri-ovulatory phase.

Discussion

Although the concentration of Igs in human cervical secretions varies because of endogenous or exogenous sex hormones *(7,15,23–25,38),* this is the first study, to our knowledge, in which both total and vaccine-specific Igs have been examined during a full menstrual cycle in ovulating women and in women taking oral contraceptives. Our data demonstrate several important results. First, intramuscular immunization of healthy, adult women with HPV16 VLPs induced relatively high titers of vaccine-specific antibodies (mainly IgG) in cervical secretions. Second, there was a statistically significant decrease in cervical vaccine–specific IgG levels during ovulation. Third, the changes in vaccine-specific IgG levels during the menstrual cycle were quantitatively similar to those in total IgG levels. Finally, the variation in vaccine-specific IgG levels in cervical secretions among ovulating women was relatively independent of the antibody levels induced in their serum, whereas a correlation was observed between the vaccine-specific IgG levels in the serum and cervical secretions among women taking oral contraceptives. The association of oral contraceptives with reduced changes in cervical Ig concentration seen during the ovulatory cycle suggests that endogenous sex hormones may be largely responsible for regulating the antibody concentration in the female genital tract.

Because antibodies of the IgG isotype are predominant in female genital-tract secretions and are largely of plasma origin, it has been assumed that systemic immunization may be an effective route of immunization for inducing humoral immune responses in cervico-vaginal secretions. Indeed, this response was observed after intramuscular vaccination with a tetanus vaccine (consisting of purified tetanus toxoid) administered with aluminum hydroxide *(14).* However, systemic immunization with a vaccine consisting of inactivated influenza virus did not lead to the detection of vaccine-specific IgGs in genital secretions *(15),* suggesting that cervical antibody response may depend on the type of vaccine used, the immunization schedule, and/or other unknown parameters.

There were several important differences in the cervical antibody profiles between the participants in the contraceptive and ovulatory groups. The vaccine-specific Ig titers in the cervical secretions of women in the contraceptive group were relatively stable. The same was also true for total Igs, which is in agreement with previous findings *(15).* However, there was a statistically significant variation in both vaccine-specific and total Igs during the ovulatory cycles, with the lowest levels of antibodies occurring around ovulation. Franklin and Kutteh *(25)* reported a fourfold difference between the pre-ovulatory and peri-ovulatory levels of total IgG and IgA during a 9-day evaluation of cervical antibody levels around ovulation (days –4 to + 4). Our total IgG results, which have confirmed and extended these observations to a full menstrual cycle and include vaccine-specific antibody responses to systemic immunization, show an even greater difference (approximately seven- to ninefold) in median IgG levels between the proliferative and peri-ovulatory phases. Interestingly, we measured only a twofold difference in the amount of cervical secretion collected by our Weck-cell procedure between the proliferative and peri-ovulatory phases. Therefore, the increased amount of cervical secretions in the peri-ovulatory phase can only partially account for the much larger decrease in Ig concentration during that phase. Although it is possible that our collection procedure may not reflect the total volume of cervical secretions, our results do raise the possibility that there may be hormone-dependent changes in the efficiency with which Igs are transudated or produced in the female genital tract. The mechanisms of transudation or diffusion through the mucosa are poorly understood and deserve further investigation. Active transport of IgG through the genital mucosa by an Fc receptor–associated mechanism *(8)* and local production of IgG *(15)* have been proposed to contribute to transudation or diffusion, and both of these mechanisms could be influenced by sex hormones, as has been demonstrated for the local production of sIgA *(16–18,39).*

Our finding that HPV16 VLP–specific IgGs are induced in the cervical secretions of all participants is consistent with the encouraging results of protection against HPV16 infection that were recently reported in women immunized with an HPV16 VLP vaccine *(36).* The vast majority of women in that trial *(36)* were taking oral contraceptives, which we have found to be associated with constant and high levels of vaccine-specific antibodies. Given our observation that ovulating women have much lower HPV16 VLP–specific antibody titers around ovulation than at other times in the menstrual cycle, it will be important to determine whether the HPV16 VLP vaccine will be as effective at protecting ovulating women from HPV16 infections as it appears to be for women taking oral contraceptives.

The decrease in vaccine-specific antibody concentration around ovulation might represent a protective mechanism that reduces the level of anti-sperm antibodies in the female genital tract at a time when conception is most likely to occur. It is also possible that the decrease in vaccine-specific antibody concentration around ovulation may predispose ovulating women to be more susceptible to some genital infections than both non-ovulating women and women taking oral contraceptives. To our

knowledge, a higher susceptibility to sexually transmitted disease during ovulation has not been reported, but it is unclear whether this issue has been directly evaluated. Susceptibility to gonococcal infection has, however, been reported to be lower at mid-cycle because of higher antigonococcal serum activity, secondary to increased complement hemolytic activity during ovulation *(40),* suggesting that other immune effectors may counterbalance the lower antibody concentration in the female genital tract around ovulation. Furthermore, higher susceptibility to chlamydial cervicitis has been associated with oral contraception *(41),* although chlamydial pelvic inflammatory disease was decreased in women using oral contraceptives *(42).*

Among ovulating women, the cervical vaccine–specific antibody concentrations appeared to be only weakly correlated with the serum-specific antibody concentrations. This finding suggests that the measurement of cervical antibody levels, in addition to serum antibody levels, might provide insight into vaccine failures and the clinical importance of vaccine-induced antibodies in cervical secretions. The latter issue is difficult to address experimentally because there is no animal model in which the sexual transmission of PV to the cervix can be readily tested. Our finding of a wide variation in cervical antibody levels during the ovulatory cycle suggests that it is essential to know when during the cycle a cervical sample has been taken; it appears that the most relevant time to collect cervical samples is when the antibody levels are lowest, i.e., during the peri-ovulatory phase.

In summary, we have shown that a candidate HPV vaccine, HPV16 VLP, has the desirable property of inducing vaccine-specific antibodies in cervical secretions in healthy, adult women, thus placing the antibodies at the site where protection is needed. It remains unclear, however, whether the observed decrease in HPV16 VLP–specific antibodies during ovulation may jeopardize the efficacy of this vaccine. In this regard, it may be worthwhile to evaluate alternative (e.g., mucosal) routes of immunization that can induce local production of specific antibodies, as has been demonstrated in a mouse model *(9).*

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NOTES

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