Antibodies against Papillomavirus Antigens in Cervical Secretions from Condyloma Patients

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Samples of cervical secretions and serum from 30 women with genital condylomas and 30 age-matched controls were tested for the presence of immunoglobulin A (IgA) and IgG antibodies against a panel of papillomavirus-derived antigens. The same cervical samples were also analyzed for presence of human papillomavirus (HPV) DNA by Southern blotting and polymerase chain reaction. By Southern blotting HPV DNA was detected in 8 of 30 patients with condylomas and 2 of 30 controls, and by the polymerase chain reaction HPV DNA was detected in 14 of 30 patients with condylomas and 5 of 30 controls. A total of 18 of 29 patients with condylomas and 8 of 28 controls had IgA antibodies in cervical secretions to an E2 synthetic peptide, and 17 of 29 patients with condylomas and 5 of 28 controls had local IgA antibodies to an E7 peptide ($P < 0.025$ and $P < 0.005$, respectively). The results suggest that measurement of local antibody production against selected HPV antigens may be useful in the study of HPV immunology and, possibly, for the diagnosis of HPV infection.

Genital papillomavirus infection (GPVI) can appear as genital warts (condylomata acuminata) associated with human papillomavirus (HPV) types 6, 11, and 42 (29) or as flat acetic white lesions (flat condylomas), where the most important types of HPV are HPV types 16 (HPV 16) and 18 because of the associated risk for progression into cervical intraepithelial neoplasia (CIN) and cervical cancer (29). HPV types 31, 33, 35, 39, 45, 51, and 52 also cause GPVI, but with a lower risk of progression to CIN and cancer (2, 29). Condylomata acuminata are diagnosed by clinical examination and mainly appear in the vulva, but in some cases they also affect the vaginal wall and portio (24). Flat condylomas can appear at any site of the lower genital tract, and a preliminary diagnosis can be made colposcopically after application of acetic acid (24). The clinical assessment of a flat condyloma can be confirmed by the presence of koilocytosis in Pap smears or by histopathological examination of biopsy specimens (24). HPV infections can also be diagnosed by the detection of viral DNA by Southern blotting (20, 21) or polymerase chain reaction (PCR) (23), but HPV DNA is commonly detected in the absence of clinical disease and about 30 to 40% of flat condylomas and other low-grade cervical lesions do not contain any known HPV type (21, 25).

In recent years there has been increasing interest in HPV serology following the development of assays for the detection of serum antibodies to HPV that are based on synthetic peptides (1, 3–5, 14) or fusion proteins (9, 12, 13). All HPV genomes have at least eight protein-encoding regions of which the early proteins are numbered E1 to E8 and the late proteins are numbered L1 and L2 (25). Serum antibodies to several early proteins are elevated in patients with CIN and cervical cancer. For example, we have previously found that patients with CIN have increased levels of immunoglobulin A (IgA) antibodies in serum to an E2-derived peptide (no. 245) compared with the levels in healthy controls (4). The HPV specificity of the response to peptide 245 has been shown by affinity purification of the antipeptide antibodies and the demonstration that these antibodies are directed against the corresponding HPV-determined protein of the expected size (48 kDa) that is present in an HPV 16-transfected cell line, whereas it is not present in the untransfected parental cell line (4). The serum IgA response to this peptide is not increased among patients with condylomata acuminata (4, 31). Similarly, 20% of patients with cervical carcinomas whereas only 4% of controls had serum antibodies to a fusion protein representing the major transforming protein E7, but these antibodies were not increased among patients in a colposcopy clinic (12). Serum antibodies against L1 and L2 are also not more prevalent among patients in a colposcopy clinic than among controls (11).

Disrupted virions of bovine papillomavirus (BPV) and HPV's share cross-reactive epitopes that have been mapped to the L1 protein (7, 10, 18). We have previously found that patients with CIN have increased levels of IgA antibodies to BPV in cervical secretions (6), and a weak increase was also found in serum (8). Antibodies to HPV 16-derived fusion proteins are also more common in cervical secretions from patients with CIN than from controls (30). Patients with condylomas but without CIN did not differ from controls in the presence of local antibodies to the broadly cross-reactive virion antigen (6). Although it thus seems clear that the antibody response to HPV among patients with condylomas is not sufficiently strong to elicit significant increases in HPV antibody titers in serum, we wished to investigate whether local IgA antibodies against HPV-derived peptide antigens were detectable and whether increased antibody prevalences could be detected among patients with clinical signs of condyloma.

MATERIALS AND METHODS

Patients and samples. Sixty women aged 17 to 39 years (mean age, 25.1 years) participated in the study. The study
was approved by the Ethical Committee on Human Research at the Karolinska Hospital, Stockholm, Sweden. Thirty women attended the Department of Gynecology, Karolinska Hospital, because of GPVI. Nineteen women were referred for treatment of condylomas diagnosed within 2 months before the actual examination, and 11 women came because of their own suspicion of disease. They all underwent clinical examination and colposcopy, and a Pap smear was taken. If colposcopy revealed a demarcated acetowhite lesion indicative of a flat condyloma or CIN, a biopsy for histology was taken. Thirteen women had lesions on the cervix, 11 women also had vulvovaginal condylomas, and 11 patients had vulvovaginal condylomas only. Six women had recently (within 2 months) been diagnosed with condylomas that had regressed at the time of sampling.

Thirty women who attended the clinic for contraceptive counseling who were matched with study subjects for age and with no previous history of GPVI and no clinical signs of GPVI served as controls. Pap smears were taken from controls if they had not been taken within the last 6 months, and Pap smears of all controls were negative.

Cervical secretions were obtained by gentle swabbing of the ectocervix with a Cytobrush (Medscand, Malmö, Sweden), trying to avoid contamination with blood. The Cytobrush was placed in a test tube containing 1 ml of phosphate-buffered saline (PBS) with 5 mM EDTA and 50 Units of penicillin and streptomycin, and the test tubes were frozen until use. Before analysis, the brush-containing tubes were thawed and centrifuged at 500 × g for 10 min and then the Cytobrush was removed gently. The supernatant (500 μl) was aspirated for enzyme-linked immunosorbent assay (ELISA) analysis. The cell pellet and the remaining supernatant were used for Southern blotting and PCR analysis of HPV DNA.

Antigens. Peptides were synthesized by the solid-phase method described previously (5). BPV was purified from bovine warts as described previously (6) and was disrupted by overnight incubation in carbonate buffer (pH 9.6).

ELISA. ELISA was performed as described previously (5). Briefly, synthetic peptides or purified BPV was coated onto microtiter plates (Costar, Cambridge, Mass.), which were subsequently blocked with 10% lamb serum in PBS (LS-PBS). Serum samples were diluted 1:20 in LS-PBS and cervical secretions were diluted 1:5 in LS-PBS, and the mixtures were incubated on the plates for 2 h at 37°C. Bound antibodies were detected with a horseradish peroxidase-labeled monoclonal antibody to human IgA (Janssen, Beerse, Belgium) or a rabbit anti-human IgG-alkaline phosphatase conjugate (Dako, Copenhagen, Denmark). For detection of secretory IgA in the cervical secretions, a monoclonal antibody to secretory piece (Sigma) and an antimouse IgG horseradish peroxidase conjugate (Southern Biotechnology) were used. Single-dilution optical density (OD) titers were calculated in the 0.2 to 1.0 OD interval (7). Prior to calculation of OD titers, the absorbance of the same sample reacted with uncoated wells was subtracted. The chi-square test was used for statistical analyses of the observed differences between patients and controls.

In order to measure the total immunoglobulin (Ig) content in the cervical secretions, ELISA plates were coated overnight at room temperature with rabbit antibodies to human IgG, IgA, and IgM (Dako) diluted 1:10 in PBS. The cervical secretions were diluted 1:100 in LS-PBS and were added to the plates for 2 h at 37°C. After five washes with PBS—0.05% Tween 20, the total bound Ig was detected by incubation with rabbit antibodies to human IgG, IgA, and IgM conjugated with alkaline phosphatase (Dako) diluted 1:500 in LS-PBS for 2 h at 37°C. Subsequent washing and development was performed as described previously (5). The ELISA ODs were compared with a standard curve obtained by using various concentrations of purified human Ig (Dako).

Southern blotting. Detection of HPV type 6, 11, 16, 18, 31, 33, or 35 DNA in the cervical Cytobrush samples was performed by Southern blotting in a kit format (Oncor Inc., Gaithersburg, Md.) according to the manufacturer’s instructions.

PCR. Some 10% of the cell pellet was digested overnight with 1.6 mg of proteinase K per ml and was incubated with a PCR mixture containing 200 μM (each) deoxynucleoside triphosphate (Boehringer Mannheim), 1.25 U of Taq DNA polymerase, and reaction buffer (Promega). HPV type-specific oligonucleotide primers (0.75 μM), which were chosen from published nucleotide sequences as described in detail elsewhere (28), were added and the reaction was carried out for 30 cycles of 30 s at 94°C (denaturation), 30 s at 50°C (annealing), and 45 s at 72°C (extension). Human lung fibroblasts and the PCR mixture without DNA templates served as negative controls. The amplified sequences were detected by hybridization under high-stringency conditions with 32P-labeled type-specific DNA probes.

RESULTS

All clinical assessments of condylomas in the 30 patients participating in the study were verified by Pap smear or biopsy, or both. No concomitant CIN was found in the histopathological examination of any of the patients. All 30 women in the control group had negative Pap smears.

HPV type 6, 11, 16, or 33 DNA was found in 8 of 30 patients and 2 of 30 controls by the Southern blotting technique (Table 1). All of the Southern blotting results were confirmed by PCR. PCR analysis also identified HPV DNA type 6, 11, 16, 18, or 31 in six additional patients and three additional controls (Table 1). Double infections were detected in three patients (Table 1).

The sera and secretions were tested for IgA and IgG antibodies against a panel of five HPV 16-derived synthetic peptides, purified, disrupted BPV, and a control synthetic peptide derived from the Epstein-Barr virus nuclear antigen type 1, which is known to be diagnostic for Epstein-Barr virus infection (19). The HPV peptides used were derived from the E2, E7, L1, or L2 open reading frames of HPV 16. The E2-derived peptide 245 was selected for testing because it was extensively characterized previously (4, 15, 17, 22, 26, 31). Comparison of the immunoreactivity of this peptide with the homologous peptides from other HPVs has shown that the peptide 245 epitope is shared by HPV types 16 and 31 and that the peptide also has significant cross-reaction with HPV types 6 and 11 (unpublished data; 31). Peptide E2-9 (GDTENMTMYTNWTH VICEK) is an E2-derived peptide that was tested because it was found to be the most promising peptide in a previous screening of overlapping peptides (3). The serotype of this peptide is not known. The E7-derived peptide used (CCKCDSTLRLCVO QSTHV DI TEDLILMGLT) is an improved version of the previously described immunoreactive E7:5 peptide (3). Preliminary analysis of the serotype of this peptide indicates that it has a broad serotype common to most genital HPVs (unpublished data). The L1 peptide (L1:31; VTSQAIACOKHTTP PKE DPL) tested in the present study is derived from the carboxy terminus of L1 of HPV 16 (5, 7). This peptide contains an epitope that is very broadly cross-reactive.
TABLE 1. IgA reactivities in cervical secretions

<table>
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<th>Patient group and sample no.</th>
<th>IgA reactivity with*:</th>
<th>HPV DNA type on portio⁵</th>
<th>Type of lesion at the following site²:</th>
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<td>E7</td>
<td>L1</td>
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<td>Subjects with condylomas</td>
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<td>30</td>
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*a* IgA reactivities in cervical secretions from 13 patients with portio condylomas, 17 patients with vulvovaginal condylomas, and 30 controls without condylomas to HPV 16-derived antigens and BPV. NA, not analyzable because of high background in ELISA.

*b* HPV DNA was detected in brush samples from the portio by Southern blotting (SB) and PCR.

⁵ The different sites of infection where condylomas were detected were the portio (Po), vagina (Va), and vulva (Vu). Flat condylomas (F) and/or condylomata acuminata (A) were found.
TABLE 2. IgA and IgG reactivities in serum and cervical secretions from 30 women with condylomas and 30 controls to synthetic peptides derived from the E2 (peptide 245), E7, L1, and L2 regions of HPV 16 and BPV

<table>
<thead>
<tr>
<th>Sample</th>
<th>Patient group</th>
<th>No. positive/no. tested with the following antigens*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peptide 245</td>
<td>E7</td>
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<td>IgA in secretions</td>
<td>Condyloma patients</td>
<td>18/29</td>
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<td>IgA in serum</td>
<td>Condyloma patients</td>
<td>8/30</td>
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<td></td>
<td>Healthy controls</td>
<td>6/30</td>
</tr>
<tr>
<td>IgG in serum</td>
<td>Condyloma patients</td>
<td>4/30</td>
</tr>
<tr>
<td></td>
<td>Healthy controls</td>
<td>5/30</td>
</tr>
</tbody>
</table>

* Positive reactions were those with ELISA titters of 1:20 or more.

among papillomaviruses since antisera against purified papillomaviruses from cows, dogs, and birds react with this peptide (7). Human sera can, however, distinguish two different serotypes at this site, with one serotype shared by HPV 6 and 11 (10, 31) and the other serotype shared by HPV 16 and 33 (unpublished data; 31). The L2 peptide (L2:49) was the most immunoreactive of a set of overlapping L2 peptides (5). The epitope found in this peptide is common to most genital HPV's (31).

The IgA antibody titers in cervical secretions were low (mean titer for E2 [peptide 245] and E7, 1:10; mean titer for L1 and L2, 1:20). Cervical secretions from one patient and two controls could not be analyzed because of a high background in the ELISA. In serum, the mean titers to E2 (peptide 245) were 1:60 and mean titers to E7 were 1:40 for both IgA and IgG antibodies, whereas the mean titers against the late antigens were higher for IgG (L1 IgG, 1:120; L1 IgA, 1:60; L2 IgG, 1:340; L2 IgA, 1:190). Low titers (mean, 1:10) of IgA to BPV were detected in secretions. IgA titers to BPV in serum were higher than IgG titers (mean, 1:140 and 1:110, respectively). In this context it should be noted that in the case of the secretions, the titers were not dilutions of the actual volume of cervical secretions, since the secretions were all collected in 1 ml of PBS before titration.

Low levels of IgG antibodies were found in cervical secretions in only a few cases, probably because of diffusion of serum antibodies into the secretions or contamination of the cervical secretions with small amounts of blood.

For 18 of 29 patients with condylomas, IgA antibodies to E2 peptide 245 were found in cervical secretions, but IgA antibodies to E2 peptide 245 were found in only 8 of 28 controls (P < 0.025) (Table 2). The antibodies tended to be preferentially found in patients with portio condylomas than in patients with vulvovaginal condylomas only (Table 1). In contrast, there were no significant differences in the IgG and IgA antibody levels to this peptide in serum between patients and controls (Table 2).

None of the controls had IgG antibodies to the other E2 peptide (E2:9) in cervical secretions, whereas five patients with condylomas did (data not shown). All E2:9-positive patients also had IgA antibodies in secretions to both the E2 peptide 245 and the E7 peptide (data not shown). No IgG antibodies to the E2:9 peptide were detected.

Seventeen of 29 patients had IgA antibodies to the E7 peptide in their cervical secretions, whereas 5 of 28 controls had IgA antibodies to the E7 peptide (P < 0.005). The antibodies to E7 were more prevalent among patients with portio condylomas (Table 1). Four of the 30 patients and none of the controls had IgA antibodies to the E7 peptide in serum. Twenty-four of the patients and 19 of the controls had IgG antibodies to this peptide in serum (Table 2).

A large proportion of both patients and controls had serum IgG, serum IgA, and local IgA antibodies against the L1 and L2 peptides (Table 2). All of the patients and all of the controls who had antibodies against the L1 peptide also had antibodies against the L2 peptide. There were no significant differences in the reactivities to BPV among patients and controls (Table 2). As expected, there were also no differences between patients and controls in their reactivities to the Epstein-Barr virus-derived peptide (data not shown).

The total Ig content in cervical secretions varied between 1.6 and 20 μg/ml (mean, 14.5 μg/ml). There were no significant differences between patients and controls, nor was the total Ig content correlated to the titers against the tested antigens.

Measurement of the secretory piece in the peptide ELISAs of cervical secretions yielded results similar to those of measurement of total IgA antibodies.

**DISCUSSION**

The present study was undertaken in order to study local antibody responses to HPV-derived antigens in patients with genital condylomas. We also wanted to evaluate whether HPV DNA and antibodies to papillomavirus antigens could both be analyzed by using the same cervical sample. We found that IgA antibodies in cervical secretions directed to papillomavirus E2- and E7-derived antigens were detectable and preferentially found among patients with condylomas of the portio. Since the antibodies detected in secretions in many cases had much lower titers than the corresponding antibodies in serum to the same antigen, leakage of serum antibodies into secretions poses a potential problem. The IgA antibodies to the E2 (peptide 245) and E7 peptides found in this study are likely produced locally rather than through diffusion from IgA in serum, since IgA antibodies to these peptides were often found in secretions in the absence of detectable IgA to the same peptide in serum. Measurement of the secretory piece in the peptide ELISAs of cervical secretions yielded results similar to those of measurement of total IgA, indicating that the IgA antibodies are actively secreted. Also, our finding that the presence of E2 (peptide 245) and E7 antibodies in secretions was correlated to the presence of condylomas, whereas the serum antibodies to the same antigens did not show this correlation, indicates that we detected locally produced antibodies. The lack of elevation of IgA in serum to peptide 245 among patients with condylomas is in agreement with previous studies (4, 17, 31). Several investigators
Fourteen, twenty-six, have found IgA levels to the 245 peptide in serum to be elevated among patients with CIN or persistent atypia. For patients with invasive cervical cancer, several studies have also found a statistically significant elevation of antibodies to peptide 245 in serum (2a, 15, 16, 26), but one study (22) found no elevation. Since the investigators (22) had previously reported a statistically significant elevation (26), their latter results (22) are puzzling. It was not clear whether the assays in their two studies were comparable or whether some type of methodological problem had occurred.

The present study demonstrates that IgA antibodies to the E2 (peptide 245) antigen are present in cervical secretions of patients with condylomas. Low-titer IgG antibodies in secretions were found only occasionally and only in patients with high IgG titers to the same antigen in serum, making leakage of IgG in serum to secretions the most probable explanation for the presence of IgG antibodies. Alternatively, although we swabbed the cervix very gently in order to avoid hemorrhage, low-level blood contamination of some samples might have occurred. Assay for the absence of IgG antibodies in the secretions may be a useful method of ensuring that antibodies in serum did not contaminate the sample.

HPV DNA was detected in only 47% of the patients with condylomas, which may seem low. However, the flat cervical condyloma is not as frequently HPV positive as is condyloma acuminata (25). For example, in a large study of 2,627 women by Lorincz et al. (21), HPV DNAs of the types tested for in this study were detected in 48% of patients with low-grade cervical lesions (condylomas and CIN type 1). Since condyloma is an HPV-associated disease, it is likely that additional uncharacterized HPV types may have caused the apparently HPV DNA-negative condylomas. It should also be noted that only one brush sample was taken from each patient and that this sample was taken from the portio, even though some of the patients had vulvar and/or vaginal condylomas and no detectable lesions on the portio. Although both the local antibodies to E2 (peptide 245) and E7 antigens were associated with a diagnosis of condyloma, there was no significant correlation between the presence of antibodies and the detection of HPV DNA. The antigens used in our test have previously been found to cross-react between different HPV types (7, 31). It is therefore possible that the antibodies to the HPV-derived antigens which were detected in patients with apparently HPV DNA-negative condylomas may have been induced by HPV types not tested for. Interestingly, a large serological study (22) reported that antibodies to the E7 antigen of HPV 16 were strongly elevated among patients with cervical cancer, but they did not correlate with detectability of HPV 16 DNA in the cervical tumor (22). Until truly type-specific HPV antigens are developed, HPV serology will not be directly comparable to HPV DNA typing.

In contrast to the findings with the peptides derived from the HPV early proteins, we could not detect any difference between patients and controls in their antibody responses to the late antigens L1 and L2. The early HPV proteins are less well conserved than the late proteins (25), and the antigens from the early region do not show the same extent of cross-reactivity with distantly related HPV types (7, 31). Also, by analogy with other viruses, antibodies to late proteins are more likely to represent previous exposure to virus, whereas antibodies to early proteins may reflect an ongoing infection (27).

In summary, we showed that a single cervical sample can be used for detection of both local antibodies to HPV antigens and HPV DNA by PCR and Southern blotting. The method is very simple and, therefore, potentially useful for large-scale studies. Although the local IgA antibodies to peptides derived from HPV 16 E2 (peptide 245) and E7 were more frequent among patients with condylomas, a full understanding of the significance of these antibodies will require thorough epidemiological studies.

ACKNOWLEDGMENT

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REFERENCES

3a. Dillner, J. Unpublished data.


