Detection of antibodies against a human papillomavirus (HPV) type 16 peptide that differentiate high-risk from low-risk HPV-associated low-grade squamous intraepithelial lesions

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Received 1 March 2004
Accepted 12 May 2004

A nonapeptide (16L1) was derived from the human papillomavirus type 16 (HPV-16) major capsid protein and tested for detection of potential cross-reactive serum IgG and cervical IgA antibodies in low- and high-risk HPV-associated low-grade squamous intraepithelial lesions (LSIL) and cervical cancer patients by ELISA. The IgG response was similar in women with low-risk HPV-associated LSIL and controls (P=0.1). In contrast, more than 90% of patients with high-risk HPV-associated LSIL were seropositive. Although tumours from cancer patients were all positive for the presence of high-risk HPV DNA, the level of seropositivity decreased significantly in this group (P<0.0001). Cervical IgA antibodies were also detected in a significantly high proportion of women with high-risk HPV-associated LSIL compared with controls. However, the proportion of IgA-positive patients was lower than the proportion of IgG seropositives. In conclusion, the 16L1 peptide appears to be a high-risk type-common epitope that induces cross-reactive antibodies in high-risk, but not low-risk, HPV-associated LSIL patients, allowing differentiation of high- and low-risk infected women at this stage of infection.

INTRODUCTION

Infection of the genital epithelium with human papillomavirus (HPV) is a common sexually transmitted disease. Epidemiological studies have demonstrated that ‘low-risk’ HPV genotypes, mainly types 6 and 11, induce benign genital warts. In contrast, ‘high-risk’ genotypes, including HPV-16, -18 and related types, are associated with the development of cervical cancer (Walboomers et al., 1999). Cervical cancer is still one of the most prevalent cancers in the developing world (Parkin et al., 1999). HPV-16 and related types (31, 33, 52, 58 and 35), together with HPV-18 and related types (45, 59, 39 and 68), are associated with 91% of cervical tumours in Central and South America (Bosch et al., 1995). Thus, early detection of genital infections by these high-risk HPV types would be of value for the prevention of cervical cancer.

Genital HPV infection frequently induces antibody-mediated immune responses, mostly directed against the viral capsid (Wang et al., 1996; Carter et al., 2000; Rocha-Zavaleta et al., 2003). The HPV capsid is composed of two structural proteins, a largely internal minor capsid protein (L2) and a major protein (L1), which is 30 times more abundant than L2 (Kirnbauer et al., 1993) and represents about 80% of the total viral protein. L1 is an important target of the immune system. Immunological assays based on the use of HPV capsids are known to detect type-restricted antibodies, possibly due to the presentation of type-specific conformational epitopes. Interestingly, the
HPV-16 capsid exposes not only type-restricted but also type-common antigenic epitopes (Heino et al., 1995). Thus, in addition to specific antibodies (Cason et al., 1992), widely cross-reactive antibodies have been found to react against peptide sequences on the L1 protein of both high- and low-risk HPV types (LeCann et al., 1995).

We have previously described a peptide (IHSMNSTIL) derived from the HPV-16 L1 protein (16L1 peptide) that binds to HLA class I allele B*3901 and elicits proliferative responses in lymphocytes from patients with cervical cancer associated with either HPV-16 or -18 (Monroy-Garcia et al., 2002). Further analysis of this peptide using Lipman and Pearson’s ALIGN program showed that the sequence presented a substantial amino acid identity with the corresponding sequence in HPV-18 (88–9 % identity), HPV-16-related types (77–8–66–7 % identity) and HPV-18-related types (88–9–66–7 % identity). Interestingly, comparison with the corresponding sequences in HPV-6 and -11 demonstrated an identity level of 55-6 %. This observation raised the question of whether antibodies generated against these sequences could cross-react with the 16L1 peptide. This study aimed to evaluate the antigen-reactivity of antibodies produced in patients with high-risk HPV-associated low-grade squamous intraepithelial lesions (LSIL) and cervical cancer and to compare it with the reaction of antibodies from women with low-risk HPV-associated LSIL to assess their potential cross-reactivity.

**METHODS**

**Study population and human samples.** The study population was selected from patients attending the National Center for Clinics of Dysplasias (CENACLID), General Hospital of Mexico, and the National Institute of Cancerology (INCAN), Mexico City, Mexico. Samples were collected between 1997 and 2002. Informed consent was obtained from all participants. Human material was handled according to institutional experimentation and safety guidelines. CENACLID provides gynaecological services to women referred for colposcopy because of abnormal cytology and women without a history of cervical abnormalities who ask for a routine examination.

In the present study, women from both groups were analysed. Women with a history of previous high-grade squamous intraepithelial lesions (HSIL) were not included in the study. All women underwent cytological and histopathological analysis of colposcopy-directed biopsies. All Pap smears were examined independently by two experienced cytopathologists. Samples with an inconsistent diagnosis were excluded from the study. Cytology diagnoses were classified according to the Bethesda system into normal, atypical squamous cells with undetermined significance (ASCUS), LSIL, HSIL and cervical carcinoma. Samples with ASCUS and HSIL were excluded from the study. Blood samples, cervical washes and colposcopy-directed biopsies were obtained from all patients. Serum was separated from blood samples by centrifugation at 9000 g for 15 min. Cervical mucus was collected by washing the uterine cervix with 1 ml sterile PBS. Cell debris was eliminated by centrifugation at 9000 g for 5 min. Cervical washes containing blood contamination were not considered for antibody detection. Cervical mucus and serum samples were stored at −70 °C until tested. Cervical biopsies were placed immediately in tubes containing sterile, contaminant-free PES (Roche Applied Science) and processed the same day.

Investigators who performed molecular and immunological assays were not aware of the clinical status of samples. To avoid contamination, cases and controls were tested in separate batches. All samples underwent molecular analysis by PCR and hybrid capture. Women who were consistently negative for both clinical and molecular tests were considered as negative controls. Only patients (LSIL and cervical carcinoma) with consistently positive results for PCR and hybrid capture tests were included in the study.

**HPV DNA detection by PCR.** All reagents used for the isolation and amplification of DNA were purchased from Gibco-BRL. Biopsies were treated with proteinase K as described elsewhere (Kaye et al., 1994). DNA was extracted with phenol/chloroform and precipitated with ethanol. HPV DNA was amplified using the general primers MY09 (5′-GTGTCMARRGGAWACTGCAG-3′) and MY11 (5′-GCMAGGWMCCATAAYAATGG-3′) (Manos et al., 1989), which amplified a conserved 450 bp fragment from the L1 gene. Genomic DNA (100 ng) was denatured by heating the reaction to 95 °C for 30 s. Annealing of primers was performed at 45 °C for 30 s and extension at 72 °C for 60 s. The cycle was repeated 30 times. PCR products were electrophoresed in 2 % agarose gels, stained with ethidium bromide and visualized in a UV transilluminator. Specific amplification of HPV-16 was achieved by using the primers Pr3 (5′-GTAAAGGCACCATGTGTC-3′) and Pr4 (5′-CATCATCATTACACGAG-3′), which amplified a 499 bp fragment covering the HPV-16 E7 gene plus fragments of the E6 and E1 genes. Amplification of HPV-18 was performed using the primers Pr1 (5′-CGAGACGGGACACGAG-3′) and Pr2 (5′-TGTTTTCTTCCTCTGAGTC-3′), which amplified a 172 bp fragment including parts of the HPV-18 E6 and E7 genes, as described previously (Karslen et al., 1996). An internal control to ensure DNA integrity was performed by amplifying the β-globin gene using the primers PC03 and PC04 as described previously (Saiki et al., 1986). As a positive control, DNA from SiHa (a cervical cancer-derived cell line containing one to two HPV-16 copies per cell) or HeLa (a cervical cancer-derived cell line positive for the presence of HPV-18) cells was run concurrently with each reaction. Additionally, negative controls to assess the presence of contaminants were carried out using purified water (Gibco-BRL) and PBS instead of template DNA. Patients whose DNA sample could not be amplified were excluded from the study.

**Detection of high- and low-risk HPV types by hybrid capture.** The HPV Test Hybrid Capture II kit (Digene) was used for the detection of high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) or low-risk types (6, 11, 42, 43 and 44) was used. DNA (250–500 ng in 20 µl) was extracted from cervical tissue and placed in a tube containing 30 µl specimen transport medium. NaOH-based denaturation reagent (25 µl) was added to each sample and the tubes were mixed vigorously and incubated at 65 °C for 45 min. Hybridization and hybrid detection were performed according to the manufacturer’s instructions. Carrier DNA and plasmid DNA containing the HPV-16 or HPV-11 genome were used as negative and positive calibrators, respectively, and were run in triplicate with each test. An assay was considered valid only when the results from the negative and positive calibrators showed a coefficient of variation ≤15 % and the positive: negative calibrator mean ratio value was ≥2.0. The cut-off value for positivity was calculated for each assay and was defined as the mean relative light units (RLU) value of the positive calibrator. Patients who were positive for both low- and high-risk types were excluded from the study.

**Peptide synthesis.** The nonamer peptide IHSMNSTIL was generated by solid-phase synthesis on a multiple peptide automatic synthesizer (Applied Biosystems Synergy Personal Peptide Synthesizer 432A; Perkin Elmer). Repeated cycles of addition of 9-fluorenlymethoxycarbonyl (Fmoc)-protected amino acids to a polystyrene resin were alternated with an Fmoc deprotection procedure.
(Gausepohl et al., 1992). After completion of the synthesis, the peptide was cleaved from the resin and the side chain-protective groups removed by treatment with trifluoroacetic acid containing 5% water. Finally, the peptide was analysed by reverse-phase HPLC for amino acid composition and purity. A 75% pure peptide preparation was lyophilized and dissolved in sterile PBS before use.

**Peptide ELISA.** Serum IgG and mucosal IgA antibodies were detected using the 16L1 peptide as the target antigen in a standard ELISA. ELISA plates (Maxisorp; Nalge Nunc) were coated with 500 ng 16L1 peptide per well diluted in sodium carbonate/bicarbonate buffer (0.1 M sodium carbonate, 0.1 M sodium bicarbonate, pH 9.6) at 4°C overnight. Plates were then washed four times with TBS containing 0.1% Tween 20 (TBS/Tween 20). Non-specific binding sites were blocked with 200 μl 2% BSA in TBS/Tween 20 for 2 h at 37°C. After washing, alkaline phosphatase-conjugated rabbit anti-human IgA or rabbit anti-human IgG (Dako), which reacts specifically with free human secretory component and secretory component bound to secretory IgA or rabbit-anti human IgG (Dako) were diluted 1:500 in sodium carbonate/bicarbonate buffer (0.1 M sodium carbonate, 0.1 M sodium bicarbonate, pH 9.6; Sigma) and added to the plates. The absorbance was read at 405 nm in an ELISA plate reader. All samples were tested in triplicate for each antibody class. The assay was considered valid only when the coefficient of variation of the triplicates was ≤10%. Additionally, all samples were tested on two wells not coated with peptide to define non-specific reactivity. The final ELISA value was calculated by subtracting the non-specific reactivity mean absorbance from the triplicate mean absorbance. To control for inter-assay variation, positive IgG and IgA controls selected from a previous study (Rocha-Zavaleta et al., 2003) were included in each plate and tested as described. Plates with an inter-assay coefficient of variation >10% were not considered valid. The cut-off value for positivity was calculated on the basis of the distribution of absorbance of the control group and was defined as the mean absorbance+3 SD after exclusion of outliers. The calculated cut-off value for IgG seropositivity was 0.440 and for IgA was 0.237.

**Statistical analysis.** To evaluate the differences between the proportions of positive samples in the different groups, data were arranged in the form of two-by-two contingency tables and analysed by Fisher’s exact test to calculate the odds ratios (OR), 95% confidence intervals (CI) and P values. The Wilcoxon signed rank test and Student’s t-test were used to compare the mean signal strength (absorbance) of the various groups. All tests were two-tailed and the basic significance level was taken as P=0.05. Sensitivity, specificity, predictive values and efficiency of the ELISA were calculated using standard medical biostatistical formulae.

## RESULTS

### Detection of IgG antibodies against the 16L1 peptide in serum samples

After clinical and molecular diagnosis, patients were divided into five groups: (A) patients with a diagnosed LSIL and infected with low-risk HPV types (n=122); (B) patients with LSIL, infected with high-risk HPV types (n=166); (C) patients with high-risk HPV-associated cervical cancer (n=182); (D) patients with a diagnosis of LSIL who were infected with HPV-16 (n=119); and (E) negative-control women without clinical or molecular evidence of HPV infection (n=115) (Table 1). Groups A, B and C consisted of independent groups of patients. Group A included patients infected with any of the low-risk HPV types detected by the hybrid capture kit. Groups B and C comprised patients infected with any of the high-risk HPV types detected by the hybrid capture test, including HPV-16. Group D contained samples that tested positive for the high-risk hybrid capture test and were subsequently proved positive for the presence of HPV-16 by PCR.

We first investigated the presence of IgG antibodies against the 16L1 peptide in serum samples. A small proportion of patients with low-risk HPV-associated LSIL (6.5%) tested positive for the presence of IgG antibodies. This proportion was similar to the proportion of seropositive individuals in the normal population (P=0.1) (Fig. 1). In contrast, more than 90% of patients infected with high-risk HPV were positive for the presence of IgG. The difference between this group and the negative controls was highly significant (P<0.0001). Moreover, the mean signal strength of antibody response in high-risk HPV-associated LSIL patients was 3.7-fold higher than the mean signal strength in control samples. PCR analysis of DNA

<table>
<thead>
<tr>
<th>HPV DNA type</th>
<th>Diagnosis</th>
<th>Number of sexual partners</th>
<th>Number of pregnancies</th>
<th>Mean age (years) [range]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative (n=115)</td>
<td>Normal</td>
<td>1.2</td>
<td>1.1</td>
<td>31.5 [16–42]</td>
</tr>
<tr>
<td>Low-risk (n=122)</td>
<td>LSIL</td>
<td>1.4</td>
<td>2.8</td>
<td>31.3 [19–49]</td>
</tr>
<tr>
<td>High-risk (n=166)</td>
<td>LSIL</td>
<td>1.5</td>
<td>3.0</td>
<td>32.0 [17–58]</td>
</tr>
<tr>
<td>High-risk (n=182)</td>
<td>Cervical cancer</td>
<td>5.5</td>
<td>4.9</td>
<td>49.9 [28–81]</td>
</tr>
<tr>
<td>HPV-16 (n=119)</td>
<td>LSIL</td>
<td>1.3</td>
<td>2.7</td>
<td>33.5 [18–53]</td>
</tr>
</tbody>
</table>

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Table 1. Characteristics of the groups of patients included in the study

The presence or absence of HPV DNA was verified using the general MY09/MY11 primers for PCR. Low- and high-risk types were determined by hybrid capture test. HPV-16 was detected using the Pr3/Pr4 type-specific primers for PCR. Clinical diagnosis was based on cytological analysis and was corroborated by histopathological analysis.
Our results demonstrated a difference in anti-peptide IgG response between low- and high-risk HPV-infected women. Our results demonstrated a difference in anti-peptide IgG (Table 2). Patients (n) 101 29 36 Seropositivity (%) 95·0 93·1 90·9 Cervical cancer Patients (n) 105 32 45 Seropositivity (%) 67·6 46·8 42·2

Fig. 1. IgG antibody responses against the 16L1 peptide in sera from women without evidence of HPV infection (control), patients with LSIL infected with low-risk or high-risk HPV types and patients with cervical cancer (CeCa) who are also positive for the presence of high-risk HPV types. The mean absorbance value of each group is represented as a solid line. The cut-off value for positivity (0·440) is indicated as a dotted line. Statistical differences between groups are shown. P values were calculated using Fisher’s exact test.

from high-risk HPV-associated LSIL samples indicated that 60·8 % of patients were infected with HPV-16, while 17·4 % were infected with HPV-18 and 21·6 % with other high-risk types. However, the proportion of seropositive subjects in each group was similar (P > 0·1) (Table 2). This seemed to indicate that most LSIL patients infected by high-risk HPV types produced antibodies that reacted against the 16L1 peptide. Thus, we next addressed the question of whether these antibodies could also be found in patients who had developed a high-risk HPV-associated cervical tumour. Results showed that only 57·7 % of cervical cancer patients had antibodies against the 16L1 peptide. The proportion of seropositive subjects in the cervical cancer group was significantly higher than that observed in the negative controls (P < 0·0001). However, seropositivity in cervical cancer patients was significantly lower than that detected in high-risk HPV-associated LSIL patients (P < 0·0001). This suggested that the presence of viral DNA in cervical cancer patients does not correlate with the presence of IgG antibodies against the peptide. Furthermore, statistical analysis revealed that the proportion of seropositive patients whose tumours were HPV-18 positive was significantly lower than the proportion in patients with HPV-16-associated tumours (P = 0·03) (Table 2).

Our results demonstrated a difference in anti-peptide IgG response between low- and high-risk HPV-infected women with a diagnosis of LSIL. Thus, we next addressed the significance of the 16L1 peptide-based ELISA for the detection of high-risk HPV infection in LSIL and cervical cancer patients. As shown in Table 3, the 16L1 peptide-based ELISA was able to detect a high proportion (91·6 %) of LSIL patients who were actually infected with high-risk HPV types and produced low levels of false-positive and false-negative results. The probability that an LSIL patient is infected with high-risk HPV types, given a positive ELISA value, was high (positive predictive value = 97·7 %). However, the probability of not being infected with high-risk genotypes, given a negative ELISA result, was lower (negative predictive value = 87·7 %). The efficiency of the

Table 2. Prevalence of IgG serum antibodies against the 16L1 peptide in patients with LSIL and cervical cancer infected with different high-risk HPV types

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>HPV-16</th>
<th>HPV-18</th>
<th>Other high-risk types*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSIL Patients</td>
<td>101</td>
<td>29</td>
<td>36</td>
</tr>
<tr>
<td>Seropositivity</td>
<td>95·0</td>
<td>93·1</td>
<td>90·9</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>105</td>
<td>32</td>
<td>45</td>
</tr>
<tr>
<td>Seropositivity</td>
<td>67·6</td>
<td>46·8</td>
<td>42·2</td>
</tr>
</tbody>
</table>

*High-risk HPV types detected by the hybrid capture test (31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68).

Table 3. Sensitivity, specificity, predictive values and efficiency of the 16L1 peptide-based ELISA for serological detection of high-risk HPV infection in LSIL and cervical cancer

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LSIL</th>
<th>Cervical cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>True positives (n)</td>
<td>132</td>
<td>105</td>
</tr>
<tr>
<td>False positives (n)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>True negatives (n)</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td>False negatives (n)</td>
<td>12</td>
<td>77</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>91·6</td>
<td>57·6</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>96·6</td>
<td>96·6</td>
</tr>
<tr>
<td>Positive predictive value (%)</td>
<td>97·7</td>
<td>97·2</td>
</tr>
<tr>
<td>Negative predictive value (%)</td>
<td>87·7</td>
<td>52·7</td>
</tr>
<tr>
<td>Test efficiency (%)</td>
<td>93·5</td>
<td>70·4</td>
</tr>
</tbody>
</table>
ELISA to detect high-risk HPV infection in LSIL patients was higher than 90%. In comparison, the ELISA was not quite as sensitive for the detection of high-risk viruses in patients with cervical cancer, having a low negative predictive value (52.7%) and, as a consequence, a reduced level of efficiency (70.4%).

**Detection of IgA antibodies against the 16L1 peptide in cervical secretions**

Since the results showed that IgG antibodies against the 16L1 peptide were present in the sera of high-risk HPV-infected LSIL patients, we next determined whether reactive IgA antibodies could also be found in cervical secretions from these women. Once more, ELISAs revealed that LSIL patients infected with low-risk HPV genotypes were largely negative for the presence of anti-16L1 peptide antibodies (Fig. 2). In addition, IgA reactivity in the high-risk HPV-associated LSIL population was significantly higher than that observed in the negative-control group and in the low-risk HPV group. HPV-16 is by far the most frequent high-risk HPV type infecting women in Latin America (Bosch et al., 1995). Thus, to determine whether mucosal IgA antibodies against the 16L1 peptide were more prevalent in women infected with HPV-16 than in women infected with any high-risk HPV type, we evaluated the presence of IgA in cervical secretions of HPV-16-associated LSIL patients. As shown in Fig. 2, the proportion of IgA-positive subjects in the HPV-16-infected group was significantly higher than that of the control group ($P<0.0001$). However, statistical comparison of the IgA positivity in the high-risk HPV- and HPV-16-associated LSIL groups indicated that the responses were similar in both groups [$OR=1.3; 95\% CI=0.8–2.1; P=0.09$].

To ascertain whether the presence of serum IgG correlated with the detection of cervical IgA, a statistical analysis of the proportion of positive subjects in each group was performed. As shown in Table 4, there was no difference in the proportion of women positive for the presence of IgG and IgA in the non-infected group ($P=0.4$). Interestingly, when all patients diagnosed with LSIL were analysed together, no difference was detected between the IgG and IgA responses ($P=0.06$). However, separate analysis of LSIL patients infected by low- or high-risk HPV types showed that the proportion of cervical IgA responders was significantly higher than the proportion of IgG seropositives in the group of patients infected by low-risk HPV types. In contrast, in the patients infected with high-risk HPV, the proportion of IgG seropositives was significantly higher than that of cervical IgA positives ($P<0.0001$). In this group, the likelihood of presenting circulating IgG antibodies to the 16L1 peptide was six times higher than that of presenting mucosal IgA antibodies against the peptide. This suggested that, during early infection by high-risk HPV genotypes, the antibody response is mediated predominantly by systemic IgG antibodies.

**DISCUSSION**

In the present work, we have shown that IgG antibodies against the 16L1 peptide can be found in sera from LSIL patients infected with high-risk HPV types. Antibodies against HPV 16L1-derived peptides have been demonstrated previously (Cason et al., 1992; Dillner et al., 1995; LeCann et al., 1995; Sharma et al., 1996). However, to our knowledge, this is the first report of a peptide-based serological assay capable of differentiating low-risk from high-risk HPV infections in LSIL patients. Previously, a conformation-dependent HPV 16L1 epitope was suggested to be the subject of the antigenic recognition of HPV-16 infected LSIL patients. Since the results showed that IgG antibodies against the 16L1 peptide were present in the sera of high-risk HPV-infected LSIL patients, we next determined whether reactive IgA antibodies could also be found in cervical secretions from these women. Once more, ELISAs revealed that LSIL patients infected with low-risk HPV genotypes were largely negative for the presence of anti-16L1 peptide antibodies (Fig. 2). In addition, IgA reactivity in the high-risk HPV-associated LSIL population was significantly higher than that observed in the negative-control group and in the low-risk HPV group. HPV-16 is by far the most frequent high-risk HPV type infecting women in Latin America (Bosch et al., 1995). Thus, to determine whether mucosal IgA antibodies against the 16L1 peptide were more prevalent in women infected with HPV-16 than in women infected with any high-risk HPV type, we evaluated the presence of IgA in cervical secretions of HPV-16-associated LSIL patients. As shown in Fig. 2, the proportion of IgA-positive subjects in the HPV-16-infected group was significantly higher than that of the control group ($P<0.0001$). However, statistical comparison of the IgA positivity in the high-risk HPV- and HPV-16-associated LSIL groups indicated that the responses were similar in both groups [$OR=1.3; 95\% CI=0.8–2.1; P=0.09$].

To ascertain whether the presence of serum IgG correlated with the detection of cervical IgA, a statistical analysis of the proportion of positive subjects in each group was performed. As shown in Table 4, there was no difference in the proportion of women positive for the presence of IgG and IgA in the non-infected group ($P=0.4$). Interestingly, when all patients diagnosed with LSIL were analysed together, no difference was detected between the IgG and IgA responses ($P=0.06$). However, separate analysis of LSIL patients infected by low- or high-risk HPV types showed that the proportion of cervical IgA responders was significantly higher than the proportion of IgG seropositives in the group of patients infected by low-risk HPV types. In contrast, in the patients infected with high-risk HPV, the proportion of IgG seropositives was significantly higher than that of cervical IgA positives ($P<0.0001$). In this group, the likelihood of presenting circulating IgG antibodies to the 16L1 peptide was six times higher than that of presenting mucosal IgA antibodies against the peptide. This suggested that, during early infection by high-risk HPV genotypes, the antibody response is mediated predominantly by systemic IgG antibodies.

**Table 4. Statistical comparison of serum IgG and cervical IgA responses against the 16L1 peptide**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Positives(n)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum IgG</td>
<td>Cervical IgA</td>
</tr>
<tr>
<td>Normal (n=115)</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>LSIL (n=288)</td>
<td>162</td>
<td>139</td>
</tr>
<tr>
<td>Low-risk HPV (n=122)</td>
<td>8</td>
<td>26</td>
</tr>
<tr>
<td>High-risk HPV (n=166)</td>
<td>154</td>
<td>113</td>
</tr>
<tr>
<td>Cervical cancer (n=182)</td>
<td>102</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not determined.
* Determined by Fisher’s exact test.
to be an immunodominant site recognized by most human sera following HPV infection (Wang et al., 1997). This site turned out to be a major neutralizing epitope on HPV-16 (White et al., 1999) that seemed to induce an antibody response in more than 75% of infected patients. Nevertheless, this epitope covers non-contiguous regions of the capsid surface, making its use difficult as a detection reagent for early HPV infection.

Serological studies, using virus-like particles (VLPs) as antigenic targets, have demonstrated that the majority of women infected with HPV-16 produce an IgG antibody response (Kirnbauer et al., 1994). Here, we found that most women with high-risk HPV type-associated LSIL generated systemic IgG antibodies against the 16L1 peptide. However, antibodies detected in VLP-based assays are mainly type restricted and directed against conformational epitopes (Christensen et al., 1994, 1996). Therefore, it might be possible that anti-16L1 and anti-VLP antibodies are generated in parallel as a response against infection. To ascertain whether these antibodies co-exist in infected humans, the population studied herein is currently being tested in a VLP-based ELISA.

It is generally accepted that IgG antibodies against HPV capsid antigens are long lasting (Af Geijersstam et al., 1998; Shah et al., 1997; Carter et al., 2000) and may be a marker of past and current infection. From the information presented in Table 3, it appears that systemic IgG antibodies against the 16L1 peptide are good indicators of current infection. Nevertheless, we cannot conclude this, because we did not analyse women with a previous history of HPV infection and the number of seropositive patients who were negative for the presence of viral DNA was too small. To ascertain whether antibodies against the 16L1 peptide are markers of prior infection, it will be necessary to test groups of currently non-infected women, who had a previous infection that was either treated successfully or cleared naturally. On the other hand, we observed that a small proportion of patients with LSIL were seronegative, regardless of the presence of HPV DNA. With reference to this, an earlier study of incident HPV infection demonstrated that some women with a persistent HPV infection never seroconverted (Carter et al., 2000). Thus, it is possible that the seronegative patients detected in this work do not produce antibodies against the peptide.

Here we demonstrated that IgG antibodies against the 16L1 peptide were detected in sera from most LSIL patients infected with high-risk HPV types and were almost absent in low-risk HPV-associated LSIL and uninfected women. In as much as HPV infection is associated with risk factors such as age and sexual activity (Giuliano et al., 1999), it could be postulated that the apparent cross-reactivity between high-risk HPV types might reflect shared epidemiological risk factors. This is unlikely, because the epidemiological data presented in Table 1 showed that mean values for age, number of sexual partners and pregnancies were similar between high- and low-risk HPV-associated LSIL and even non-infected women. This suggests strongly that the antibodies detected are not associated with these risk factors. Another possibility might be that patients included in the high-risk HPV-associated LSIL group were previously infected with HPV-16. To determine whether previous HPV-16 infection occurred in these women, a study of the presence of HPV-16 DNA in archive tissue samples is warranted.

A more likely explanation might be that the difference in antibody responses is associated with the level of sequence conservation of the peptide region. This hypothesis is supported by previous work in which antibodies raised against a peptide sequence containing the 16L1 peptide reacted with HPV-16 VLPs but not with HPV-11 VLPs (Heino et al., 1995). The 16L1 peptide is located within the structure of the h2 and h3 α-helices of the C-terminal laterally projecting domain of L1 (Chen et al., 2000). They form, together with the h4 helix, the surface of contact with other L1 monomers. Consequently, this site is important for the assembly of viral capsids and is relatively conserved among different HPV types. However, comparison of the corresponding sequences in the high- and low-risk types detected by the hybrid capture test used in this work showed interesting differences. For instance, all five low-risk types differed at three or more amino acid positions from the 16L1 peptide sequence. In contrast, only three of the 12 high-risk types differed at more than three amino acid positions from the 16L1 peptide. Moreover, changes at positions 1, 5 or 8 were found in four of five low-risk types, while only four of 12 high-risk types had these changes.

We also observed that a high proportion of patients with cervical cancer were positive for the presence of systemic IgG antibodies against the 16L1 peptide. In an earlier study (Dillner et al., 1990), IgG responses to peptides covering the complete amino acid sequence of the HPV-16 L1 protein were examined in sera from patients with cervical cancer. Interestingly, a peptide region that included the 16L1 peptide sequence was demonstrated to be largely unreactive. This divergence might be attributable to differences in the amino acid sequences. In the work by Dillner et al. (1990), the 16L1 peptide sequence was included as a part of a 20 amino acid peptide, which meant an extension of nine amino acids at the N terminus and one amino acid at the C terminus of the 16L1 peptide. In this respect, there is evidence to indicate that recognition and binding of antibodies to a particular epitope are highly influenced by the extension of a peptide sequence. In fact, extension of a single amino acid can induce a reduction in monoclonal antibody recognition of two to three orders of magnitude (Uray et al., 2003), while an extension of 13 amino acids can result in complete loss of antibody binding (Calderon-Aranda et al., 1999). Therefore, extensions of the 16L1 peptide might account for the lack of response observed in the former report.

Interestingly, patients with high-risk HPV-associated invasive cervical cancer showed a reduced antibody response...
against the 16L1 peptide compared with high-risk HPV-associated LSIL. In agreement with our results, previous studies have shown that IgG responses against different L1-derived peptides are significantly prevalent in patients with premalignant lesions (Sharma et al., 1996), but are not associated with cervical cancer (Dillner et al., 1995). Reduction of anti-peptide IgG responses in cervical cancer may be due to a natural decline in antibody titres that occurs over time in infected individuals (Andersson-Ellstrom et al., 1996; Carter et al., 2000), or may be associated with a lack of antigen expression. It is known that transcription of the L1 gene is restricted to terminally differentiated keratinocytes and that the level of cellular differentiation decreases as the grade of neoplasia increases (Stoler et al., 1992). Consequently, the expression of the L1 protein is significantly reduced in advanced lesions associated with high-risk HPV genotypes (Melsheimer et al., 2003). Furthermore, integration of HPV DNA in cervical cancer may cause disruption of the L1 gene (Walboomers & Meijer, 1997), affecting the expression of the L1 protein. Indeed, integration of viral DNA occurs more frequently in HPV-18 than in HPV-16-associated tumours. This is consistent with our observation that the prevalence of antibodies was lower in patients with HPV-18 than in patients with HPV-16-associated cervical cancer.

In conclusion, our study indicates that the 16L1 peptide might be a high-risk type-common epitope capable of inducing cross-reactive antibodies in high-risk HPV-associated LSIL patients. Importantly, antibodies in LSIL patients infected with low-risk HPV types did not react against the peptide, suggesting that the reaction was restricted to high-risk virus types. Although more extensive studies to investigate antibody responses against the 16L1 peptide are still required, our primary results suggest that the 16L1 peptide might be useful for the development of serological assays for the study of natural and vaccine-induced immune responses against high-risk HPV, as well as for the early detection of oncogenic HPV infections.

ACKNOWLEDGEMENTS

We thank Mr Ranullo Pedraza for excellent technical assistance. This study was supported by DGAPA-PAPIIT-UNAM grants IN210501 and IN215703-2 and CONACyT grant 34835-M.

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