Differential T Helper Cell Responses to Human Papillomavirus Type 16 E7 Related to Viral Clearance or Persistence in Patients with Cervical Neoplasia: A Longitudinal Study


ABSTRACT

T-cell-mediated immune responses against oncogenic human papillomaviruses (HPVs) are believed to play a role in the prevention of cervical carcinogenesis. The in vitro production of interleukin 2 by CD4+ T helper (Th) cells in response to overlapping 20-mer peptides covering the HPV-16 E7 oncoprotein sequence was determined in 72 women with cytological evidence of premalignant cervical intraepithelial neoplasia (CIN) who participated in a nonintervention follow-up (FU) study. In addition, 15 HPV-16 + cervical carcinoma patients were tested. Positive Th cell reactivity was restricted to patients infected by HPV-16 and related types and showed a strong association with viral persistence and disease progression, as evidenced by the high frequency of positive responders among women with persistent HPV-16 infections who ended FU with high-grade CIN III lesions [14 of 15 (93%)]. Women with cervical carcinoma showed responses at a significantly reduced rate [7 of 15 (47%); P = 0.014]. Over the FU period (10–34 months), the level of E7-induced interleukin 2 production from the lymphocytes of CIN patients who had cleared HPV-16 infection showed an inverse correlation with time relative to the last positive HPV DNA test, with 8 of 13 of these patients showing positive responses after clearance. By contrast, among women with persistent HPV-16 infections and developing CIN III lesions (n = 8), there was a rise in Th cell activity over the course of FU. The majority of women responded to an immunogenic region in the carboxyl terminus of the E7 protein (amino acids 67–98). The observed HPV-16 E7-specific Th cell responses may develop as a consequence of increased antigen availability resulting either from clearance or from progression of cervical lesions.

INTRODUCTION

Persistent infection with oncogenic HPV1 types, of which HPV-16 is the most prevalent, represents a major risk factor for the development of cervical cancer and may result from the inability of the immune system to raise effective T-cell-mediated antiviral immune responses (1, 2). Evidence for this can be found in studies reporting an increased prevalence of HPV-induced cervical lesions in hosts with impaired cellular immune effector functions such as renal transplant recipients and HIV-seropositive women (2). The precise targets and effector subsets responsible for such natural immunity are unknown. Much effort has focused on the E7 oncoprotein, because it is expressed in all stages of cervical disease development and thus represents a viable target for immunotherapy. Several studies have shown lymphocytes from cervical neoplasia patients to proliferate (3–6) or produce IL-2 (7) or generate cytotoxic activity (8) after specific E7 peptide stimulation. To further investigate the role of such cellular immunity in the natural defense against HPV infection, this study examines E7 peptide-induced IL-2 production by CD4+ Th lymphocytes from patients at different stages in the natural course of the disease. By using IL-2 production as a read-out, the activation of a subset of Th cells with particular importance for cellular immunity may be detected. Moreover, this assay proved to be more sensitive and less laborious than the restimulation method we previously used to detect E7-specific proliferation (5).

We tested 15 HPV-16+ patients with cervical carcinoma and 72 patients with CIN from a prospective nonintervention cohort study of patients with abnormal cervical cytology (9). This allowed for a longitudinal analysis in relation to HPV-16 infection patterns (cleared or persistent) and histologically determined disease outcome.

MATERIALS AND METHODS

Controls and Patients. Samples of umbilical cord blood mononuclear cells (n = 13) were used as immunologically naive controls. A control group of nine women from Amsterdam with HPV-16 infections but normal cervical cytology (Pap 1 or Pap 2) was also included in the study (mean age, 36.6 years; SD, 9.6 years. These donors participated in a FU study of HPV-positive women with normal cervical cytology and had no history of previous cervical lesions.

The study of patients with CIN described in this paper was nested within a larger prospective nonintervention cohort study that was designed and conducted to determine the relationship between HPV infection patterns and CIN disease course (9). Women presenting with abnormal cervical cytology (mild to moderate dyskaryosis), were referred to the gynecological outpatient clinic of the Free University Hospital in Amsterdam, the Netherlands and, after informed consent, were enrolled in this study. The study design was approved by the ethics committee of the hospital.

Clinical FU consisted of cytological and colposcopic examinations that were performed every 3–4 months. Biopsies were not taken during the FU period to avoid interfering with the natural course of the disease. Results from the cytomorphological tests were classified according to a modified Pap system as used in the Netherlands (9): (a) Pap 1, no cytomorphological abnormalities; (b) Pap 2, inflammation; (c) Pap 3a, mild to moderate dyskaryosis; (d) Pap 3b, severe dyskaryosis; (e) Pap 4, carcinoma in situ; and (f) Pap 5, (microinvasive) carcinoma. Colposcopy was used to indicate the predicted grade of dysplasia and to determine the extent of the observed lesions in cervical quadrants. At the end of the FU period, biopsies were taken, and a histological diagnosis was made (< CIN, normal or metaplastic epithelium; CIN I, 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 FU was reported by Remmink et al. (9).

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Table 1: Composition of the patient and control groups in the cross-sectional analysis

<table>
<thead>
<tr>
<th>Patients and controls</th>
<th>n</th>
<th>Group</th>
</tr>
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<tbody>
<tr>
<td>Umbilical cord control group</td>
<td>13</td>
<td>A</td>
</tr>
<tr>
<td>Normal cytology, HPV-16+</td>
<td>9</td>
<td>B</td>
</tr>
<tr>
<td>CIN, HPV-1</td>
<td>15</td>
<td>C</td>
</tr>
<tr>
<td>CIN, HPV-16 cleared</td>
<td>17</td>
<td>D</td>
</tr>
<tr>
<td>CIN, HPV-16-related infection cleared</td>
<td>4</td>
<td>E</td>
</tr>
<tr>
<td>CIN, HPV-16 persistent</td>
<td>23</td>
<td>F</td>
</tr>
<tr>
<td>CIN, HPV-16-related infection persistent</td>
<td>5</td>
<td>G</td>
</tr>
<tr>
<td>Cervical carcinoma, HPV-16+</td>
<td>15</td>
<td>H</td>
</tr>
</tbody>
</table>

*Groups were defined on the basis of disease and HPV status. Normal cytology, Pap I or 2; CIN, patients participating in cohort study, originally diagnosed with abnormal cervical cytology; cervical carcinoma, FIGO stage I-III; HPV typing by DNA PCR analysis, related HPV types included were HPV-31, HPV-33, HPV-35, HPV-52, and HPV-58.

Every 3–6 months, 40 ml of heparinized peripheral blood was drawn. Blood samples from 187 patients who consented to one or more blood donations were collected. For this study, 72 patients were selected to represent the following groups with differing HPV status.

(a) Fifteen patients who were negative for HPV during the entire FU period and for at least 12 months (mean length of time, 28.9 months; range, 14–41 months) before immunological testing constituted the HPV − group (mean age, 39.2 years; SD, 8.4 years; mean FU, 28.9 months; SD, 8.7 months).

(b) In total, 40 patients were tested with either cleared or persistent HPV-16 infections. Patients with fluctuating HPV-16 DNA positivity were excluded from this study, because it was impossible to determine whether the observed fluctuation was due to actual viral clearance and reinfection or to a persistent infection with low viral load fluctuating around the PCR detection level. We therefore decided to focus on two more clear-cut HPV-16 infection patterns, i.e., clearance or persistence.

The HPV-16 clearance group included 17 patients (mean age, 36.8 years; SD, 9.7 years; mean FU, 45.7 months; SD, 14.3 months) who were consistently found to be negative for HPV-16 DNA for at least 12 consecutive months after having been HPV-16 DNA + (mean time since the patients were last determined to be positive for HPV-16 DNA, 28.6 months; range, 14–65 months).

Disease outcome, as determined by histology, varied between CIN II and no discernable abnormalities.

The HPV-16 persistence group consisted of 23 patients (mean age, 34.9 years; SD, 8.7 years; mean FU, 36.1 months; SD, 12.5 months) who were consistently positive (in 4–11 consecutive PCR tests) for HPV-16 DNA over a period of 15–51 months before immunological testing. Fifteen of these patients ended FU with CIN III, and eight ended FU with lower-grade lesions.

(c) Patients with a current or past infection of an HPV type other than HPV-16. This group consisted of nine patients with infections of HPV-16-related types (HPV-31, HPV-33, HPV-35, HPV-52, and HPV-58) and eight patients with unrelated types (HPV-6, HPV-18, HPV-39, HPV-43, HPV-44, HPV-51, HPV-54, and HPV-66). The patients with HPV-16-related types were subdivided into patients with cleared infections (n = 4; mean age, 30.0 years; SD, 2.8 years; mean FU, 36.0 months; SD, 7.6 months) and patients with persistent infections (n = 5; mean age, 33.4 years; SD, 6.7 years; mean FU, 33.6 months; SD, 13.7 months). Because these groups behaved similarly to the respective HPV-16 cleared and persistent groups, both in clinical and in immunological terms, they were combined with these groups in the final analysis of the data. The patients with HPV-16-unrelated types had a mean age of 31.8 years (SD, 6.5 years) and a mean FU of 31.8 months (SD, 8.5 months).

A total of 15 patients with invasive cervical carcinoma who were PCR positive for HPV-16 DNA and staged according to recommendations made by the FIGO were also included in the study (mean age, 49.4 years; SD, 14.6 years). Eight of these patients visited the Free University Hospital in Amsterdam, the Netherlands (all were diagnosed with FIGO stage I cervical carcinoma), and seven attended the Christie Hospital National Health Service Trust (Manchester, United Kingdom one with FIGO stage I cervical carcinoma, four with stage II carcinoma, and three with stage III carcinoma). Blood samples were collected immediately before treatment.

The composition of the groups and codes (A–F) that were used in the analysis of the data are summarized in Table 1.

Table 2: HPV-16 E7 synthetic peptides: sequences and subpools

<table>
<thead>
<tr>
<th>Subpool</th>
<th>Peptide</th>
<th>no.</th>
<th>aa</th>
<th>aa sequence</th>
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</thead>
<tbody>
<tr>
<td>sp1 (aa 1–32)</td>
<td>1</td>
<td>1–20</td>
<td>MGIDTPTLTHEMLQLQPE1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7–26</td>
<td>TLHEMLQLQPTDSDLVCY8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13–32</td>
<td>LDQPGPDLELCVYQDL8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>19–38</td>
<td>TTDLVCYQIGASQEDDE1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>25–44</td>
<td>QQALDDEEPLTPQ11</td>
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<td></td>
<td>6</td>
<td>31–50</td>
<td>SSEEEDIEQPAPEQIPRA1</td>
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<td>7</td>
<td>37–56</td>
<td>EIDQPAPEQ IPA NAHY11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>43–62</td>
<td>GQAEFHRAVQTVFTCP2C</td>
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<tr>
<td>sp2 (aa 43–80)</td>
<td>9</td>
<td>49–68</td>
<td>RQHIN1VTPFCXCDGL1</td>
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<tr>
<td></td>
<td>10</td>
<td>55–74</td>
<td>VTFCCDKCSSDLTLCQG8</td>
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<td>11</td>
<td>61–80</td>
<td>CDSRLTLCYQSTVDIRL8</td>
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<tr>
<td>spv (aa 67–98)</td>
<td>12</td>
<td>67–86</td>
<td>LCQVSTWIFITQDLMOT10</td>
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<td>13</td>
<td>73–92</td>
<td>HVDQFTLEDLMLG11</td>
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<td>14</td>
<td>79–98</td>
<td>L6DLGMGTLG11VFC15SQK</td>
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</table>

*Numbersed from the amino terminus to the carboxyl terminus.
sorting magnetic microbeads (Miltenyi Biotec). All antibodies were dialyzed against PBS before use to remove Na3SO4, CD4 and CD8 fluorescence-activated cell-sorting analyses were performed after double staining with FITC and phycoerythrin-conjugated antibodies (Simulset; Becton Dickinson) to verify the depletion of the respective subsets.

**IL-2 Bioassay.** IL-2 production in the culture supernatants was measured in a bioassay with the IL-2-dependent cell line HT2 (14). HT2 cells (1 × 10^4 cells well) were cultured for 24 h in Iscove’s modified Dulbecco’s medium supplemented with 50 units/ml penicillin-streptomycin, 1.6 mm L-glutamine, 0.01 mm β-mercaptoethanol, and 10% FCS, with PBMC culture supernatants at final dilutions of 1:2, 1:4, and 1:8. Triplicate wells were set up per test condition and supernatant dilution. During the last 4 h, the cells were incubated with (³H)thymidine (0.4 μCi/well; Amersham, Aylesbury, United Kingdom). The cells were harvested onto fiberglass filters, and (³H)thymidine incorporation was determined using a flatbottom liquid scintillation counter (Wallac, Turku, Finland). IL-2 titration curves were included in each assay (100, 50, 25, ..., 0.375, 0 IU/ml IL-2 (Cetus, Amsterdam, the Netherlands)). Counts in the E7 test wells never exceeded the linear range of the titration curves (usually between 12.5 and 0.375 IU/ml). Samples were considered positive when the mean HT2 proliferation (in cpm) in the test wells exceeded proliferation in the medium control wells by a factor of two (SIₑT₂ > 2) for at least two of the tested culture supernatant dilutions, with a difference of at least 500 cpm between the means of the sets of triplicate test and medium control wells.

**Statistical Analysis.** Frequencies of positive IL-2 responders between the different groups were compared using a 2 × 2 table analysis and Fisher’s exact test. Comparisons between sets of SIₑT₂ values were carried out using the Mann-Whitney U test. Trends in disease development were tested using 2 × 4 table analyses and the χ² test for trend. Differences and trends were considered significant when P < 0.05.

**RESULTS**

**HPV-16 E7-specific IL-2 Production by Th Cells in Relation to HPV Infection Patterns and Cervical Disease Outcome: Cross-Sectional Analysis.** IL-2 production in response to HPV-16 E7 peptides was related to cervical disease outcome and HPV status in the CIN patients in a cross-sectional analysis at the end of FU and was compared to IL-2 reactivities from HPV-16+ women with normal cervical cytology or cervical carcinoma. The different patient and control groups are listed in Table 1. Because comparable levels of IL-2 responsiveness were found to be associated with HPV persistence and clearance for CIN patients with either HPV-16 or HPV-16-related infections (data not shown), these groups were combined (Table 1). This was not the case for patients with HPV-16-unrelated infections, who showed significantly lower E7-specific IL-2 reactivities than patients with HPV-16-related infections (P = 0.0498; data not shown) and were excluded from further analysis.

All PBMC samples tested showed strong IL-2 production in response to PHA. Fig. 1 shows the IL-2 production by lymphocytes from the different test groups after stimulation with a pool of E7 peptides. The frequency of positive responders is also given for each group. It can be seen that lymphocytes from the umbilical blood, presumably unprimed by HPV (Fig. 1A), women with HPV-16 infections but without CIN (Fig. 1B), and women with no detectable HPV but with a recent history of CIN (Fig. 1C) show no significant IL-2 responses. Fig. 1D shows the responses of CIN patients who have cleared a HPV-16 or HPV-16-related infection; within a range of 14–65 months after clearance, there is hardly any evidence of IL-2 production. This contrasts markedly with the CIN patients with persistent HPV-16 or HPV-16-related infections (Fig. 1E) and the HPV-16+ carcinoma patients (Fig. 1F), who show a high frequency of IL-2 production after E7 peptide stimulation. Three CIN patients with different HPV-16 infection patterns and disease outcomes were further investigated to establish that the assay used reflected Th cell activity. In all cases, E7 peptide-induced IL-2 production was inhibited by a monoclonal antibody to HLA class II molecules or the removal of CD4+ cells from PBMCs by magnetic microbeads but was unaffected by the removal of CD8+ cells (data not shown).

Table 3 summarizes the statistical analysis of both reactivity and responder rates for the different groups. The absence of any responses in the umbilical cord control group (A) strongly suggests that the HPV-16 E7-specific IL-2 production detected in the other test groups (D–F) stems from memory Th cells. The observed Th cell reactivities seem to result from HPV exposure, because responder frequencies were significantly higher among women with either cleared or persistent HPV-16 or HPV-16-related infections (26 of 49) as compared to women who had remained HPV− during FU (0 of 15; P = 0.0002). However, a significant difference in responder frequencies between HPV-16+ women with normal cervical cytology (0 of 9) and HPV-16 or HPV-16-related positive CIN patients (26 of 49; P = 0.0029) suggests that besides exposure to the virus, the generation of HPV-16 E7-specific IL-2-producing memory Th cells requires the presence of cervical dysplasia. Moreover, the patients with CIN and persistent HPV-16 or HPV-16-related infections showed significantly higher reactivity and responder rates than patients with cleared infections.
codes for the different groups, as defined in Table 1, are given in parentheses. CIN 16, QN patients with cleared (L6C) or persistent (L6P) HPV-16 or HPV-16-related (HPV-31, HPV-33, HPV-35, HPV-52, and HPV-58) infections; UN neg, patients with a difference of at least 500 rpm between the means of the sets of triplicate test and medium because the responder frequency in the HPV-16+ cervical cancer HT2 stimulation indices exceeding 2 in two different culture supernatant dilutions with a with HPV-16+ CIN ifi lesions (14 of 15; 93.3%; P < 0.014).

To identify different immunogenic regions within the E7 protein and establish any possible correlations to disease development, IL-2 production in response to four sets of E7 synthetic peptides (spl-IV; see Table 2) was determined in 28 HPV-16 DNA+ women with different cervical disease outcomes who had previously shown positive Th cell reactivity against the total E7 peptide pool and who were typed for HLA-DR and HLA-DQ alleles. Eight of the 28 tested women recognized the amino-terminal region aa 1—32 (spl), and 5 and 10 of 28 women recognized the peptide sets splII (aa 19—56) and splIII (aa 43—80), respectively. The most commonly recognized peptide set was splIV (17 of 28 positive responders), covering the carboxy-terminal region of E7 (aa 67—98). This carboxyl-terminal region was more often recognized among women with cleared infections (6 of 8, 75.0%) than among women with viral persistence (10 of 17, 58.8%) and among women with low-grade CIN I lesions or normal cervical histology (10 of 13, 76.9%) than among women with CIN III and cervical carcinoma patients (7 of 15, 46.7%). However, these differences did not reach statistical significance. Nor were any associations found between particular HLA-DR or HLA-DQ alleles and the recognition of specific sets of E7 peptides (data not shown).

(3). Responses seem to decline in patients with cervical cancer, because the responder frequency in the HPV-16+ cervical cancer group (7 of 15; 46.7%) was significantly lower than that in women with HPV-16+ CIN III lesions (14 of 15; 93.3%; P = 0.014).

When the responses found in CIN patients with current or past infections of HPV-16 or HPV-16-related infections (n = 49) were related to cytopathological and histopathological status at the end of FU, an association with a more severe disease status became apparent (Fig. 2). Significant trends of rising responder frequencies with higher cytological classification (Fig. 2a, P < 0.01) and increasing histological grades of dysplasia (Fig. 2b, P < 0.001) were observed.

HPV-16 E7-specific Th Cell Reactivity Over Time in CIN Patients with Persistent or Cleared HPV-16 Infections: Longitudinal Analysis. Of the 40 HPV-16+ patients included in this analysis, 27 were available for longitudinal studies at two to five time points per patient. HPV-16 E7 Th cell reactivity was assessed in relation to HPV-16 infection and disease course in 13 CIN patients with cleared HPV-16 infections (mean Th FU, 21.8 months; SD, 5.9 months) and in 14 CIN patients with persistent HPV-16 infections (mean Th FU, 20.5 months; SD, 7.1 months). Start of FU of the patients with viral clearance ranged from 4 months before to 34 months after the last HPV-16 DNA+ test (median, 4.5 months after the last HPV-16 DNA+ test) and ended at biopsy showing resolved lesions (n = 4), CIN I (n = 7), or CIN II lesions (n = 2). Whereas 8 of 13 (61.5%) patients were initially responsive to E7, only 2 of 13 (15.4%) still showed responses at the end of FU, which is similar to the responder frequency found in the larger cross-sectional analysis at that time (14.3%). When all FU data of the positively responding clearance patients were combined and shown in temporal order relative to the time of biopsy (end of FU), a significant downward trend in E7-specific IL-2 production was revealed by linear regression analysis (Fig. 3a, r = −0.54; P = 0.002). This downward trend was observed on an individual basis for all patients included in the analysis. By contrast, 4 of 14 HPV-16 persistent patients were initially responsive to E7, and 11 of 14 were positive by the end of FU (4 of 6 CIN I or II and 7 of 8 CIN III). Linear regression analysis of the Th cell reactivities from the positively responding women who developed CIN III lesions revealed a significant increase over time (Fig. 3b, r = 0.44; P = 0.035). This increase was observed for all individual patients included in this analysis. Five HPV—CIN patients were also tested over a mean FU period of 19 months (SD, 6.2 months). Four of these patients remained negative for E7-specific IL-2 production throughout FU, whereas one showed a single positive response.
Th RESPONSES TO HPV-16 E7 IN RELATION TO CERVICAL NEOPLASIA

DISCUSSION

We have determined HPV-16 E7-induced IL-2 production by CD4+ T cells in a nonintervention cohort study of CIN patients. This study design allowed us to relate the observed responses to the natural CIN disease course over time. In a cross-sectional analysis at the end ofFU, we found an association of Th cell IL-2 production with viral persistence and high-grade premalignant cervical lesions. These findings are in keeping with data from a previous study in which we determined HPV-16 E7-specific T-cell proliferation (5).

The interpretation of these results is made difficult by the inability to accurately determine the time of HPV infection and the level of E7 expression, which may determine the generation and longevity of anti-E7 Th cell activity. In general, T-cell responses have been shown to lag behind viral infections, reaching peak frequencies in peripheral blood when the viral load is decreasing (15). In this study, the level of E7-induced IL-2 production from the lymphocytes of patients who had cleared HPV-16 infection correlated inversely with the time relative to the last HPV DNA+ test. Thus, in a period of 10–34 months preceding the end of FU, 61.5% of these patients showed a positive IL-2 response, with higher responses found around the time of clearance and with a decline afterward. These results are consistent with our previous hypothesis that HPV-16 E7-specific IgG2 reactivity observed in patients with HPV-16 clearance might result from E7-specific Th1-like responses around the time of viral clearance (16).

Moreover, they raise the possibility of an involvement of HPV-16 E7-specific Th cells in viral clearance and disease regression. Alternatively, such immunity may not contribute directly to the resolution of cervical lesions but may result from other factors or events critical to the natural history of cervical neoplasia.

The association of increased Th cell activity with persistent HPV infections and the development of high-grade CIN III lesions and carcinomas may correlate with levels of E7 production sufficient for immune activation (17–19). Papillomaviruses replicate in the suprabasal layers and are shed in the relatively remote superficial layers of the squamous epithelium. This may hinder contact between HPV-derived antigens and the afferent arm of the immune system (15) and would explain why many of the tested patients showed evidence of HPV infection and HPV-induced disease for years before E7-specific T-cell responses became detectable. Indeed, antigen load may be a decisive factor in the generation of E7-specific Th cell responses. In a murine model, grafts of E7-transfected keratinocytes on skin led to priming of E7-specific T cells that were detectable after challenge in a delayed-type hypersensitivity reaction (20). The strength of these delayed-type hypersensitivity responses was found to correlate with the number of E7 expressing keratinocytes that were grafted. Thus, the observed cellular immunity in patients with resolving or progressing lesions may be reconciled by the common factor of antigen concentration reaching a threshold, either as part of the natural resolution of the disease or its progression.

In apparent contrast with this study, Kadish et al. (6) recently reported that T-cell proliferation to HPV-16 E6 and E7 peptides is predictive for the subsequent clearance of HPV DNA. However, the results can be reconciled between the studies by considering the nature of the patient cohort and the timing of the testing in the cervical disease course. The patients described in our study were not biopsied until the end of clinical FU, at which point a considerable number with HPV-16 persistence were diagnosed with CIN III. Because such patients show increasing Th cell responses against E7 toward the end of FU, and the opposite is the case for patients with HPV clearance, it is not surprising that we found an association in the cross-sectional analysis with HPV persistence. In contrast, patients from the study by Kadish et al. (6) were biopsied before testing and were mostly found to have milder CIN II lesions. It is therefore possible that E7-specific T-cell responses in these patients were generated as a consequence of taking the biopsy and had an adjuvant effect on maintaining viral clearance. These responses may be comparable to the responses we observed in the present study in patients around the time of viral clearance. In both instances, the detected responses may be a consequence rather than the cause of HPV and disease clearance, although in our study, the viral clearance would be due to natural processes rather than therapeutic or diagnostic intervention.

An association of HPV-16 E7-specific T-cell responses with HPV-16+ high-grade CIN lesions and a reduction of these responses in cervical cancer patients has been reported by Luxton et al. (4). This study also shows a significantly reduced frequency of E7-specific IL-2 responses in patients with cervical carcinoma as compared with those in women with CIN III. This effect may be a reflection of a generalized immunosuppressed state that is often observed in cancer patients, although the IL-2 production in response to PHA stimulation observed in this group was comparable to responses found in the other test groups. In contrast to our results, Tsukui et al. (7) observed a decrease in the frequency of IL-2 responses to HPV-16 E7 peptides with increasing severity of premalignant cervical lesions. It is conceivable that the patients with high-grade premalignant lesions described in the study of Tsukui et al. (7) had not yet developed E7-specific T-cell responses but would have done so, had they been further tested as in our longitudinal analysis. Alternatively, by the use of smaller overlapping peptides of 15 aa (7), longer Th epitopes may have been missed.
A differential Th cell responsiveness to HPV-derived antigens could correlate with certain HLA-DR and HLA-DQ alleles and haplotypes previously associated with susceptibility to HPV-16-related CIN and cervical carcinoma (21–23). However, this was not found for the E7-induced IL-2 production in our HPV-16+ CIN patients, nor were there any associations of HLA-DR or HLA-DQ alleles with the recognition of particular regions within the E7 protein sequence.

We did not have sufficient material to perform large-scale single-peptide analyses to pinpoint the reactive epitopes, but preliminary data from four patients specifically recognizing spl, II, III, or IV showed the following peptides to contain Th epitopes: aa 1–20; aa 19–38; aa 37–56; aa 49–68; and aa 67–86 (data not shown; for sequences see Table 2). Four of these regions (aa 1–20, aa 19–38, aa 37–56, and aa 67–86) overlap almost completely with sequences that were previously reported to induce E7-specific T-cell proliferation (3, 4, 24), whereas aa 37–56 overlaps considerably with a peptide to which responses were observed by Luxton et al. (aa 45–64; Ref. 4). Moreover, the immunodominant region in the carboxyl terminus (aa 67–98) to which a majority of patients infected by HPV-16 showed responses was also identified as such by three previous independent studies (3, 4, 7). This region was recognized at some time by 77% of the E7-reactive patients who ended FU with CIN I or completely resolved lesions (< CIN) and by 75% of the E7-reactive women who cleared HPV-16. This is in keeping with results reported by Kadish et al. (6), who found that the presence of proliferative T-cell responses against this region was predictive for the subsequent clearance of HPV and CIN. However, if these responses reflect an effective natural immunity to HPV-16, then they do not seem to be sufficient in patients with progressive lesions.

Whether the induction of E7-specific T-cell immunity by vaccination in CIN patients may prevent long-term viral persistence and the development of high-grade lesions (CIN III) remains to be determined. Animal vaccination studies have shown that E7 can function as a tumor rejection antigen (2, 25), and the first E7 vaccination trial in humans reported E7-specific CTL responses in a cervical carcinoma patient (26). A potential role for Th cells in immunity to HPV-16 E7-expressing tumors is evidenced by the enhanced protection afforded by vaccination of mice with an E7-LAMP-1 vaccinia virus construct compared to a vaccinia virus containing just the E7 gene (27). The resulting E7-LAMP-1 chimeric protein was targeted to the lysosomal and endosomal compartments in the MHC class II antigen processing pathway and the resulting E7-specific Th cell responses led to the enhancement of specific CTL and antibody responses (28). However, this study shows an association of E7-specific Th responses with malignant and potentially malignant cervical lesions, which suggests that such responses are no guarantee for natural immunity. This does not rule out a potential therapeutic influence of these responses for the resolution of cervical lesions associated with HPV-16, but they are unlikely to be sufficient. Any immunization would need to consider the additional (local) factors that might frustrate the generation of successful T-cell and possibly other types of immunity. These factors may include immunosuppressive cytokine expression (29), MHC class I down-regulation on neoplastic keratinocytes (30, 31), and CD3 γ-chain down-regulation (32) and may have to be remedied for vaccination to be effective.

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