Immunoglobulin G Responses Against Human Papillomavirus Type 16 Virus-Like Particles in a Prospective Nonintervention Cohort Study of Women With Cervical Intraepithelial Neoplasia


Background: Infection with cancer-linked human papillomavirus (HPV) types such as HPV type 16 (HPV16) is the most important risk factor in the development of cervical cancer. It has been shown that immunoglobulin G (IgG) antibody responses against HPV16 virus-like particles (VLPs) are specifically associated with genital HPV16 infection. Purpose: The aim of this study was to determine the temporal relationships between the presence of HPV16 VLP-specific IgGs, HPV16 infection patterns, and the course of premalignant cervical disease. Methods: Plasma samples from 133 women who had been diagnosed originally with mild to moderate cervical dyskaryosis and enrolled in a prospective non-intervention cohort study conducted in Amsterdam, The Netherlands, from 1991 through 1996 were analyzed for the presence of HPV16 VLP-specific IgGs by use of an enzyme-linked immunosorbent assay. A detailed analysis was performed on 43 women with different HPV16 infection patterns during a follow-up period of 10-34 months. Progression or regression of cervical intraepithelial neoplasia (CIN) lesions was monitored by cytologic and colposcopic testing at intervals of 3-4 months. HPV typing in cervical smears was performed by use of a polymerase chain reaction-based assay. Statistical analysis of the serologic data was performed by use of the Mann–Whitney U test or 2 × 2 table analyses. Results: The presence of HPV16 VLP-specific IgGs in the plasma of the patients was found to be associated with the presence of HPV16 DNA in the cervical smear. Significantly higher proportions of patients with persistent HPV16 infections (i.e., who were polymerase chain reaction positive in three to 11 consecutive tests) than of patients with cleared HPV16 infections were found to be positive for the presence of HPV16 VLP-specific IgGs (18 [69.2%] of 26 versus nine [28.1%] of 32, respectively; P = .003). HPV16 VLP-specific IgGs were consistently detected in all women (n = 11) who were persistently HPV16 DNA positive during follow-up and whose disease ultimately progressed to CIN III (histologically diagnosed severe dysplasia or carcinoma in situ). Conclusion: HPV16 VLP-specific IgG responses are present in the plasma of a majority of patients with persistent HPV16 infections and histologically confirmed high-grade lesions but only in a smaller subset of patients with cleared HPV16 infections and either normal cervical histology or low-grade CIN lesions. Implications: These results suggest that HPV16 VLP-specific antibodies are not responsible for the clearance of virally induced CIN lesions but that they might, in patients with persistent HPV16 infections, be indicative of an increased cervical cancer risk. [J Natl Cancer Inst 1997;89:630-8]

Compelling evidence has accumulated during recent years that shows infection by high-risk human papillomavirus (HPV) types such as HPV type 16 (HPV16) to be the most important risk factor in the development of carcinoma of the uterine cervix (1-3). A lack of HPV-specific immunity may contribute substantially to the persistence of HPV in the cervix and to the subsequent development of premalignant or malignant lesions. The observation of increased incidences of HPV-associated lesions in immunocompromised individuals argues in favor of this hypothesis (4-6).

Our group is currently evaluating HPV16-specific immune responses in a nonintervention cohort study of patients originally diagnosed with initial mild to moderate cervical dyskaryosis (7). This study design allows us to study the relationship between immune responses with HPV infection patterns and disease development over time. It complements several recent studies (8-10) designed to investigate seroconversion in relation to the initial acquisition of HPV infection.

Immune responses against HPV during early stages of infection may be important in blocking reinfection, inhibiting viral spread, and preventing viral persistence. Virus-neutralizing antibodies at the mucosal surface, directed against native virion epitopes, may play an important role in this regard. However, the temporal relationship between virion antibody response and the course of cervical disease has not been examined critically. In-
formation about naturally occurring immune responses against HPV16 capsids might have important implications for possible vaccine strategies.

The lack of an in vitro culture system for high-risk HPV types has hindered the production of sufficient amounts of virions to test for specific antibody responses. Recently, HPV16 virus-like particles (VLPs), consisting of the major capsid protein L1 alone or L1 in combination with the minor capsid protein L2, were obtained through self-assembly in a baculovirus expression system and were used to detect serologic responses against epitopes on the capsid (11-14). Serum immunoglobulin G (IgG) responses against conformational epitopes on HPV16 VLPs, as determined in an enzyme-linked immunosorbent assay (ELISA) format, were shown to be associated with HPV16 infection in patients with cervical intraepithelial neoplasia (CIN) (15). HPV16 VLPs are also excellent candidates for use in prophylactic vaccination. Indeed, vaccination with papillomavirus capsid-like structures was recently shown to confer protection from a subsequent experimental viral challenge and to prevent the outgrowth of papillomavirus-induced lesions in several animal models (16-18).

In this prospective study, we determined the relationship between HPV16 VLP IgG reactivity, viral clearance or persistence, and the course of disease in patients with premalignant cervical lesions.

**Subjects and Methods**

**Patient Cohort and Study Design**

Our study was nested within a larger prospective nonintervention cohort study of patients with CIN that was designed and conducted to examine the relationship between HPV infection patterns and the course of CIN disease (7). Women (n = 352) presenting with abnormal cervical cytology (mild to moderate dyskaryosis) were referred to the gynecologic outpatient clinic of the Free University Hospital in Amsterdam, The Netherlands; after giving written informed consent to participate, they were enrolled in the study. The study design was approved by the ethics committee of the hospital.

Clinical follow-up consisted of cytologic and colposcopic examinations that were performed every 3-4 months. Biopsy specimens were not taken during the follow-up period to avoid interfering with the natural course of the disease. Results from the cytomorphologic tests were classified according to a modified Papanicolaou (Pap) system as employed in The Netherlands (7); i.e., Pap 1 = no cytomorphologic abnormalities, Pap 2 = inflammation, Pap 3a = mild to moderate dyskaryosis, Pap 3b = severe dyskaryosis, Pap 4 = carcinoma in situ, and Pap 5 = microinvasive carcinoma. Colposcopy was used to indicate the predicted grade of dysplasia (termed “CINc”) and to determine the extent of the observed lesions in cervical quadrants. At the end of follow-up, biopsy specimens were taken and a histologic diagnosis was made (no CIN = normal or metaplastic epithelium, CIN 1 = mild dysplasia, CIN II = moderate dysplasia, and CIN III = severe dysplasia or carcinoma in situ). A detailed description of the tight clinical surveillance of the patients and the evaluation of clinical follow-up were reported by Remmink et al. (7).

The design of this study is presented in Fig. 1. Of the 352 patients with CIN who participated in the study, 227 were still in clinical follow-up when plasma collection was started. Plasma samples were collected from 187 patients who consented to one or more blood donations. Patients infected by unidentified HPV types (HPV-X; n = 54) were excluded. Plasma samples from the remaining 133 patients (including all HPV16-positive patients; n = 72) were tested for IgG reactivity against HPV16 VLPs in a cross-sectional analysis after 10-56 months of clinical follow-up (mean length of follow-up, 31.2 months). HPV16-positive patients from whom multiple plasma samples were available were studied serologically and clinically over a follow-up period of 10-34 months (mean length of follow-up, 20.7 months; n = 43; Fig. 1). Of the total of 72 tested HPV16-positive patients, 54 had reached the end of clinical follow-up before this study was concluded. For these patients, histologic data were available that could be related to the IgG status determined (Fig. 1). The 133 CIN patients included in the cross-sectional analysis ranged in age from 19 to 56 years (mean age of the patients: 35.1 years; standard deviation [SD], 8.6 years). These patients were assigned to different test groups on the basis of HPV typing and infection patterns over time up to the moment of serologic testing.

A group of 30 women with HPV infections and normal cervical cytology (Pap 1 or Pap 2) was included in the cross-sectional analysis. These healthy donors participated in a follow-up study of HPV-positive women with normal cervical cytology. Like the CIN patients, they were derived from the Greater Amsterdam area at a mean age of 35.3 years (SD = 9.1 years). These women had the following HPV infections: HPV6 (n = 3), HPV16 (n = 16), HPV6/HPV16 (n = 1), HPV31 (n = 1), and HPV-X (n = 9).

Plasma samples from a group of 50 women were taken; these samples had been stored at the Laboratory of Clinical Immunology of the Free University Hospital. These women visited the hospital during the period from 1991 through

Fig. 1. Schematic representation of the cervical intraepithelial neoplasia (CIN) study design. After 10-56 months of clinical follow-up (varying among patients), plasma was available from 187 patients. After the exclusion of 54 patients with human papillomavirus (HPV) type-X infections (i.e., infections with unidentified HPV types), 133 patients with CIN (HPV negative [HPV-neg.], n = 29; HPV non-16 [HPV type 16 negative but positive for other HPV types], n = 32; and HPV16, n = 72) were tested for immunoglobulin G (IgG) reactivity to HPV type 16 (HPV16) virus-like particles (VLPs) in a cross-sectional analysis at the start of plasma collection. HPV16-positive patients, from whom multiple plasma samples were available (n = 43), were subsequently tested for VLP-specific IgG reactivity during follow-up (length of follow-up time being 10-34 months, varying among patients). At the end of the study, histologic data were available for 37 patients tested for VLP-specific IgG reactivity during follow-up and for 17 HPV16-positive patients tested only once in the cross-sectional analysis. Clinical disease course, histologic data from biopsy specimens taken at the end of clinical follow-up, and HPV infection patterns over time were related to the observed IgG antibody responses to HPV16 VLPs.

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**No plausible explanation provided.**
1996 with symptoms unrelated to cervical disease and were found to have no immunologic or other clinical abnormalities. They were included in the study as sex-matched, healthy control subjects with unknown HPV and cytologic status and were selected to match the age distribution of the patient test groups (mean age of the patients: 35.6 years [SD, 10.2 years]).

There were no significant differences in the age distributions of the patients with CIN, the HPV-positive women with normal cervical cytology, and the healthy control population (patients with CIN versus HPV-positive women with normal cytology, \( P = .955 \); patients with CIN versus healthy controls, \( P = .791 \); healthy control subjects versus HPV-positive women with normal cytology, \( P = .895 \); two-sided, unpaired Student’s \( t \) test).

**HPV Detection and Genotyping**

HPV detection and genotyping were performed on cellular material from cervical smear samples, as described elsewhere (7,19). Briefly, cells obtained from the same cervical smears used for cytology were collected in phosphate-buffered saline (PBS)–0.05% Merthiolate (Sigma Chemical Co., St. Louis, MO), pelleted, resuspended in 1 mL 10 mM Tris (pH 7.5), and frozen at \(-70^\circ \text{C}\). For the polymerase chain reaction (PCR) assay, 10-\( \mu \)L aliquots were taken. These suspensions were boiled for 10 minutes, cooled on ice, and centrifuged at room temperature for 1 minute at 3000 \( \times \) \( g \) before being used in an HPV general primer-mediated PCR. General primer-mediated PCR-positive samples were further tested in an HPV type-specific PCR for the relatively common genital HPV types 6, 11, 16, 18, 31, and 33. General primer-mediated PCR-positive products of samples that tested to be negative in the type-specific PCR were hybridized stringently and successively after general primer-mediated PCR to full-length cloned DNA from genital HPV types 13, 30, 35, 39, 40, 42, 43, 44, 45, 51, 52, 54, 55, 56, 57, 58, 59, 61, and 66 (20). HPV general primer-mediated PCR-positive smears that could not be typed in this manner were called HPV-X.

**CIN Patient Groups Divided by HPV Infection Patterns**

The 133 CIN patients included in this study were divided into different test groups on the basis of HPV infection patterns as determined by PCR during clinical follow-up. HPV16 DNA was found to be present in the cervical smear material of 72 patients at or before plasma collection. These patients were assigned to the HPV16 group, which was further subdivided into the following three groups: 1) The HPV16-clearance group included 32 patients who were found consistently to be negative for HPV16 DNA for at least 12 consecutive months after having been HPV16 DNA positive (mean time since the patients were last determined to be positive for HPV16 DNA: 29.6 months [range: 12-54 months]; mean age of the patients: 35.5 years [SD, 8.7 years]). 2) The HPV16-fluctuation group included 14 patients with intermittent HPV16 DNA positivity (mean age of the patients: 35.1 years [SD, 9.6 years]). 3) The HPV16-persistence group included 26 patients who were consistently positive (in three to 11 consecutive PCR tests) for HPV16 DNA over a period of at least 12 months before serologic testing (mean age of the patients: 33.7 years [SD, 7.9 years]).

Patients who were negative for HPV during the entire follow-up period and for at least 12 months (mean length of time: 28.7 months [range: 20-45 months]) prior to serologic testing constituted the HPV-negative group. Plasma from 29 such patients was available for this study (mean age of the patients: 38.3 years [SD, 8.4 years]).

Patients with a current or past infection of HPV types other than HPV16 constituted the so-called HPV non-16 group (\( n = 32 \), mean age of the patients: 33.5 years [SD, 8.2 years]). This group consisted of 12 patients with infections of HPV16-related types (HPV types 31, 33, 52, and 58) and 20 patients with HPV16-unrelated types (HPV types 6, 18, 43, 45, 51, 54, 61, and 66).

There were no substantial differences in the age distribution of the total HPV16-positive group compared with the HPV-negative group or the HPV non-16 group or between the age distributions of the HPV16-clearance, HPV16-fluctuation, and HPV16-persistence groups.

**Preparation of Plasma**

Plasma derived from heparinized blood was diluted 1:1 in PBS containing 0.5\% bovine serum albumin and was stored at \(-20^\circ \text{C}\). Before use, the plasma samples were centrifuged at 2300 \( g \) for 10 minutes at room temperature to remove protein debris.

**HPV16 VLP Enzyme-Linked Immunosorbent Assay**

Self-assembled HPV16 L1/L2 VLPs were expressed in baculovirus-infected SI-9 insect cells, isolated, and purified by cesium chloride gradient, as previously described (12). These VLPs were used in an ELISA to determine specific plasma IgG reactivity, using a modification of the assay reported previously by Kirnbauer et al. (15). Approximately 50 ng per well of VLPs in PBS was added to 96-well ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) and incubated at 4°C for 1.5 hours. Subsequently, the plates were washed three times with PBS, blocked for 2 hours at 37°C with PBS containing 0.1% Tween-20 (Genfarma bv, Maarssen, The Netherlands) and 1% newborn calf serum (Gibco BRL, Paisley, Scotland), and again washed three times with PBS. The plates were then incubated at room temperature, while gently rocking, with human plasma diluted 1:10 in PBS containing 0.1% Tween-20 plus 1% newborn calf serum (100 \( \mu \)L per well). This plasma dilution consistently yielded optical densities in the linear part of the resulting curves when plasma samples from five different patients were tested in a dilution range from 1:2 to 1:50. After five washes with PBS, 100 \( \mu \)L horseradish peroxidase-labeled rabbit ahuman IgG (DAKO-patts, Glostrup, Denmark) diluted 1:1000 in PBS containing 0.1% Tween-20 plus 1% newborn calf serum was added per well. The plates were left for 1 hour at room temperature and again washed five times with PBS. Finally, 0.2 g/mL o-phenylenediamine (Dako), dissolved in 0.64% \( \text{KH}_2\text{SO}_4 \) plus 0.05% \( \text{Na}_2\text{PO}_4 \) (pH 5.4) containing 0.02% \( \text{H}_2\text{O}_2 \), was added (100 \( \mu \)L per well). The reaction was stopped by the addition of 100 \( \mu \)L 1 N \( \text{H}_2\text{SO}_4 \) per well. Subsequently, the optical density at 492 nm (\( \text{OD}_{492} \)) was measured by use of an ELISA plate reader (ICN Biochemicals, Inc., Costa Mesa, CA). Duplicate tests were performed for all plasma samples. From the optical densities obtained for the test wells of each plasma sample, the background reactivity found in the corresponding negative control wells coated with plain PBS and blocked with PBS containing 0.1% Tween-20 plus 1% newborn calf serum was subtracted. IgG reactivities were expressed as the mean optical density of the duplicate tests. Plasma samples were retested when discordant results were found between duplicates. To compensate for interassay variability, we adjusted \( \text{OD}_{492} \) values in relation to optical densities obtained for a set of internal control plasma samples (with both positive and negative IgG reactivities) that were included in each plate. A cutoff level, above which optical density values (\( \text{OD}_{492} \)) were considered positive, was based on the distribution of optical densities found in an age-matched control population of 50 healthy women from the same geographic area as the women in the test groups (sera diluted 1:10). This cutoff value was reached by adding three times the standard deviation to the mean optical density, after exclusion of one outlier (outlier: \( \text{OD}_{492} = 0.246 \); cutoff = 0.211).

**Statistical Analysis**

Proportional analysis between patient groups was performed with the chi-squared or Fisher’s exact test (\( 2 \times 2 \) table analysis) where appropriate. Odds ratios and 95\% confidence intervals were calculated with chi values derived from \( 2 \times 2 \) table analyses. IgG reactivities between groups were compared with the Mann–Whitney \( U \) test. All tests were two-sided, and results were considered significant when \( P<.05 \).

**Results**

IgG Reactivity to HPV16 VLPs in Relation to HPV Infection Patterns Before Plasma Collection

At the start of serologic follow-up, a cross-sectional analysis was performed. In this analysis, patient test groups were divided by HPV infection patterns. These patterns were determined during clinical follow-up (over a period of 10-56 months) before IgG testing.

IgG reactivities found for the healthy control women, the HPV-positive women with normal cervical cytology, and the HPV-negative, the HPV non-16-positive, and the HPV16-positive patients with CIN are shown in Fig. 2, A. Significantly higher VLP-specific IgG reactivities were found in the HPV16-positive CIN group than in the HPV non-16 group or the HPV-negative and the HPV non-16 CIN test groups combined (Fig. 2, A). Moreover, when the HPV16-positive CIN patient group was further subdivided into patients with a cleared, a fluctuating, or
a persistent HPV16 infection, an association between anti-VLP reactivity and HPV16 persistence became apparent (Fig. 2, B). HPV16 VLP-specific IgG reactivities in the control group of healthy women were significantly lower than those in the group of patients with CIN, which included the HPV-negative, the HPV16-positive, and the HPV non-16-positive patients (P<.001). A significant difference was also found with the group of women with normal cervical cytology and HPV infections; this result suggests that IgG responses against the HPV16 capsid protein can be generated and maintained in the absence of cytologic abnormalities (P = .003; Fig. 2, A).

Based on the distribution of IgG reactivities within the control group of healthy women, a cutoff level was calculated [mean OD<sub>492</sub> + (3 × SD), one outlier (OD<sub>492</sub> = 0.246) excluded]. IgG reactivities exceeding this value (OD<sub>492</sub> = 0.211) were considered positive. Table 1 shows the frequency of positive responders for all the different test groups.

Overall, 40 (30.1%) of 133 patients who had been tested were positive for HPV16 VLP-specific IgGs. The observed frequency in the HPV16-positive CIN group was significantly higher than that in the other groups of patients with CIN (i.e., HPV-negative or HPV non-16-positive). All three patients in the HPV non-16 CIN group showing positive responses had cleared infections of HPV types not related to type 16 (i.e., types 6, 18, and 61, respectively). None of the 12 patients with ongoing or past infections of HPV16-related types showed a positive IgG reactivity (maximum OD<sub>492</sub> = 0.118).

The highest frequency of women with positive IgG responses

### Table 1. Immunoglobulin G (IgG) positivity against human papillomavirus (HPV) type 16 (HPV16) virus-like particles in patients with cervical intraepithelial neoplasia (CIN) and in normal control subjects: cross-sectional analysis

<table>
<thead>
<tr>
<th>Test group*</th>
<th>No. positive responders/total†</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy women</td>
<td>1/50</td>
<td>2.0</td>
</tr>
<tr>
<td>Normal control subjects—HPV-positive‡</td>
<td>9/30</td>
<td>30.0</td>
</tr>
<tr>
<td>Patients with CIN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-negative</td>
<td>5/29</td>
<td>17.2</td>
</tr>
<tr>
<td>HPV16§</td>
<td>32/72</td>
<td>44.4</td>
</tr>
<tr>
<td>HPV16 clearance</td>
<td>9/32</td>
<td>28.1</td>
</tr>
<tr>
<td>HPV16 fluctuation</td>
<td>5/14</td>
<td>35.7</td>
</tr>
<tr>
<td>HPV16 persistence]</td>
<td>18/26</td>
<td>69.2</td>
</tr>
<tr>
<td>HPV non-16¶</td>
<td>3/32</td>
<td>9.4</td>
</tr>
</tbody>
</table>

*Healthy women: age-matched control subjects with unknown cervical HPV and cytological status. Normal subjects—HPV-positive: women with HPV infections but normal cervical cytology. Patients with CIN grouped on the basis of HPV patterns: HPV16 = HPV16 positive at least once during follow-up (HPV16 persistence + HPV16 clearance + HPV16 fluctuation).
†The cutoff used was derived from IgG reactivities in healthy women and based on three standard deviations.
‡Versus healthy women: P<.001 (two-sided Fisher’s exact test); seven (41.2%) of 17 HPV16 positive and two (15.4%) of 13 HPV16 negative but positive for other HPV types.
§Versus CIN HPV-negative: P = .019 (two-sided chi-squared test). |
¶Versus HPV16 clearance: P = .003 (two-sided Fisher’s exact test).
¶HPV non-16 = HPV16 negative but positive for other HPV types. Versus HPV16: P = .001 (two-sided chi-squared test); 0 (0%) of 12 HPV16 related and three (15.0%) of 20 not related.

**Fig. 2.** Cross-sectional analysis of immunoglobulin G (IgG) reactivity against human papillomavirus (HPV) type 16 (HPV16) virus-like particles (VLPs) (expressed as the detected optical density at 492 nm [OD<sub>492</sub>]). All tested patient groups are shown. Each dot represents IgG reactivity from one patient. Mean reactivities for all groups are indicated. Significant differences between groups are indicated by the corresponding P values (two-sided Mann–Whitney U test). Women were assigned to the different groups by disease and HPV DNA status. A) Control groups: healthy women (unknown HPV and cytologic status). NORM: HPV-positive women with normal cervical cytology. Cervical intraepithelial neoplasia (CIN) patient groups—HPV-negative: polymerase chain reaction negative for HPV during entire clinical follow-up prior to testing; HPV non-16: infected by HPV type other than type 16; HPV16: patients with current or past HPV16 infections. B) HPV16-positive patients with CIN divided by HPV16 infection patterns. 16C: HPV16 clearance. 16F: HPV16 fluctuation. 16P: HPV16 persistence.
against HPV16 VLPs was found in the HPV16-persistence group (69.2%); this frequency was significantly higher than the frequency observed in the HPV16-clearance group alone (28.1%; \( P = .003 \)) or in the HPV16-clearance group combined with the HPV16-fluctuation group (30.4%; \( P = .003 \)) (Table 1).

Within the HPV16-clearance group, a significant difference in IgG positivity was observed between patients who were found to be HPV16 PCR positive once during follow-up (five [19.2%] of 26) and patients who were PCR positive more than once during follow-up (four [66.7%] of six; \( P = .040 \)). A frequency of 30% positive responders was found in the group with normal cervical cytology and HPV infections (HPV16 positive: seven [41.2%] of 17; HPV non-16-positive: two [15.4%] of 13); this frequency differed significantly from that found in the control group of healthy women (2.0%; \( P < .001 \)).

### IgG Reactivity to HPV16 VLPs in Relation to HPV16 Infection Patterns

IgG reactivity against HPV16 VLPs was determined in 43 patients with CIN who had different HPV16 infection patterns and from whom multiple plasma samples were available over a period of 10-34 months (mean serologic follow-up: 22.5 months). Plasma samples were available from two to seven different time points. Results from 20 HPV16-clearance-group, 17 HPV16-persistence-group, and six HPV16-fluctuation-group patients are shown in Fig. 3. IgG reactivities in the persistence and fluctuation groups are shown relative to the time that passed since the start of clinical follow-up. Data for the clearance group are shown relative to the time that passed since the last positive HPV16 DNA test. In the clearance group, seven (35%) of 20 patients had HPV16 VLP-specific IgG reactivities, which generally decreased over time. For five patients, plasma samples were available covering the actual time of clearance. Of these patients, one was IgG positive (see Fig. 3, 16C). Thirteen (76.5%) of 17 persistence-group patients were found to be positive for the presence of HPV16 VLP-specific IgGs; 11 of these patients were consistently positive during follow-up, while the other two patients had IgG reactivities fluctuating around the cutoff level. Finally, three (50.0%) of six patients with fluctuating HPV16 DNA positivity over time were consistently positive for HPV16 VLP-specific IgGs during follow-up.

### VLP IgG Reactivity in Relation to Disease Status

Table 2 presents cytologic and colposcopic data in relation to the frequency of HPV16 VLP-specific IgG positivity for all HPV16-positive patients at the start of plasma collection. The frequencies of VLP-specific IgG positivity were also determined for the different histologic CIN classifications ultimately found for 54 HPV16-positive patients at the end of clinical follow-up. Fig. 4 shows a higher frequency of IgG positivity in patients whose disease had ultimately progressed to CIN III than in HPV16-positive patients with CIN I or II or without CIN lesions, both in the total HPV16-positive group (HPV16 clearance, fluctuation, and persistence) (\( P = .002 \); odds ratio = 10.4; 95% confidence interval = 2.1-51.4) and in the subgroup of patients with HPV16 persistence (\( P = .023 \); odds ratio = 15.0; 95% confidence interval = 1.04-215.5). Similar results were found in 37 patients who were tested for HPV16 VLP-specific IgG reactivity over time and for whom histologic data were available by the end of serologic follow-up. All patients whose disease had progressed to CIN III over the course of follow-up had a persistent HPV16 infection and showed IgG positivity against HPV16 VLPs (\( n = 11 \)). In contrast, only 10 of 26 patients without lesions or with CIN I/II lesions were positive (\( P < .001 \)). Even within the HPV16-persistence group, a significant difference was found between the patients whose disease had progressed to CIN III (11 of 11) and those whose disease did not (two of five; \( P = .018 \)).
Discussion

In this study, we have shown that HPV16 VLP-specific IgG reactivity is type specific and is associated with both past and ongoing HPV16 infections in patients with CIN. These results are in agreement with earlier reports that anti-VLP responses occur more frequently in HPV16-positive patients with CIN and that IgGs raised against VLPs show type restriction (15,21-23).

Although we observed IgG responses against HPV16 VLPs in a considerable number of patients whose HPV16 infections cleared with time (nine [28.1%] of 32), there was a much stronger association between these antibody responses and HPV16 persistence (18 [69.2%] of 26 patients who were IgG positive) in the same cohort of patients. All 11 patients with CIN, whose disease over a follow-up period of 10-31 months progressed to histologically confirmed CIN III (severe dysplasia or carcinoma in situ), had persistent HPV16 infections and showed consistently positive IgG reactivities to HPV16 VLPs at two to seven different time points over the course of follow-up.

In a recently reported case-control study of patients with CIN III or cervical cancer in Colombia and Spain, Nonnenmacher et al. (24) showed high frequencies of HPV16 VLP-specific IgG positivity in the CIN III groups from both countries (81% and 73%, respectively). This is comparable to the 87% that we found in the cross-sectional analysis for patients whose disease ultimately progressed to CIN III (Fig. 4). It is interesting that the matched control groups from Colombia showed significantly higher frequencies of HPV16 VLP-specific IgG positivity than the control groups from Spain, which corresponded to an eightfold higher incidence of cervical cancer in Colombia. These data demonstrated an association between the presence of anti-HPV antibodies in the serum and increased cervical cancer risk. Another recent study by Lehtinen et al. (25) showed IgG reactivity against HPV16 VLPs to have predictive value for the development of cervical cancer. These results are in line with our own findings of an association between IgG reactivity against HPV16 VLPs and progression to CIN III and obviously raise the question whether VLP IgG reactivity might be useful in screening to aid in the determination of cervical cancer risk. Inasmuch as IgG reactivity against HPV16 VLPs appears to define a subset of patients in the HPV16 DNA-persistence group with an increased risk of disease progression to CIN III (Fig. 4), it might prove to be a valuable addition to the existing HPV DNA detection methods of screening for past and present HPV infections.

Nonnenmacher et al. (24) found significantly lower frequen-

![Fig. 4. Frequencies of patients with cervical intraepithelial neoplasia (CIN) who were positive for the presence of human papillomavirus type 16 (HPV16) virus-like particle (VLP)-specific immunoglobulins G (IgGs) in the cross-sectional analysis, in relation to histologic grading at the end of clinical follow-up. Data from two test groups are shown: HPV16 = the total HPV16-positive group (n = 54); HPV16 P = the HPV16-persistence group (n = 22). CIN classifications are indicated: no CIN = no dysplasia (blank bar); CIN I = mild dysplasia (hatched bar); CIN II = moderate dysplasia (cross-hatched bar); CIN III = severe dysplasia (black bar). The total number of patients in each subgroup is indicated at the top of the bars.](image-url)
cies of positive IgG responders in patients with cervical carcinoma than in patients with CIN III. We also observed a lower frequency in a small number of tested HPV16-positive patients with cervical carcinoma (three [21.4%] of 14 being positive for HPV16 VLP-specific IgG; data not shown). This observation may be explained by a decreased expression of the L1 and L2 capsid proteins accompanying the transition from CIN to invasive cervical carcinoma (26). The transcripts encoding L1 and L2 have been reported to be primarily present in low-grade CIN lesions (CIN I). Minimal L1 and L2 messenger RNA expression was observed in progressed, high-grade cervical lesions (26,27).

These observations are supported by our finding that six of 11 patients with disease progressing to CIN III during follow-up already showed a decrease in HPV16 VLP-specific IgG reactivity (Fig. 5, D).

What then underlies the apparent association between anti-VLP IgG positivity and the progression to CIN III?

First, the duration of HPV16 infection may be an important factor. Progression of cervical lesions occurs in a subset of patients with a persistent infection of high-risk HPV types (7,28). We found a clear association between anti-VLP IgG positivity and HPV16 persistence. Even patients with a cleared HPV16 infection were more likely to be IgG positive when they were PCR positive for HPV16 more than once before clearance. These findings are in agreement with a recent report by Wideroff et al. (21), who found that patients who were positive for the presence of HPV16 DNA by PCR at two consecutive time points showed a considerably higher frequency of positive anti-VLP IgG responses (83%) than patients who were positive at only one time point (22%).

Long-lasting, persistent infections may increase the likelihood of contact between HPV capsid proteins and the immune system and the subsequent generation of specific immune responses. Inasmuch as persistence is associated with disease progression, anti-VLP IgG reactivity may thus also be associated indirectly with the development of CIN III lesions.

Second, low-grade CIN lesions with productive HPV infections may still be present in patients with cervical lesions that had progressed to CIN III. Indeed, seven of 11 patients with CIN who were ultimately diagnosed with CIN III lesions during the period of follow-up by serologic testing also had focal CIN I or II lesions as determined by histologic examination. These lesions may still produce capsids and thus provide antigenic stimulation to maintain the detected humoral responses. Because these milder lesions tend to disappear with further progression, IgG levels may start waning. Immunohistochemical staining of L1 and/or L2 in cervical biopsy specimens from IgG-tested women may confirm this hypothesis.

Third, viral load may also be a crucial factor in the generation of humoral responses against HPV capsids. This is suggested by the finding of higher frequencies of IgG responders in cytologi-
cally normal women found to be HPV16 positive by both the Viratype assay (Digene Diagnostics, Beltsville, MD) and the more sensitive PCR than in women found to be positive by PCR alone (15). Rates of IgG positivity were also higher among patients with CIN who had been found to be HPV16 DNA positive by hybrid capture than among patients found to be positive by PCR (27). Perhaps women with a higher viral load are more likely both to produce higher amounts of capsids (leading to stronger antibody responses) and to develop cervical lesions. To examine this possibility, we are now in the process of quantifying HPV16 DNA load in cervical smears from the patients tested in this study in a PCR–ELISA format developed in our laboratory (29). Differences in viral load after initial infection may also explain the absence of detectable IgG responses in a considerable proportion of CIN patients with current or past HPV16 infections.

From our data on HPV16-positive patients with normal cervical cytology (seven [41%] of the 17 women were also positive for the presence of HPV16 VLP-specific IgG), it can be inferred that the presence of detectable neoplastic lesions is not a prerequisite for raising IgG responses against HPV16 capsid proteins. This is in keeping with earlier studies showing that HPV16 VLP-specific seroconversions occur within months after a new HPV16 infection in cytologically normal women with multiple sex partners (8,9). A recent follow-up study of university students in the United States (10) showed that such positive IgG reactivities can persist for years and have a certain predictive value for the subsequent development of premalignant cervical lesions.

Only one of five patients who underwent HPV16 clearance showed HPV16 VLP-specific IgG positivity at that time (Fig. 3). This finding suggests that other immune mechanisms may be involved in clearing established lesions.

Assuming that neutralizing antibodies are indeed detected with the test employed in this study, then what implications do these findings have for possible vaccination strategies?

Several studies employing animal models demonstrated the usefulness of VLPs for prophylactic vaccination. Vaccination with cottontail rabbit papillomavirus VLP preparations protected domestic rabbits from experimental challenge with this virus and prevented the development of cutaneous warts (16). Similarly, vaccination with canine oral papillomavirus and bovine papillomavirus type 4 prevented the development of mucosal warts after experimental challenge (17,18). VLP-specific antibodies, detected after vaccination, were cross-reactive with native virions and were presumed to be neutralizing and responsible for protection. Passive transfer of protection by serum or purified IgG supports this assumption (16,17). However, VLPs were not found to be effective in therapeutic vaccination. They did not induce regression of established lesions (18). This result appears to be in line with our findings. HPV16 VLP-specific IgG responses are present in patients with viral clearance and may have neutralized virions in normal or mildly dysplastic epithelium, but they are ineffective in patients with persistent infections. In fact, in the latter group, they are associated with progressive CIN lesions. These data suggest that HPV16 VLPs may be effective as a prophylactic vaccine, but not suitable for therapeutic vaccination. Protection could be mediated by two classes of antibodies. It has been proposed that a critical level of serum IgG can neutralize mucosal pathogens by exudation onto mucosal surfaces such as the surface of the cervix (30). In addition, local immunoglobulin A responses could be protective. In future studies, the presence of HPV-specific antibody responses in the genital mucosa should be examined in the context of cervical disease development.

References


Notes

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