Human papillomavirus type 16 E2-specific T-helper lymphocyte responses in patients with cervical intraepithelial neoplasia

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T-cell-mediated immune responses against mucosal oncogenic types of human papillomaviruses (HPV) are thought to play a role in the control of the virus infection and its associated cervical lesions. The in vitro production of interleukin-2 by T-helper (Th) cells in response to the C-terminal and N-terminal domains of the HPV-16 E2 protein was determined in 74 women with cytological evidence of premalignant cervical epithelial neoplasia who participated in a non-intervention follow-up (FU) study. Cross-sectional analysis at the end of FU showed that Th cell responses against the C-terminal domain were associated with evidence of previous or present HPV-16 infection as compared to patients with no evidence of any HPV infection (18.9% versus 0%, P = 0.039). Th cell responses against the N-terminal domain were not associated with evidence of HPV-16 infection. No association with disease outcome was observed with Th cell responses against either of the E2 protein domains. However, longitudinal analysis revealed that Th cell responses against the C-terminal domain frequently occur at the time of virus clearance. Whether these responses are responsible for the clearance of the virus is not known.

Introduction

Human papillomaviruses (HPV) make up a large group of DNA viruses that exclusively infect epithelia. Infection with certain high risk HPV types is associated with cervical intraepithelial neoplasia (CIN) and invasive cervical carcinoma (Zur Hausen, 1994). The E6 and E7 viral oncoproteins interfere with cell cycle control (Morris et al., 1993; Slebos et al., 1995), which may ultimately lead to the oncogenic transformation of keratinocytes. Transcription of the E6 and E7 open reading frames (ORFs) is regulated by the p97 early promoter within the long control region of the virus (Cripe et al., 1987). The 45 kDa product of the E2 ORF has been shown to both activate (Bouvard et al., 1994; Ushikai et al., 1994) and repress (Bouvard et al., 1994; Demeret et al., 1994) virus transcription. The E2 protein has a domain structure similar to other transcription factors (Ham et al., 1991), i.e. a DNA-binding/dimerization C-terminal domain linked by a flexible hinge to a transactivation or transcription N-terminal domain. Expression of the E2 protein in HPV-positive cervical cancer cell lines has been shown to repress the expression of the viral oncoproteins and to lead to cell cycle arrest (Dowhanick et al., 1995). In cervical cancers, the HPV genome is frequently integrated into the host cell genome, often leading to disruption of the E2 ORF, resulting in loss of functional E2 expression (Schwarz et al., 1985; Yee et al., 1985). The elimination of the transcriptional repression by E2 upon virus integration may result in overexpression of E6 and E7, which may lead to progression of cervical dysplasia. However, E2 is not always completely lost, since in both premalignant lesions (Cullen et al., 1991) and invasive carcinomas (Matsukura et al., 1989) episomal circles of HPV DNA are often observed.

A higher incidence of HPV-associated disease in immunocompromised individuals suggests that the host immune response plays a role in controlling HPV infection and its
associated lesions (Laga et al., 1992; Schafer et al., 1991). Cellular immune responses against E6 and mainly E7 have been studied extensively (reviewed in Man, 1998). While E6 and E7 are attractive targets to study in high grade dysplasia and invasive carcinoma, immune responses directed against E2 might be expected during the early stages of cervical dysplasia. E2-specific immune responses potentially play a role early on because E2 is necessary for virus replication and is therefore expected to be expressed in productive lesions, furthermore expression is expected to be reduced in more advanced lesions when virus integration may have occurred.

Serum immunoglobulin A (IgA) and IgG responses to either an E2-derived peptide or an Escherichia coli-derived fusion protein were prevalent in CIN (Reeves et al., 1990) and cervical carcinoma patients (Dillner et al., 1994); however, this was not a general observation (Mann et al., 1990; Dillner et al., 1995). IgA responses to the native protein, produced in a baculovirus system, were associated with low grade CIN, while response rates went down in CIN III and cervical carcinoma patients (Rocha-Zavaleta et al., 1997). Recently, it was shown that primary cytotoxic T-lymocyte responses can be induced in healthy donors against an HLA-A2-binding peptide epitope derived from the E2 protein (Könlya et al., 1997). However, to date nothing is known about the natural T-cell response against E2 in HPV-infected individuals. We have studied T-helper (Th) cell responses, by measuring interleukin-2 (IL-2) release, against the C-terminal and N-terminal parts of the HPV-16 E2 protein in patients participating in a non-intervention cohort study of women with abnormal cytology. In a cross-sectional analysis at the end of follow-up (FU), E2-specific IL-2 release was correlated to virus infection patterns and disease outcome.

**Methods**

### Patients

The patients studied participated in a non-intervention cohort FU study of patients with cervical dysplasia that was designed and conducted to determine the relationship between HPV infection patterns and CIN disease course (Remmink et al., 1995). From this cohort, 74 patients were selected on the basis of availability of sufficient numbers of peripheral blood mononuclear cells (PBMC). Patients presenting with abnormal cytology were enrolled in the study after giving written informed consent to participate. The study design was approved by the ethics committee of the hospital. Patients entered the prospective study with mild to severe dyskaryosis and every 3–4 months HPV typing and cytological analysis were performed on cervical scrapes. Cytomorphological classification was done according to a modified version of the Papanicolaou (Pap) system (Kopac) as commonly employed in The Netherlands. Furthermore, colposcopical evaluation was done to describe colposcopically the predicted grade of dysplasia without histological intervention and to determine the extent of the lesions. At the end of FU, biopsies were taken for histological diagnosis (CIN 0, normal or metaplastic epithelium; CIN I–III, mild to severe dysplasia or carcinoma in situ). Details of the cohort study are described by Remmink et al. (1995). Patients who had an HPV-16-positive smear at least once during FU (n = 37) were analysed in this study as well as patients who had been HPV-negative during the complete FU of at least 12 months (group III, n = 22, mean age 38.8 years, mean FU time 35.4 months). The HPV-16-positive group was stratified according to HPV-16 infection status as previously defined (de Gruijl et al., 1998). Briefly, patients showing HPV-16 clearance (group IV, n = 16, mean age 37.1 years, mean FU time 50.6 months) were consistently negative for HPV-16 DNA over a period of at least 12 months and three consecutive PCR tests after having been HPV-16 DNA-positive, and patients with a persistent infection (group V, n = 21, mean age 35.6 years, mean FU time 41.4 months) were consistently positive for HPV-16 DNA over a period of at least 12 months and three consecutive PCR tests before Th cell responses were measured. In addition, patients infected with HPV types other than HPV-16 (group II, n = 15, mean age 34.7 years, mean FU time 47.6 months), including nine patients infected with HPV-16-related types and six patients infected with HPV-16-unrelated types, were analysed. A group of eight women (group I, mean age 37.5 years) from Amsterdam with HPV-16 infection but normal cytology was also included in the study. These women participated in a FU study of HPV-positive women with normal cervical cytology and had no history of previous cervical lesions.

### HPV genotyping

Cervical scrapes were evaluated for the presence of HPV DNA using the general primer (Gp5+/Gp6+)–based PCR method as previously described (de Roda Husman et al., 1995). Positive samples were subsequently subjected to a type-specific PCR to identify 27 mucosotropic HPV types including HPV-16 (de Roda Husman et al., 1994).

### Isolation of PBMC

PBMC were isolated from 40 ml of heparinized blood by density centrifugation with Lymphoprep (Nycomed Pharma) and cryopreserved in RPMI 1640 medium supplemented with 0.01 M HEPES buffer, 50 U/ml penicillin–streptomycin and 1.6 mM l-glutamine, containing 10% DMSO and 10% FCS.

### E2 proteins

The N-terminal and C-terminal domains of the HPV-16R E2 protein were cloned in the pET15b plasmid vector, which encodes an 12 kDa protein containing a small fragment of E. coli. The stretch of six histidine residues allowed purification by nickel binding. Purification by nickel binding was followed by anion exchange (N-terminal domain) or heparin binding (C-terminal domain). The proteins were at least 99% pure according to the following criteria: the proteins generated a single band on SDS–PAGE, a single peak on BioLogic trace and a single band on Western blot using a rabbit polyclonal antibody. Proteins were dissolved in a 20 mM HEPES buffer containing 100 mM NaCl, 20% glycerol, 0.2 mM EDTA and 5 mM DTT (pH 7.9 at 4°C). The N-terminal protein was a 29.3 kDa protein containing a large fragment of the hinge region (bp 2750–3517); the C-terminal protein was a 12.5 kDa protein containing a small fragment of the hinge region (bp 3584–3853) (Sanders et al., 1995).

### T-cell stimulation

PBMC were seeded in round-bottomed 96-well culture plates (Nunc/Corning) at 2 x 10^5 per well in Iscove’s modified Dulbecco’s medium containing 10% human pooled serum (CLB), 50 U/ml penicillin–streptomycin, 1.6 mM l-glutamine and 0.01 mM β-mercaptoethanol. Cells were stimulated by the addition of the C-terminal or N-terminal protein or phytohaemagglutinin (PHA) as a positive control (Murex) and cultured for 7 days at 37°C in an incubator with a 5% CO₂ humidified atmosphere. The anti-CD25 monoclonal antibody TB30 (a kind gift from R. van Lier, CLB) was added to all wells to prevent IL-2 consumption (hybridoma supernatant at a final dilution of 1:25).

Optimal stimulatory concentrations for both E2 protein fragments were determined in titration experiments. Both proteins were added to the cultures at a final concentration of 4 µg/ml. PHA was used at a concentration of 30 µg/ml. PBMC cultured in medium containing the
same amount of buffer as the protein supplemented conditions served as a negative control. All culture conditions were carried out in triplicate wells. After 7 days, the culture supernatants were harvested, pooled per test condition and stored at $-20\,{}^\circ\mathrm{C}$ until further use.

**IL-2 bioassay.** IL-2 production in the culture supernatant was measured in a bioassay with the IL-2-dependent cell line HT2. HT2 cells were cultured at $\times 10^4$ cells per well for 24 h in Iscove’s modified Dulbecco’s medium supplemented with 50 U/ml penicillin–streptomycin, 1-6 mM l-glutamine, 0.01 mM $\beta$-mercaptoethanol and 10% FCS, with the culture supernatants at final dilutions of 1: 2, 1: 4 and 1: 8. Triplicate wells were set up per test condition and per supernatant dilution. During the last 4 h, the cells were incubated with $[^3H]$thymidine ($0.4\,\mu$Ci per well; Amersham). The cells were harvested onto fibreglass filters and $[^3H]$thymidine incorporation was determined using a flatbed liquid scintillation counter (Wallac). IL-2 titration curves were included in each assay (100, 50, 25, 12.5, 6, 3, 1.5, 0.75, 0.375, 0 IU/ml IL-2; Cetus). Counts in the E2 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 c.p.m.) in the test wells exceeded proliferation in the buffer control wells by a factor of two ($\exp_{HT2}$) for at least two of the tested culture supernatant dilutions.

**Statistical analysis.** Frequencies of positive Th cell responders between the different groups were compared using $2 \times 2$ table analysis and Fisher’s exact test (FE). Comparisons between sets of SI (HT) between the different groups were compared using the Mann–Whitney U-test (MWU). Differences were considered significant when $P < 0.05$.

**Results**

**HPV-16 E2-specific IL-2 production by Th cells in relation to HPV-16 infection patterns and disease outcome; a cross-sectional analysis at the end of FU**

IL-2 production in response to HPV-16 E2 C-terminal and N-terminal proteins 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-positive women with normal cytology. All samples tested showed strong IL-2 production in response to PHA (not shown). Fig. 1 shows the IL-2 production by PBMC from the different test groups after stimulation with the C-terminal protein (Fig. 1A) and the N-terminal protein (Fig. 1B). The frequency of positive responders is also given for each group. Women with HPV-16 infection but without CIN (group I), women infected with HPV types other than HPV-16 (group II) and women with no detectable HPV but with a recent history of CIN (group III) showed no significant IL-2 responses against the C-terminal protein (Fig. 1A) and only in one case in each of these groups against the N-terminal protein (Fig. 1B). Responder frequencies against both proteins are low and do not differ significantly between the HPV-16 clearance (group IV) and HPV-16 persistence (group V) groups; 12.5% and 23.8% respectively against the C-terminal protein (Fig. 1A), and 18.8% and 14.3% against the N-terminal protein (Fig. 1B).

Responses against the C-terminal protein were significantly associated with evidence of previous exposure to HPV-16 infection, because both Th cell reactivities and responder frequencies against the C-terminal protein were significantly higher among women with either cleared (group IV) or persistent (group V) HPV-16 infections compared to women who had remained HPV-negative (group III) during FU (MWU, $P = 0.033$; FE, $P = 0.039$). The difference in Th cell reactivities and responder frequencies between the group with a cleared or persistent HPV-16 infection and the group of women infected with an HPV type other than HPV-16 (group II) did not reach statistical significance (MWU, $P = 0.055$; FE, $P = 0.093$). There were no significant differences in Th cell reactivities and responder frequencies against the C-terminal protein between the HPV-16-positive group with CIN and the HPV-16 group without CIN, nor between the HPV-16 clearance and the HPV-16 persistence group. No significant differences were observed between the different test groups for Th cell reactivities and response rates against the N-terminal protein.

When the responses found in CIN patients with current or past HPV-16 infections ($n = 37$) were related to the cytopathological and histopathological status at the end of FU no clear associations with resolved lesions or low grade lesions were observed (not shown).

**HPV-16 E2-specific Th cell reactivity over time in CIN patients: longitudinal analysis**

Of the 59 HPV-16-positive and HPV-negative patients, 29 were available for longitudinal analysis at two to six time-points per patient. The mean number of time-points tested per patient was comparable in the different patient groups, i.e. 3-3, 3-9, 3-8 in the HPV-16 persistence, HPV clearance and HPV-negative groups respectively. Th cell reactivity was measured in relation to HPV-16 infection and disease course in seven CIN patients who had no evidence of HPV infection (mean Th FU, 18±1 months), in 11 CIN patients with cleared HPV-16 infections (mean Th FU, 25 months) and in 11 CIN patients with persistent HPV-16 infections (mean Th FU, 19±7 months). Start of FU of the clearance patients ranged from 5 months to 43 months after the last HPV-16-positive DNA test (median, 17 months after the last HPV-16-positive DNA test). Th cell responses against the N-terminal protein were detected during FU in 4/7 HPV-negative patients, in 5/11 HPV-16 clearance patients and in 7/11 HPV-16 persistence patients (Table 1). In nine cases these responses occurred at a single time-point, while in seven cases responses were detected at two consecutive time-points. It is important to note that exposure to HPV-16 prior to FU cannot be excluded in the HPV-negative group. In the HPV-negative group, responses were found at a mean of 22 months after start of FU, while in the clearance group responses were detected at a mean of 23-6 months after the last positive HPV-16 DNA test ($P = 0.73$). In the HPV-negative group, no responses were detected against the C-terminal protein, while in 6/11 clearance patients and in 4/11 persistence patients Th cell responses could be detected during FU (Table 1). Responses against the C-terminal protein...
Persistence
Clearance
Longitudinal
Longitudinal
Cross-sectional
Cross-sectional

Table 1. T-helper cell responses against HPV-16 E2 and E7; longitudinal and cross-sectional analyses

<table>
<thead>
<tr>
<th>Protein‡</th>
<th>Negative*</th>
<th>Cross-sectional</th>
<th></th>
<th>Clearance†</th>
<th>Cross-sectional</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Longitudinal‡</strong></td>
<td><strong>End FU</strong></td>
<td><strong>End FU</strong></td>
<td><strong>Longitudinal‡</strong></td>
<td><strong>End FU</strong></td>
</tr>
<tr>
<td>E2-NT</td>
<td>During FU</td>
<td>57% (4/7)</td>
<td>14% (1/7)</td>
<td>45% (1/22)</td>
<td>18.8% (3/16)</td>
</tr>
<tr>
<td>E2-CT</td>
<td>0% (0/7)</td>
<td>0% (0/7)</td>
<td>0% (0/22)</td>
<td>12.5% (2/16)</td>
<td>28% (4/14)</td>
</tr>
<tr>
<td>E7</td>
<td>20% (1/5)</td>
<td>0% (0/5)</td>
<td>15% (2/13)</td>
<td>14.3% (3/21)</td>
<td>78% (6/11/14)</td>
</tr>
</tbody>
</table>

* CIN patients with no HPV DNA detected during FU.
† CIN patients with a cleared HPV-16 infection.
‡ CIN patients with a persistent HPV-16 infection.
§ Results from the longitudinal analysis of Th cell responses during FU and at the end of FU.
¶ Results from the cross-sectional analysis at the end of FU.
†† Target protein: E2-NT, the N-terminal domain of the HPV-16 E2 protein; E2-CT, the C-terminal domain of the HPV-16 E2 protein; E7, a pool of 14 20-mer synthetic peptides covering the entire HPV-16 E7 sequence.
sectional analysis (12.5% and 14.3% respectively). In the persistence group, responses against the C-terminal protein were detected at single time-points during or at the end of FU with similar frequencies (36.4% and 27.3% respectively, and 23.8% in the cross-sectional analysis). However, Th cell response rates against the E7 protein increased from 28.6% during FU to 78.6% at the end of FU (82.1% in the cross-sectional analysis).

**Discussion**

We have investigated IL-2 production by Th cells in response to the C-terminal or the N-terminal domain of the HPV-16 E2 protein in a non-intervention cohort study of patients with CIN. This study design has allowed us to relate the observed Th cell responses to the natural course of HPV-16 infection and CIN disease course over time. In a cross-sectional analysis at the end of FU, we found an association of Th cell IL-2 production in response to the C-terminal domain of HPV-16 E2 with evidence of previous or current exposure to HPV-16. Although a higher Th cell responder frequency directed against the N-terminal domain was observed in the HPV-16-positive group, this frequency was not significantly different from the HPV-negative group or the group of patients infected with HPV types other than HPV-16. In a longitudinal analysis, 57.1% of the HPV-negative patients showed Th cell responses against the N-terminal domain at one or two time-points. This may be due either to cross-reactivity with an unknown protein or to previous exposure to HPV-16 before the start of FU. Indeed the mean FU time at which these responses were detected in the HPV-negative group (22 months) was similar to the mean FU time after clearance in the Th cell-positive clearance patients (23-6 months). Alternatively, these responses may result from a short transient HPV infection between HPV DNA tests.

Th cell responses against the C-terminal domain were frequently detected at the last HPV-16-positive DNA test or within 6 months of the last HPV-16-positive DNA test. These findings correlate with the previous published Th cell responses against HPV-16 E7 (de Grujil et al., 1998). Th cell responses against HPV-16 E7 were higher around the time of clearance than at the end of FU in a group of HPV-16 clearance patients from the same cohort study. It is not clear whether these Th cell responses are involved in virus clearance and disease regression or whether these responses result from other events important in the natural history of cervical neoplasia. In contrast, Th cell responses against the E7 protein increase as the virus persists, and 82.5% of the patients with a persistent infection had E7-specific Th cells (de Grujil et al., 1998; Table 1), while Th cells against the E2 C-terminal domain are incidentally detected in patients with a persistent infection, during FU and at the end of FU at similar frequencies. While E7 expression is likely to increase as the virus persists and the CIN lesion progresses to CIN III, E2 expression is likely to decrease probably as a result of virus integration and loss of productive virus infection (Schwarz et al., 1985; Yee et al., 1985).

Because both capsid proteins and E2 are expressed during a productive infection we compared virus-like particle (VLP)-specific IL-2 production and IgG-specific antibodies with the Th cell responses to both E2 domains. We found no correlation between either of these different immune responses (data not shown).

Since Th cell responses directed against either the C-terminal domain or the N-terminal domain in patients with a persistent HPV-16 infection occur mainly at single time-points during FU, these may depend on E2 protein expression during the virus life-cycle. E2 is required for virus replication and the protein would therefore be expected to be expressed in productive lesions, expression levels may fluctuate during the virus life-cycle and during the natural history of CIN disease. We have recently shown that E2 expression is mainly found in koilocytosis and CIN I lesions, while expression was decreased in CIN II lesions and absent in CIN III lesions as detected with a polyclonal antibody directed against the C-terminal domain (Maitland et al., 1998). In concordance with these results, IgA levels to the native HPV-16 E2 protein were high in patients with low grade lesions and decreased in CIN III patients (Rocha-Zavaleta et al., 1997). However, in the cross-sectional study Th cell responses did not correlate with disease outcome. This may be because low grade productive lesions frequently exist in close proximity to high grade lesions. Alternatively, the lack of an association with disease outcome may be due to the relatively small CIN III group or the overall relatively low response rates. The reason for the low responder frequency may be due to the fact that expression of the E2 protein is mainly found in koilocytes (Maitland et al., 1998), which are usually located in the top layers of the squamous epithelium. This relatively remote localization could hamper the transport of E2 by migrating dendritic cells to the draining lymph nodes, which has been proposed as a crucial step in the generation of virus-specific T-cell responses (Zinkernagel, 1996).

E2 has been suggested as a candidate protein for vaccination (Tindle, 1996) and has been shown to be successful in animal models (Selvakumar et al., 1995). Although the results of the present study might suggest that, in some cases, Th cell responses against the C-terminal domain of E2 may play a role in virus clearance, the overall low response rates and the transient character of these responses would imply that E2 is less attractive than, for example E7, to which high persisting Th cell responses have been reported (see also Table 1). However, vaccination with the E2 protein may induce T-cell responses at sufficient levels. In a prophylactic/early therapeutic vaccination setting, an E2 and VLP combined vaccination may be beneficial. While neutralizing antibodies, induced by the VLP, may prevent infection (Nardelli-Hefliger et al., 1997), E2-specific T-cells may eliminate keratinocytes infected with HPV particles which have escaped the neutralizing antibodies.
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