CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Mar. 1997, p. 122–126 Vol. 4, No. 2
1071-412X/97/$04.00+0


# Prevalence of Antibodies to Human Papillomavirus (HPV) Type 16 Virus-Like Particles in Relation to Cervical HPV Infection among College Women

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Received 21 June 1996/Returned for modification 4 September 1996/Accepted 5 November 1996

**A human papillomavirus type 16 (HPV-16) virus-like particle (VLP)-based enzyme-linked immunosorbent assay (ELISA) was used to measure serum antibody to capsid proteins in 376 sexually active college women who were also screened for the presence of genital HPVs by PCR and interviewed for demographic and behavioral risk factors for HPV infection. The seroprevalence was 46% in women with HPV-16 DNA in the genital tract. The corresponding values for women who harbored other HPV types or no HPV in the genital tract were 30 and 19%, respectively (HPV-16 group versus no-HPV group; odds ratio [OR], 3.7; 95% confidence interval [CI], 1.5 to 8.9). The antibody response was significantly higher among women with a high viral load than among those with a low viral load (median optical density value, 0.838 versus 0.137, $P = 0.009$). Comparable levels of seroreactivity were observed among women infected with HPV types distantly or closely related genetically to HPV-16. Seroreactivity was significantly associated with an age of 25 to 30 years (OR, 2.3; 95% CI, 1.2 to 4.4), three or more lifetime sexual partners (OR, 2.9; 95% CI, 1.1 to 10), and history of a sexually transmitted disease other than HPV (OR, 3.1; 95% CI, 1.5 to 6.3). The percent seropositivity increased linearly with number of lifetime sexual partners until reaching a plateau at 35% for women with more than six partners ($\chi$ for linear trend, $P < 0.001$). The low sensitivity of HPV-16 VLP-based ELISA may limit the usefulness of the assay as a diagnostic test for HPV-16 infection. However, the assay appears to have adequate specificity and should be useful as an epidemiological marker of HPV-16 infection and sexual behavior.**

Genital human papillomaviruses (HPVs) are among the most common sexually transmitted infections. A majority of these infections are clinically inapparent and produce no cytological abnormalities (27). HPVs are also etiologically linked to invasive carcinoma of the cervix and other anogenital cancers. Studies of the immune response to HPV have progressed slowly, in part due to the lack of suitable reagents for immunologic assays. The observation that HPV capsid proteins generated in eukaryotic expression systems self-assemble into virus-like particles (VLPs) has suggested their use as reagents for studies of the immune response to HPV (8, 12–14, 17, 18, 20, 22, 23, 26, 29, 32, 33). A number of studies have demonstrated that human sera react with HPV VLPs and that the reactivity is largely HPV specific and type specific (2, 3, 6, 7, 10, 15, 19, 24, 30, 31). Assessment of HPV seroprevalence in populations with different risks for HPV infection and HPV-associated disease will be important for establishing the utility of VLP-based enzyme-linked immunosorbent assays (ELISAs) for further epidemiological and clinical studies. The purpose of this study was to determine the prevalence of antibodies to HPV type 16 (HPV-16) capsid proteins by ELISA in relation to cervical HPV infection and to demographic and behavioral risk factors for HPV infection in a population of sexually active college women. The women were characterized virologically for a large number of HPV types. These women had a high prevalence of HPV infection, but unlike the subjects in many other HPV-16 serology studies (6, 15, 19, 30), the women had a low prevalence of HPV-associated cytological abnormalities.

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## MATERIALS AND METHODS

**Study subjects.** The subjects were college women enrolled in a prospective longitudinal study of HPV infection (16). The women were undergraduate and graduate students between 18 and 40 years of age (mean age, 22 years) who sought routine gynecological care at the Women's Health Clinic of the University of Maryland at College Park. Participants were asked to make regular visits to the clinic every 4 months for up to 2 years. At enrollment and at each follow-up visit, subjects completed a self-administered questionnaire that elicited information on demographic characteristics and sexual behavior. In addition, exfoliated cervical cells for a Papanicolaou smear and for HPV DNA PCR were collected from the endocervix and transformation zone with a Christmas tree cytobrush. HPV nucleotide sequences in the specimens were identified after PCR amplification with consensus L1 primers (11). After amplification, the PCR products were analyzed by dot hybridization with a biotin-labeled generic HPV probe and 25 biotin-labeled, type-specific HPV oligonucleotide probes. Specimens were scored as strongly positive or weakly positive on the basis of the signal strength of the type-specific probe. A strongly positive signal correlated closely with positivity by Hybrid Capture (Digene Diagnostics, Rockville, Md.), which is a non-amplification-based method (unpublished data). Serum specimens for antibody assays were collected at enrollment and every 12 months thereafter. The protocol was approved by the institutional review board, and all subjects gave written informed consent prior to enrollment. Serum samples from 22 healthy children between 15 and 60 months of age were used to establish a cutoff for the ELISA. Samples were taken from these children as part of a screening program to identify volunteers for phase I testing of new vaccines.

**Preparation of baculovirus-expressed VLPs.** A recombinant baculovirus expressing L1 and L2 proteins of HPV-16 (gift of J. T. Schiller, National Cancer Institute, Bethesda, Md.) was used to prepare HPV-16 VLPs in our laboratory. Briefly, Sf9 cells at a density of $10^6$/ml in 245- by 245-mm square petri dishes were infected at a multiplicity of infection of 10 with the recombinant baculovirus. After incubation for 72 h at 27°C, the cells were harvested and lysed by sonication. VLPs were purified by cesium chloride density gradient centrifugation as

described by Kirnbauer et al. (14). Total protein was measured by the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, Ill.). The purified particle preparation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electron microscopy. The only visible band in a Coomassie blue-stained gel was a 55-kDa protein (expected size of the L1 protein). Electron micrographs of the purified particle preparation showed the presence of multiple particles of the correct size and shape (spherical particles approximately 50 nm in diameter).

**ELISA.** A total of 435 serum specimens from 430 subjects were analyzed. Wells of a 96-well polystyrene microtiter plate (catalogue no. 25802; Corning-Costar, Cambridge, Mass.) were coated with 50 μl of purified VLP preparation at a total protein concentration of 10 μg/ml in phosphate-buffered saline (PBS) and held overnight at 4°C. Control wells were coated with an equivalent amount of the particle preparation in 0.06 M carbonate buffer, pH 9.6. Treatment of VLPs with an alkaline buffer is known to disrupt the particles (15). The disrupted particle preparation served as a control for binding to epitopes on free, unassembled L1 and L2 capsid proteins and may serve as a control for binding to contaminating baculovirus and host cell proteins. After the plate was washed five times with PBS–0.05% Tween 20 (PBS-T), 50 μl of a 1:10 dilution of the test serum in PBS–0.5% nonfat milk was added to each of two wells containing either intact or disrupted particles. The plate was incubated for 2 h at 37°C and then washed five times with PBS-T. VLP-reactive antibodies were detected with horseradish peroxidase-conjugated recombinant protein G (Zymed Laboratories, San Francisco, Calif.) diluted 1:20,000 in PBS-T after 30 min of incubation with the conjugate, followed by 30 min of incubation with freshly prepared 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) and hydrogen-peroxide solution (Kirkegaard and Perry, Gaithersburg, Md.). Optical density (OD) was read in a microtiter plate reader (Molecular Devices Corp., Menlo Park, Calif.) at 405 nm. An OD value of the serum was calculated by subtracting the mean OD of wells coated with VLP in carbonate buffer (disrupted particles) from the mean OD of wells coated with VLP in PBS (intact particles). Three human serum samples previously found to be reactive in assays performed in our laboratory and a mouse monoclonal antibody directed against conformational epitopes on intact HPV-16 virions (gift of Shin-je Ghim, Georgetown University School of Medicine, Washington, D.C.) were included on each plate as positive controls. The mouse monoclonal antibody was detected with horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (Zymed Laboratories). Reactivity of the monoclonal antibody exclusively with intact particles was confirmed by ELISA. The mean (1 standard deviation [SD]) OD value was 0.741 (0.079) in wells containing antigen diluted in PBS (intact particles), compared to a mean (1 SD) OD of 0.054 (0.0007) in wells containing antigen diluted in carbonate buffer (disrupted particles). The negative control was PBS–0.5% nonfat dry milk, the diluent for serum samples. Individual serum samples were retested if there was more than a twofold difference in the OD values of duplicate wells. On the basis of this criterion, 43 (11%) of the samples were retested. The identity of serum samples was masked until completion of the study. Intra-assay variability was evaluated by testing three serum samples 16 times on the same plate. Interassay variability was evaluated by testing three reactive serum samples on eight separate days, and interassay reproducibility was evaluated by testing 44 serum samples on two separate days. Paired ELISA results for the 44 serum samples tested on two separate days showed a high correlation ($r = 0.68$, $P < 0.001$; Spearman rank correlation). The intra-assay coefficient of variation ranged from 5 to 21% (average, 13%), and the interassay coefficient of variation ranged from 9 to 44% (average, 27%).

**Statistical analysis.** Distributions of OD values were compared by the Kruskal-Wallis test, with corrections for multiple comparisons (Dunn's method). In addition, sera were categorized as antibody positive or negative, with a cutoff value of the mean plus 3 SDs for negative control sera after one outlier in the control sera was excluded. When this method was applied to the 22 children's sera, the cutoff OD value was 0.166. This value did not differ significantly from that obtained by the same method with sera from a subset of women in the study who did not have HPV DNA detected in the genital tract on any visit, had no cytological abnormalities, and reported 0 or 1 sexual partners (cutoff OD value of 0.143). Odds ratio (OR) estimates with 95% confidence intervals (CIs) were obtained by $\chi^2$ or Fisher's exact test to assess the magnitude of the associations between HPV seropositivity and HPV DNA positivity and between HPV seropositivity and risk factors for HPV infection. When multiple comparisons were performed, $P$ values were corrected by the Bonferroni method. The association between seropositivity and number of lifetime sex partners was assessed by the $\chi^2$ test for trend.

## RESULTS

**Characteristics of the study population.** Between 19 October 1992 and 17 December 1993, 482 women were approached, and 414 women were enrolled in the natural history study. Of the 414 women in the cohort, 376 (91%) provided at least one serum specimen. The serum sample was obtained from 352 women at enrollment, from 16 women at the second visit, and from 8 women at the third visit. The demographic characteristics and sexual behavior of the women who provided a serum sample are summarized in Table 1. The mean age of the women was 22.5 years. The majority of subjects (78%) were white, and nearly all the women (99%) were sexually active. Fourteen percent of women reported having had sexually transmitted diseases (STDs), including 4.3% with a history of genital warts. No women reported having human immunodeficiency virus infection.

TABLE 1. Demographic characteristics and sexual behavior of 376 college women

| Characteristic | No. (%) |
|---|---|
| Age (yr) | |
| 17–19 | 60 (16) |
| 20–22 | 184 (49) |
| >23 | 132 (35) |
| Ethnicity | |
| White | 293 (78) |
| African American | 42 (11) |
| Other | 41 (11) |
| No. of lifetime partners | |
| 1–2 | 124 (33) |
| 3–10 | 214 (57) |
| >10 | 38 (10) |
| Age at first intercourse | |
| <17 | 135 (36) |
| 17–19 | 184 (49) |
| >19 | 57 (15) |
| History of STD | |
| Genital warts | 16 (4.3) |
| Chlamydia | 34 (9) |
| Herpesvirus | 8 (2.2) |
| Gonorrhea | 2 (0.5) |

At enrollment, HPV DNA was found in the genital tracts of 129 (34%) of the 376 women from whom serum samples were available. Twenty-four of the virus-positive specimens (18.6%) could not be typed with the available probes. Of the 105 typeable infections, HPV-16, HPV-51, and HPV-45 were the most common HPV types identified (26.7, 12.4, and 12.4%, respectively, of women with typeable infections). Other frequently detected types (>5%) included HPV-18, -6, -31, -53, -59, and -66 and PAP155. Infection with multiple HPV types occurred in 28 (22%) of HPV-infected women.

**Association between antibodies to HPV-16 VLPs and detection of HPV DNA in genital tract specimens.** The 376 women were classified according to their HPV PCR test results from cervical specimens collected on or before the date of the first serum specimen as follows: group 1 ($n = 247$), negative for HPV; group 2 ($n = 101$), positive for an HPV type other than 16; group 3 ($n = 28$), HPV-16 positive. The distribution of the OD values in the ELISA for the three groups is shown in Fig. 1. The median (interquartile range) OD values for groups 1, 2, and 3 were 0.0045 (0.000 to 0.0874), 0.0145 (0.00 to 0.218), and 0.158 (0.044 to 0.659), respectively. The differences in OD values were statistically significant in comparisons of group 3 (HPV-16) with the other two groups ($P < 0.001$).

The association of seropositivity with HPV DNA positivity was also analyzed as dichotomous comparisons of antibody prevalence. The seroreactivity of children between 15 and 60 months of age was used to set the cutoff point. The highest antibody prevalence was observed with sera from the HPV-16 group (46%). The antibody prevalence in women with no HPV DNA in the genital tract (19%) was significantly lower than

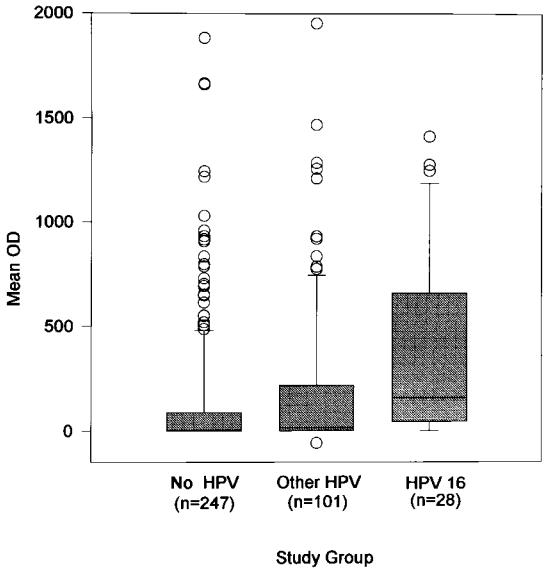


FIG. 1. Reactivity of sera from 376 college women in the HPV-16 VLP ELISA. The college women were classified according to their HPV PCR test results from cervical specimens collected on or before the date of the first serum specimen as follows: negative for HPV (No HPV), positive for an HPV type other than 16 (Other HPV), and HPV-16 positive (HPV 16). The HPV-16 VLP ELISA OD values ($10^{-3}$) are displayed in box plots. The length of each box corresponds to the interquartile range, with the upper boundary of the box representing the 75th and the lower boundary representing the 25th percentile. The horizontal line in the box indicates the median value. The 90th percentile is shown by the small bar at the end of the line extending upward from the box plot. Each outlier value is shown as an open circle.

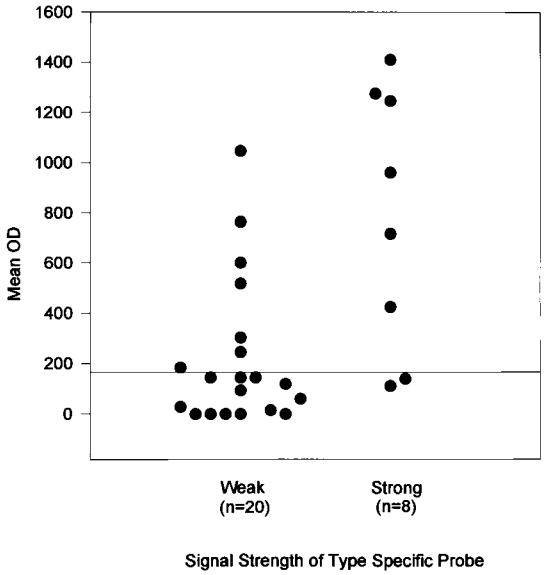


FIG. 2. OD values ($10^{-3}$) for seroreactivity to HPV-16 VLPs among college women by HPV-16 viral load in the genital tract. HPV-16 DNA was detected in cervical specimens by L1 consensus primer PCR. After amplification, the amount of HPV-16-specific PCR product was assessed by categorizing the signal strength of a type-specific oligonucleotide probe as weak or strong. The horizontal line indicates the cutoff for seropositivity (OD, ≥0.166).

that in the HPV-16 group (OR, 3.7; 95% CI, 1.5 to 8.9) (Table 2). The seroprevalence among women who harbored other HPVs in the genital tract (30%) was lower than that of women with HPV-16 infection (OR, 0.5; 95% CI, 0.2 to 1.25) but greater than that in women who were HPV DNA negative (OR, 1.8; 95% CI, 1.02 to 3.16). In the subset of women who were HPV DNA negative, had no cytological abnormalities, and reported 0 or 1 sexual partner, the prevalence of antibody to HPV-16 was 8.5%.

A majority of women with a documented HPV-16 infection did not have detectable serum antibodies to HPV-16 VLP. A possible explanation for this observation is that the viral load was too low to elicit a detectable antibody response. Therefore, viral load, as assessed by the intensity of the signal with the type-specific oligonucleotide probe, was correlated with the antibody response. The median (interquartile range) of OD values for women with a high viral load (strong signal) was significantly higher than that for women with a low viral load (weak signal) (0.838 [0.283 to 1.260] versus 0.137 [0.008 to 0.275], $P = 0.009$) (Fig. 2). The prevalence of antibody was also higher among the women with a high viral load than among those with a low viral load (75 versus 35%, $P = 0.009$).

TABLE 2. Association of HPV-16 VLP seropositivity and HPV cervical infection in 376 college women

| HPV group | No. (%) positive | No. negative | OR (95% CI) |
|---|---|---|---|
| No HPV | 47 (19) | 200 | 1.0 (reference) |
| Other HPV[a] | 30 (30) | 71 | 1.8 (1.02–3.16) |
| HPV-16 | 13 (46) | 15 | 3.7 (1.5–8.9) |

[a] Other than HPV-16.

If viruses that have the greatest amino acid similarity are serologically cross-reactive, then sera from women infected with HPV-16-related viruses would be expected to react more strongly with HPV-16 VLPs than sera from women infected with more distantly related viruses. However, the median (interquartile range) OD values for 22 women infected with HPV-16-related viruses (type 31, 33, 52, 58, or 53) and for 79 women infected with other HPV types were not significantly different (0.00 [0.00 to 0.239] versus 0.020 [0.00 to 0.214], $P = 0.28$).

**Association between antibodies to HPV-16 VLPs and risk factors for HPV infection.** Seroreactivity in the HPV-16 VLP ELISA was analyzed in relation to previously identified risk factors for prevalent HPV infection (Table 3). Seropositivity was significantly associated with multiple (three or more) lifetime sexual partners, history of an STD (other than HPV), and age (25 to 30 years) but not with history of genital warts, age at first sexual intercourse, frequency of condom use, or low-grade cytological abnormalities. The association of seropositivity and the number of sexual partners showed a dose response effect, increasing from 7.8% among women with one lifetime partner to 15.5, 19.9, and 35.7% among women with 2, 3 to 5, and 6 to 9 lifetime partners, respectively ($\chi$ for linear trend, $P < 0.001$). A plateau was reached at a level of 35% seropositivity.

## DISCUSSION

The production of VLPs composed of HPV capsid proteins represents a major advance in efforts to develop reagents for immunologic studies of HPV. Viral surface proteins are commonly targets of the immune response to infection, and these proteins frequently display neutralizing and protective epitopes. Previously, the only available reagents for studies of the immune response to HPV L1 and L2 capsid proteins were fusion proteins expressed in *Escherichia coli*, HPV-derived synthetic peptides, or HPV virions produced in the mouse xenograft system (reviewed in reference 28). The epitopes displayed

TABLE 3. Association of HPV-16 VLP seroreactivity and risk factors for HPV infection

| Risk factor | No. (%) positive | No. (%) negative | OR | 95% CI[a] |
|---|---|---|---|---|
| Age (yr) | | | | |
| <25 | 61 (20) | 240 (80) | 1.0 | Reference |
| 25–30 | 22 (37) | 37 (63) | 2.3 | 1.2–4.4* |
| >30 | 6 (37) | 10 (63) | 2.4 | 0.7–7.5 |
| Age at first intercourse | | | | |
| >20 | 4 (14) | 24 (86) | 1.0 | Reference |
| 19–20 | 18 (25) | 53 (75) | 2.0 | 0.6–9.1 |
| 17–18 | 33 (25) | 101 (75) | 2.0 | 0.6–8.3 |
| <17 | 34 (24) | 109 (76) | 1.9 | 0.6–7.9 |
| No. of lifetime sex partners | | | | |
| 1 | 5 (8) | 59 (92) | 1.0 | Reference |
| 2 | 9 (16) | 49 (84) | 2.2 | 0.6–8.7 |
| 3–5 | 33 (20) | 133 (80) | 2.9 | 1.1–10.1* |
| 6–9 | 20 (36) | 36 (64) | 6.6 | 2.1–>24* |
| >9 | 19 (35) | 36 (65) | 6.2 | 2.0–>22.9* |
| Previous STD[b] | | | | |
| No | 77 (23) | 265 (77) | 1.0 | Reference |
| Yes | 12 (35) | 22 (65) | 3.1 | 1.5–6.3* |
| Condom use | | | | |
| Not always | 77 (25) | 233 (75) | 1.0 | Reference |
| Always | 12 (18) | 54 (82) | 0.7 | 0.3–1.4 |
| History of genital warts | | | | |
| No | 82 (23) | 278 (77) | 1.0 | Reference |
| Yes | 7 (44) | 9 (56) | 2.6 | 0.9–8.0 |
| Cytological abnormality[c] | | | | |
| No | 85 (23) | 278 (77) | 1.0 | Reference |
| Yes | 5 (38) | 8 (62) | 0.5 | 0.14–1.77 |

[a] *, $P < 0.05$, Fisher's exact or $\chi^2$ test, applying the Bonferroni correction when multiple comparisons were made.
[b] History of STDs other than papillomavirus (chlamydia, gonorrhea, genital herpes, or syphilis).
[c] Low-grade squamous intraepithelial lesion.

on synthetic peptides and *E. coli*-expressed proteins are predominantly linear, while antibodies elicited by natural infection are frequently directed toward conformational epitopes. In contrast, VLPs resemble native virions in size and shape and react with polyclonal and monoclonal antibodies directed against conformational epitopes on intact virions, an indication that VLPs display conformational epitopes (4, 9, 23). Immunization with bovine papillomavirus type 1 and HPV-11 VLPs has been shown to induce neutralizing antibodies as efficiently as immunization with authentic virions (13, 24). Thus, the principal neutralizing epitopes are also present on the surface of VLPs.

The reactivity of human sera with VLPs has been demonstrated in several studies (2, 3, 6, 7, 10, 15, 19, 24, 30, 31). Serum antibodies to HPV-6 and HPV-11 VLPs can be detected by ELISA in approximately half of patients with genital warts (3, 24). In control populations without evidence of current HPV infection, seroprevalence ranges from 0 to 19%. Kirnbauer et al. reported that 59% of women who harbored HPV-16 DNA in the genital tract as demonstrated by PCR had antibodies to HPV-16 VLP as demonstrated by ELISA, compared to 6% of women who were cytologically normal and HPV DNA negative (15). Among women with HPV-16 infections associated with severe dysplasia, the antibody prevalence was 75%. In a study of women enrolled in a case control study of incident cervical neoplasia, Wideroff et al. observed a seroprevalence of 48.6% among women who were HPV-16 DNA positive, compared to 15.7% among HPV-16 DNA-negative women (30). Seroprevalence increased with repeated detection of HPV-16 DNA, from 22.2% in women who were DNA positive once to 83.3% in women who were DNA positive twice. Among women with low- and high-grade squamous intraepithelial lesions, the seroprevalence was 30.8 and 52.4%, respectively, compared to 16.6% among matched controls.

In our study, antibodies to HPV-16 VLPs among sexually active college women were also significantly associated with the presence of HPV-16 DNA in the genital tract as demonstrated by PCR. The prevalence of antibody (46%) was very similar to that reported by Wideroff et al. There was also a significant number of antibody-positive women among those with other HPV types detected in the genital tract (30%) and among those without detectable HPV DNA (19%). This result may indicate that the antibody-positive women had been infected with HPV-16 in the past. The similar seroreactivity to HPV-16 VLPs among women infected with closely and distantly related HPVs indicates that closely related viruses are not more cross-reactive serologically than distantly related viruses and is consistent with the notion that the antibody response to HPV capsid proteins is type specific. Studies with polyclonal and monoclonal antibodies of known specificity have also shown that HPV VLP ELISAs are largely type specific (4, 21, 25). The association between seropositivity and previously reported risk factors for prevalent HPV infection, including multiple lifetime sexual partners, history of STDs, and an age of 25 to 30 years, provides further support for the HPV specificity of seroreactivity to HPV-16 VLPs (1, 5). The dose response observed between seropositivity and number of sexual partners is consistent with the sexual transmission of HPV. Using an ELISA with baculovirus-expressed HPV-16, HPV-18, or HPV-33 capsids, Dillner et al. also observed a linearly increasing HPV seroprevalence with increasing numbers of sex partners (7). The absence of an association between HPV-16 seropositivity and cytological abnormalities may be explained by the small number of women with HPV-16 infection (3 of 13 women) and the fact that they had low-grade cytological abnormalities.

In our study, as well as in other studies reported in the literature, not all women with an HPV infection had detectable antibody to the infecting HPV type in ELISAs with VLPs. The weak antibody response to HPV capsid proteins differs markedly from the strong humoral immune response elicited by many other infectious agents. The reasons for the weak antibody response are likely to be multiple and may include lack of sensitivity of the assay, a constitutively low antibody response in some women, a weak antibody response due to low levels of viral replication, or detection of viral DNA early in the infection, prior to seroconversion. In support of the latter two possibilities, we observed higher levels of antibody in women with a high viral load than in women with a low viral load. We also documented a rise in antibody titer in two women (data not shown), suggesting that in these cases, PCR detected infection prior to seroconversion. One practical consequence of the modest sensitivity of serological assays for current infection is that they may have limited usefulness as a diagnostic test for HPV infection and as a way to estimate the cumulative prevalence of HPV infection. Nevertheless, the assays appear to be specific for HPV-16 antibodies, which should make them useful for calculations of relative risk in epidemiological studies.

## ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service grants RO1 CA56514 from the National Cancer Institute and RO1AI28711 from the National Institute of Allergy and Infectious Diseases.

We thank Steven S. Wasserman for statistical advice and Richard Daniel for technical assistance.

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