Cell-mediated immune responses to E7 peptides of human papillomavirus (HPV) type 16 are dependent on the HPV type infecting the cervix whereas serological reactivity is not type-specific

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Forty-two women attending a colposcopy clinic for evaluation of abnormal cervical cytology and 13 normal controls were studied for the presence of lymphocyte proliferation (LP) cell-mediated immune (CMI) responses and serological reactivity to E7 peptides of human papillomavirus type 16 (HPV-16). HPV was typed by Southern blot hybridization of exfoliated cervicovaginal cell DNA. Positive LP responses (stimulation index > 5.0) to one or more E7 peptides were observed in 28.6% (12 of 42) of patients and 23.1% (three of 13) of controls. Of patients infected with HPV-16, -31 or -33, 63.6% (seven of 11) showed a positive LP response compared with 14.3% (two of 14) of women infected with other HPVs (P = 0.04), 5.9% (one of 17) of women negative for HPV (P = 0.02) and 7.7% (one of 13) of controls (P = 0.05). HPV-16 group-specific LP responses of borderline significance were also observed against E7 peptides 103, 105 and 108 (17-37, 37-54 and 62-80) (P = 0.07). ELISA reactivity (IgG) to E7 peptide 109 (72-97) was present in 7.7% (one of 13) of controls, 35.3% (six of 17) of HPV-negative patients, 42.9% (six of 14) of patients infected with other HPVs, and only 9.1% (one of 11) of patients infected with HPV-16, -31 or -33. CMI responses to C-terminal HPV-16 E7 peptide 109 (72-97) were thus significantly related to ongoing cervical infection with HPV-16 and closely related types, whereas serological reactivity to E7 peptides was not HPV type-specific.

Introduction

Human papillomavirus (HPV) infection has been shown to be the major risk factor for the development of both premalignant disease and cancer of the uterine cervix (Morrison et al., 1991; Koutsky et al., 1992; Reeves et al., 1989). Of the HPV types known to infect the genital tract, of which there are more than 20, several types including HPV-16, -33 and 18 have been associated with the development of high-grade precancerous disease and cancer (Kadish et al., 1992; Lorincz et al., 1992). Of women with squamous intraepithelial lesions (SILs), a large proportion undergo regression, whereas others have persistent disease and a small number progress to invasive carcinoma (Schneider & Koutsky, 1992). Epidemiological studies demonstrating increased prevalence and severity of HPV-induced lesions in immunosuppressed patients (Vermund et al., 1991; Kiviat et al., 1990; Ho et al., 1994), in addition to histological studies of regressing genital HPV-induced lesions (Rogoziński et al., 1988) suggest that immune responses to HPV are critical in mediating the regression of disease.

Studies of immune responses to HPV have been hampered by the difficulty in obtaining sufficient quantities of native HPV antigens. Bacterially expressed fusion proteins and synthetic peptides corresponding to the products of HPV open reading frames (ORFs) have been used to demonstrate cell-mediated immune (CMI) and humoral immune responses to HPV-encoded antigens in both human and murine systems (Dillner, 1990; Müller et al., 1990; Jenison et al., 1991; Jochmus-Kudielka et al., 1989; Tindle et al., 1990, 1991). The HPV-16-transforming proteins E6 and E7, which are abundantly expressed in precancerous and malignant cervical lesions (Stoler et al., 1992), have been shown to be tumour rejection antigens in murine systems (Chen et al., 1991, 1992). Human serological responses to HPV-16 E6 and E7 proteins have been reported to occur most
frequently in women with cervical cancer, with only partial HPV type specificity (Jochmus-Kudielka et al., 1989; Krchňák et al., 1990; Müller et al., 1992; Mandelson et al., 1992). Several human T cell epitopes have been identified in the HPV-16 E6 and E7 proteins (Altmann et al., 1992; Strang et al., 1990), yet little is known about the clinical relevance and type specificity of cellular immune responses to these proteins in women with ongoing genital HPV infection.

To study human CMI responses to the HPV-16 E7 protein, we have performed lymphocyte proliferation (LP) assays using as antigens overlapping peptides representing the entire E7 ORF. We present here evidence that LP responses to HPV-16 E7 peptides in women with ongoing genital HPV infection and cervical SILs were dependent on genital infection with HPV-16 and closely related types, whereas serological reactivity (detected by ELISA) to the same peptides was not HPV type-specific.

Methods

Patients. Following hospital institutional review board approval, blood samples were obtained from 42 women aged 17 to 53 attending a municipal hospital colposcopy clinic for a history of recent abnormal Papanicolaou smears. Forty women had abnormal colposcopic findings and underwent cervical biopsy for diagnosis. Biopsy diagnoses included cervical intraepithelial neoplasia grade 1 (CIN I) (n = 6), CIN II (n = 21), CIN III (n = 1) and biopsy negative for CIN (n = 12). Controls included 13 female laboratory workers aged 23 to 55 with no known history of cervical disease. The HPV status of these donors was unknown.

Detection of HPV. DNA was purified from exfoliated cervicovaginal cells (CVCs) obtained by cervicovaginal lavage (Burk et al., 1986). HPV DNA was identified and typed in coded specimens by Southern blot hybridization using a mixed probe containing radiolabelled DNAs of HPV-11, -16 and -18 as previously reported (Morrison et al., 1991). Nine patients were positive for HPV-16 (21.4%), three for HPV-18 (7.1%), one each (2.4%) for HPV-6, -31, -33, -42 and -56, seven (40.4%) were negative for HPV.

Peptides. Overlapping synthetic peptides covering the entire HPV-16 E7 ORF were used as antigen (Fig. 1). Peptides were synthesized using Fmoc chemistry on an Applied Biosystems 431A synthesizer as previously reported (Tindle et al., 1990, 1991). The amino acid composition, toxicity and mitogenicity of all peptides were checked prior to use.

LP assay. Peripheral blood leucocytes (PBLs) were obtained by venipuncture and Ficol-Paque sedimentation (Pharmacia). PBLs were cultured in RPMI supplemented with 6% human AB serum in round-bottomed 5 ml capped tubes at 1 x 10^6 PBL/ml. As a control for T cell reactivity, cells were also cultured with concanavalin A (Con A; Sigma) for 3 days and with Candida antigen (Dermatophyton O; Holliston-Stier) for 5 to 6 days. In preliminary experiments using HPV-16 E7 peptides, standard 5 to 6 day LP assays done in microtiter plates yielded low level and frequently inconclusive results (data not shown). To obviate this difficulty, 3-week 'bulk' cultures were done (Strang et al., 1990). PBLs were cultured with E7 peptides (10 to 15 µg/ml) and with medium alone for 1 week. Fresh peptides as well as irradiated autologous cryopreserved antigen-presenting cells were added weekly. Recombinant human interleukin-2 (15 µg/ml) (Boehringer Mannheim) was added on days 14 and 21. [3H]Thymidine (1 gCi) was added to 100 µl aliquots of the bulk cultures in flat-bottomed 96-well microtitre plates for 8 to 16 h at 14 and 21 days. Cultures were harvested and counted in a scintillation counter. Stimulation indices (S.I.) were calculated as c.p.m. in the presence of antigen/c.p.m. with medium alone. A positive response was defined as an S.I. > 5.0.

Serological assays. Peptides were dissolved in carbonate–bicarbonate buffer (pH 9.5) for coating of Probind plates (Falcon, Becton Dickinson) as previously reported (Beiss et al., 1991). Peptides were added at 1 µg per well, incubated overnight at 37°C and plates were blocked overnight with 5% non-fat dry milk. Test sera were then added to plates (100-fold dilution) and incubated for 1 h at room temperature. Peroxidase-conjugated anti-human IgG or IgA (Sigma) was added (1 h), followed by a 30 min incubation with ABTS substrate [2,2'-azino-di-(3-ethylbenzthiazoline sulphonate); Kierkegaard & Perry]. Plates were read in an ELISA reader at 405 nm. A405 read with buffer alone was subtracted from A405 read with peptides. Adequate binding of all peptides to plates was confirmed using specific peptide antisera (data not shown). As negative controls, sera from women with histories of negative Papanicolaou smears and no HPV detectable by Southern blotting were included in each ELISA experiment. A test serum was considered positive when the A405 was 3 S.D. above the mean for negative controls. High and low positive sera from patients with cervical neoplasia and HPV-16 infection were included in each experiment as positive controls for peptide 109. Experiments in which the low positive serum failed to give a positive result were not included. Western blot assays using the cII-E7 fusion protein were performed as reported by Jochmus-Kudielka et al. (1989).

Results

Cell-mediated immunity to E7 peptides

Of 55 blood donors tested for LP responses, 50 (90.9%) had positive proliferative responses to Con A or to Candida antigen. Fifteen subjects (27.3%) responded to one or more E7 peptides (S.I. > 5.0). Of the positive responses, three (20%) were detected by day 14 of culture and the rest by day 21. Of 42 colposcopy clinic patients with recent histories of abnormal Papanicolaou smears, 12 (28.6%) responded to one or more E7 peptides, compared to three of 13 (23.1%) normal controls (difference not significant, NS). Of the 15 responders, three (20%) were controls (HPV status...
Human immune responses to HPV-16 E7 peptides

**Fig. 2.** LP responses to HPV-16 E7 peptides. Proliferative CMI responses to HPV-16 E7 peptides among colposcopy clinic patients infected with HPV-16 (b, c and e), HPV-31 (a), HPV-33 (d) and HPV-18 (f). A positive response is defined as an S.I. > 5.0.

**Table 1. CMI reactivity to HPV-16 E7 peptides***

<table>
<thead>
<tr>
<th></th>
<th>Any positive*</th>
<th>Peptide 103</th>
<th>Peptide 105</th>
<th>Peptide 108</th>
<th>Peptide 109</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls†</td>
<td>3/13 (23.1)</td>
<td>1/13 (7.7)</td>
<td>1/13 (7.7)</td>
<td>2/13 (15.4)</td>
<td>1/13 (7.7)</td>
</tr>
<tr>
<td>Colposcopy clinic‡</td>
<td>12/42 (28.6)</td>
<td>5/42 (11.9)</td>
<td>3/42 (9.1)</td>
<td>4/42 (9.5)</td>
<td>7/42 (16.7)</td>
</tr>
<tr>
<td>HPV-16</td>
<td>5/9 (55.6)</td>
<td>2/9 (22.2)</td>
<td>2/9 (22.2)</td>
<td>2/9 (22.2)</td>
<td>3/9 (33.3)</td>
</tr>
<tr>
<td>HPV-16, -31, -33§</td>
<td>7/11 (63.6)</td>
<td>3/11 (27.3)</td>
<td>3/11 (27.3)</td>
<td>3/11 (27.3)</td>
<td>5/11 (45.5)</td>
</tr>
<tr>
<td>Other HPV¶</td>
<td>2/14 (14.3)</td>
<td>0/14 (0.0)</td>
<td>0/14 (0.0)</td>
<td>0/14 (0.0)</td>
<td>1/14 (7.1)</td>
</tr>
<tr>
<td>HPV-negative</td>
<td>3/17 (17.6)</td>
<td>2/17 (11.8)</td>
<td>0/17 (0.0)</td>
<td>1/17 (5.9)</td>
<td>1/17 (5.9)</td>
</tr>
</tbody>
</table>

* S.I. > 5.0 in LP assay with one or more HPV-16 E7 peptides (percentage positive shown in parentheses).
† Women with no history of cervical disease. HPV status not known.
‡ All colposcopy clinic patients with recent histories of abnormal Papanicolaou smears.
§ Patients with HPV-16 (n = 9), -31 and -33 (one each).
¶ Patients with HPV types other than 16, 31 and 33.

unknown) and 12 (80.0%) were colposcopy clinic patients. Five of the responders (33.3%) were infected with HPV-16, one was infected with HPV-31, one with HPV-33, one with HPV-18, one with HPV-42, and no HPV was identified for three of them. Representative LP assays from six responders are shown in Fig. 2.

When LP assay results were correlated with HPV DNA type identified in CVCs of individual subjects, it became clear that there was a relationship between HPV type and CMI reactivity (Table 1). Of nine patients infected with HPV-16, five (55.6%) responded to one or more E7 peptides, compared to three of 17 (17.6%) HPV-negative patients (P = 0.06), four of 16 (25%) infected with HPV types other than 16 (P = 0.08) and three of 13 (23.1%) controls (NS). When patients infected with HPV-16 and closely related types (HPV-31
Serological reactivity to HPV-16 E7 peptides

Sera from all 55 subjects were tested for humoral immune responses to the HPV-16 E7 peptides. The most frequently reactive peptide in ELISA was peptide 109 (72–97), as it was for LP assays (Fig. 3, 4). Although IgA reactivity was occasionally seen, virtually all positive responses were seen when testing for IgG. Thirteen of 42 colposcopy clinic patients (31.0%) demonstrated positive IgG reactivity to peptide 109 in ELISA compared to one of 13 (7.7%) control subjects (P = 0.09). Six of 14 patients infected with HPV types other than 16, 31 or 33 (42.9%), and six of 17 patients with no HPV DNA identified (35.3%) responded to peptide 109, compared with only one of 11 patients infected with HPV-16, -31 or -33 (9.1%) (P = 0.07).

Of six subjects with high positive ELISA reactivity to peptide 109 (A405 > 0.700), two showed positive Western blot reactivity to the HPV-16 E7 fusion protein (cII-E7; kindly provided by Dr I. Jochmus-Kudielka, German Cancer Research Centre, Heidelberg, Germany). Reactive bands were eliminated by absorption with E7, but not with the E4 fusion protein. In one case, competition with excess peptide 109, but not with other peptides, removed the E7 band (data not shown). No positive Western blot reactivity to the E7 fusion protein was found among 10 subjects negative for ELISA reactivity to E7 peptides.

Occasional low positive ELISA responses (A405 < 0.100) were observed against peptides 101 to 105 in the N-terminal half of E7 (data not shown). These
low-level responses were seen with approximately equal frequency between controls and colposcopy clinic patients and did not show an HPV type-specific or disease-specific pattern.

There was no correlation between ELISA and CMI reactivity to E7 peptides. In no case was the same donor responsive to any peptide in both LP assays and ELISA. One HPV-16-infected patient (SPV 95) gave a positive response to peptide 109 in ELISA, and a positive LP response to peptides 104 and 105 (Fig. 2e). All other subjects with positive LP responses to HPV-16 E7 peptides were negative to all peptides by ELISA.

**Discussion**

Human serological immune responses to HPV have been documented in several studies, yet the clinical significance and HPV type specificity of these responses is unclear (Dillner, 1990; Krchňák et al., 1990; Müller et al., 1990). Studies of T cell responses in patients with well-characterized genital HPV-induced disease have not been reported, so that the correlation between cell mediated immunity to specific HPV antigens, clinical and HPV status is as yet unknown. To address this issue, we studied cellular and humoral immune responses to HPV in women with a history of recent abnormal cervical cytology attending a municipal hospital colposcopy clinic. LP CMI responses probably mediated by CD4+ T cells and serological responses (determined by ELISA) to overlapping HPV-16 E7 peptides were studied. HPV genomes were detected by Southern blot hybridization of DNA from CVCs which is indicative of a relatively high DNA copy number consistent with active or recent virus replication (Morrison et al., 1991).

We have demonstrated HPV group-specific CMI responses to HPV-16 E7 peptides in women with active HPV infection and cervical disease. Accurate HPV typing was critical for demonstration of type-specific immune reactivity. When HPV types identified in CVCs were analysed, positive LP responses to E7 peptides (S.I. > 50) were significantly more common among patients infected with HPV-16 and the closely related types 31 and 33 than among control subjects (HPV status unknown), patients infected with other HPV types, and patients in whom no HPV was detected. Although it is possible that we have identified primary in vitro sensitization of human T lymphocytes to specific peptide epitopes, the observed correlation with HPV type-specific genital infection makes this unlikely. It is more likely that low numbers of circulating memory T cells reactive to HPV antigens in the blood of women with active HPV infection were amplified in our long-term culture system.

Several HPV-16 E7 peptides reactive in LP assays were identified. C-terminal peptide 109 (72-97) was the most reactive and elicited type-specific LP responses in 45.5% of patients (five of 11) infected with HPV-16, -31 or -33, compared with only 6.4% (two of 31) of colposcopy clinic patients infected with other HPV types or negative for HPV and 7.7% (one of 13) of control subjects (HPV status unknown). Other E7 peptides that were recognized more often by PBLs from women infected with HPV-16, -31 or -33 than by other subjects included peptides 103 (17-37), 105 (37-54) and 108 (62-80), but the type specificity of these responses did not reach statistical significance in this small group of patients (Table 1). The exact sequences of the HPV-16 E7 epitopes observed are as yet unknown and it is possible that some of the peptides used (18 to 26 amino acids in length) may contain more than one T cell epitope. Specific epitope mapping as well as HLA-DR restriction analysis must await T cell cloning studies which have been difficult to accomplish while dealing with an inner city municipal hospital population such as ours, where getting patients back for repeat studies was difficult.

The majority of responders to E7 peptide 109 in LP experiments were infected with HPV-16, -31, or -33 which show extensive similarity (> 70%) in their C-terminal sequences (Table 2). HPV-16 was the single most common genotype identified in this group of patients, as it was in our previous studies of colposcopy clinic patients in whom infection with HPV-16, -31 or -33 was found for 31% of women with HPV identified in CVCs (Kadish et al., 1992). Whether the apparent HPV-16 group specificity observed for LP responses to E7 peptide 109 was due to the high prevalence of HPV-16 infection in our population, or to the higher prevalence of high-grade SILs among HPV-16 group-induced lesions, with increased exposure of immunocytes to E7 epitopes in the cervical mucosa, or is truly HPV-16 type-specific, must await more extensive studies.

Only a few studies of human CMI responses to HPV-antigens have been reported. LP responses to HPV-16 E4 and E6 fusion proteins have been described in patients with CIN and in normal controls, with no significant differences between groups (Cubie et al., 1989). HPV-16 E6 and L1 synthetic peptides, selected by using an algorithm predicting T cell epitopes, were successfully used to detect LP responses in PBLs from normal individuals (Strang et al., 1990). HLA-DR restriction was confirmed, and HPV-16 type specificity was demonstrated for two of the five epitopes tested. Altmann et al. (1992) described LP responses to HPV-16 E7 peptides in human donors known to be seropositive to HPV-16 E7 fusion proteins in Western blots. The LP responses reported in these donors with no known history of HPV-induced disease were similar to the LP responses of women actively infected with HPV observed in this study. Both N-terminal (5-18 and 17-38) and C-terminal
Table 2. Sequence homologies of C-terminal segment of E7 proteins

<table>
<thead>
<tr>
<th>Type</th>
<th>Similarity/Identity* (%)</th>
<th>Sequence†</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>100/100</td>
<td>THVDRTLEDLLMGTLG1VCPICSQKF†</td>
</tr>
<tr>
<td>31</td>
<td>74/63</td>
<td>TQVDRLGELLMGSG1VCPNCSTRL</td>
</tr>
<tr>
<td>33</td>
<td>73/58</td>
<td>TASDLRFTDLLMGTVK1VCPCTACQ</td>
</tr>
<tr>
<td>18</td>
<td>52/33</td>
<td>SADDLRFTQLLLNLSCPCCASQ</td>
</tr>
</tbody>
</table>

* Amino acid sequence similarity or identity to HPV-16 72-98 using alignment method of Myers & Miller (1988). Similarity score denotes percentage of residues that can theoretically be interchanged without modifying biological activity of protein. Residues defined as similar: R, K and H; I, L, V and M; E and D; A, S and T.
† Residues not similar or identical to the corresponding HPV-16 E7 residue are underlined.
‡ Circles are present above amino acids that are identical between HPV-16, -31 and -33; plus signs denote similar residues between these types.

E7 peptides (69–86) yielded LP responses from donors of different HLA haplotypes. Cytotoxic T cell responses were also observed to both C-terminal (73–86 and 69–82) and N-terminal epitopes (25–35).

Using an algorithm which identifies amphipathic helical sequences (Margalit et al., 1987), possible T cell epitopes in HPV-16 E7 17–30, 23–36, 34–46 and 71–88 have been predicted (J. A. Berzofsky, personal communication); this analysis predicted several of the peptides reactive in our studies. The human LP responses to HPV-16 E7 peptides in the current study were also similar to those previously reported in murine experiments. The 'public' T cell epitope DRAHYNI (48–54) described by Tindle et al. (1991) was included within one of the peptides (105) frequently recognized by our patients in an HPV-16 group-specific manner. The HPV-16 E7 T cell epitopes T1 (20–29), T2 (45–54), T3 (60–79) and T4 (85–94) identified in studies of mice immunized with E7 fusion proteins by Comerford et al. (1991) and peptides 17–32, 42–59, 62–77 and 87–98 observed by Shepherd et al. (1992) were similar to those observed in this study of human 'naturally induced' LP responses to HPV-16 E7. HPV-16 type-specific delayed hypersensitivity reactions mediated by CD4+ T cells have recently been reported in mice engrafted with syngeneic keratinocytes transfected with HPV-16 E7 DNA (McLean et al., 1993), supporting the type-specific CMI reactivity reported here for humans with natural HPV infection.

Whereas CMI reactivity to HPV-16 E7 peptides yielded an HPV-16 group-specific pattern, serological responses to E7 peptides in our patient population were not HPV-16 type-specific. None of the responders to E7 peptides in either CMI responses or ELISA responded to the same peptide in both tests. The apparent discrepancy between CMI and serological reactivity (Fig. 4) might be explained by activation of different CD4+ subsets such as TH1 and TH2 (Salgame et al., 1991), although no data are yet available to establish this hypothesis.

The high prevalence of serological IgG reactivity to peptide 109 in colposcopy clinic patients who were negative for HPV and those infected with other types is likely to be caused by previous infection with HPV-16 in this patient population at risk of HPV infection and cervical disease. Alternatively, ELISA reactivity to peptide 109 may be due to cross-reactive B cell epitopes related to infection with other HPV types. Even among patients with invasive cervical cancer, the correlation between seroreactivity to HPV-16 E7 fusion proteins in Western blots was only 50% (Mandelson et al., 1992). Although seroreactivity to HPV-16 E7 peptides may have some diagnostic relevance in identifying patients with a high antigen burden and cervical cancer (Müller et al., 1992; Jochmus-Kudielka et al., 1989), our data would suggest that ELISA reactivity to E7 peptides may not be useful in the discrimination of HPV types.

The observation that C-terminal peptide 109 was the most frequently reactive E7 peptide is different from results published by other investigators (Krchnák et al., 1990; Müller et al., 1990; Dillner, 1990). This may be due partly to the relatively longer C-terminal peptide 109 used in our studies (26 amino acids) than those used by others, or to other technical differences. Several subjects with ELISA reactivity to peptide 109 showed positive Western blot reactivity to an HPV-16 fusion protein (cII-E7). Absorption with the E7 fusion protein in addition to competition with peptide 109 specifically eliminated the reactive E7 band, suggesting that in some patients serological reactivity to C-terminal sequences is responsible for Western blot reactivity to the entire fusion protein.

Both the HPV-16 E6 and E7 proteins have been shown to be tumour rejection antigens capable of inducing CD8+ cytotoxic T cells and inducing rejection of syngeneic tumours in mice (Chen et al., 1991, 1992). In our study, CMI responses to HPV-16 E7 peptides occurred more often in women with low-grade SILs...
infected with HPV-16, -31, or -33 (all of three) than in those with high-grade SILs (four of eight) and occurred in patients who subsequently underwent regression of disease. One patient, a 17-year-old female with multiple vaginal, cervical and vulvar condylomata infected with HPV-31, underwent spontaneous simultaneous regression of all lesions. A positive LP response to peptide 109 was noted 2 to 3 weeks after regression (Fig. 2a). Three months later, repeat experiments failed to show positive LP responses to E7, suggesting that these responses may be only transiently detectable. ELISA reactivity was negative for all E7 peptides at the time of positive LP response to peptide 109, whereas a positive serological response to peptide 108 occurred several months after regression, when CMI reactivity was negative. This finding, in addition to the observation that patients with active infection with HPV-16, -31 or -33 were usually seronegative to E7 peptides, whereas many were reactive in LP assays, suggests that serological reactivity may occur after regression of disease.

We have thus identified human CMI responses to HPV-16 E7 peptides that appear to be HPV-16 group-specific. These studies suggest that CMI responses and not serological reactivity may correlate with ongoing genital HPV infection, and possibly with regression of premalignant disease. Further studies to map specific epitopes and to delineate more clearly clinical and HPV type specificity are in progress.

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