Immune responses against human papillomavirus (HPV) type 16 virus-like particles in a cohort study of women with cervical intraepithelial neoplasia

I. Differential T-helper and IgG responses in relation to HPV infection and disease outcome

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T-helper (Th) cell-dependent IL-2 production and plasma IgG responses to virus-like particles consisting of the human papillomavirus type 16 (HPV-16) major capsid protein L1 (L1-VLP) were determined in patients with cytological evidence of cervical intraepithelial neoplasia (CIN) participating in a non-intervention prospective cohort study. IgG responses were associated with HPV-16 persistence and high-grade CIN lesions, while high frequencies of Th responses were observed in patients with both virus clearance and virus persistence, irrespective of CIN grade. The IgG response was found in conjunction with an IL-2 response to L1-VLP in 87% of the patients. Recognition of the HPV-16 L1 Th epitope (amino acids 311–335) was found to be more closely associated than recognition of L1-VLP as a whole to HPV exposure and CIN development. Among the HPV-16+ patients included in this study, those showing a Th response to amino acids 311–335 were more likely to carry the HLA DRB1*11/DQB1*0301 haplotype, while those showing an IgG response to L1-VLP were more likely to carry DRB1*0101/DQB1*0501. However, neither cell-mediated nor humoral immune responses against HPV-16 L1 appear to be sufficient for the natural control of HPV infection and CIN development.

Introduction

Mucosal, oncogenic human papillomaviruses (HPV), of which HPV-16 is the most prevalent, play an important role in the development of cancer of the cervix uteri and its precursor lesions (IARC, 1995). Failure of the host immune system to control HPV infections may contribute to virus persistence and the concomitant development and progression of premalignant cervical lesions (Frazer, 1996; Tindle, 1996).

The possibility of immunological intervention in cervical neoplasia has been widely discussed (Tindle, 1996) and protocols for prophylactic and/or therapeutic immunization against HPV-derived proteins are in development (Borysiewicz et al., 1996; Tindle, 1996). For prophylactic vaccination, the use of the HPV-16 capsid proteins, particularly in the form of virus-like particles (VLP) comprising either L1 or L1 in combination with L2, to induce neutralizing antibodies has been proposed (Schiller & Roden, 1995; Frazer, 1996; Tindle, 1996; Kirnbauer et al., 1993; Le Cann et al., 1994). However, the presence of IgG antibody responses against HPV-16 capsid proteins in humans does not appear to correlate with the prevention or clearance of HPV-induced cervical lesions (Carter et al., 1996; Wikström et al., 1995; Chua et al., 1996; de Gruijl...
et al., 1997) and can be correlated with increased risk of the development of cervical cancer (Nonnenmacher et al., 1995; Lehtinen et al., 1996).

Patients with cervical intraepithelial neoplasia (CIN) show evidence of T-helper (Th) cell responses to HPV-16 L1-derived peptide sequences in a proliferation assay (Shepherd et al., 1997) and delayed-type hypersensitivity reactions after cutaneous injection of a recombinant HPV-16 L1 fusion protein (Höppf et al., 1991). How these responses influence HPV infection and disease outcome is unknown. This study was performed to determine the relationship between cellular (Th cell-dependent IL-2 release) and humoral (plasma IgG reactivity) immune responses against HPV-16 L1 VLPs (L1-VLP) and the possible influence of these responses on the clearance of virus infection and the behaviour of premalignant cervical lesions in a non-intervention follow-up study of patients with CIN.

**Methods**

**Controls, patients and study design.** Sixteen umbilical cord blood samples were included in the study as immunologically naive negative controls (UC controls). Nine HPV-16+ women attending a general city hospital in Amsterdam and participating in a follow-up study of HPV-positive women, with normal cervical cytology (Pap 1 or Pap 2) and no history of previous cervical lesions, were included in the study as controls for disease status (mean age 36.6 years; SD 9.6).

The study of women with cervical dysplasia described in this paper was nested within a larger prospective non-intervention cohort study of Dutch women with CIN that was designed and conducted to study the relationship between HPV infection patterns and the natural disease course of premalignant cervical lesions (Remmink et al., 1995). Women with cytologically determined mild to moderate cervical dyskaryosis were referred to the gynaecological outpatient clinic of the Free University Hospital in Amsterdam and, after giving written, informed consent, were enrolled in the study. The study design was approved by the ethics committee of the hospital.

Clinical follow-up consisted of cytological and colposcopical examinations that were performed every 3–4 months. Biopsies were not taken during the follow-up period, to avoid interference with the natural course of the disease. Results from the cytomorphological tests were classified according to a modified Papanicolaou system, as employed in the Netherlands (Remmink et al., 1995): Pap 1, no cytomorphological abnormalities; Pap 2, inflammation; Pap 3a, mild to moderate dyskaryosis; Pap 3b, severe dyskaryosis; Pap 4, carcinoma in situ; Pap 5, (microinvasive) carcinoma. Colposcopy was employed to indicate the predicted grade of dysplasia and to determine the extent of the observed lesions in cervical quadrants. At the end of follow-up, biopsies were taken and a histological diagnosis was made (CIN 0, normal or metaplastic epithelium; CIN I, mild dysplasia; CIN II, moderate dysplasia; CIN III, severe dysplasia or carcinoma in situ). A detailed description of the tight clinical surveillance of the patients and the evaluation of clinical follow-up was reported previously (Remmink et al., 1995).

Every 3–6 months, 40 ml heparinized peripheral blood was drawn. Blood collection started 10–56 months (varying for each patient) after enrolment in the study (de Gruijl et al., 1997, 1998). Blood samples from 187 patients, who consented to one or more blood donations, were collected. From these patients, 63 women were selected to be tested in a cross-sectional analysis at the end of follow-up (i.e. at the time when biopsies were taken) on the basis of HPV infection patterns and to represent the following groups: patients who were negative for HPV during the entire follow-up period and for at least 12 months (n = 14, HPV−); patients with a current or past infection of HPV types other than HPV-16 (n = 13, HPV non-16: HPV-18, -31, -33, -51, -52, -58 or -66); patients with cleared HPV-16 infections (n = 15, HPV-16 C; mean time since the patients were last determined to be positive for HPV-16 DNA, 28.5 months; range 12–65 months); and patients with persistent HPV-16 infections (n = 21, HPV-16 P, positive for HPV-16 DNA in 4–11 consecutive PCR tests over a period of 13–59 months). Mean ages, clinical follow-up times and histologically determined disease outcome for all the CIN patient groups are presented in Table 1.

The CIN patients tested in the cross-sectional analysis (n = 63) had a mean clinical follow-up period of 37.1 months (range 13–68). Of these women, five who were HPV−, 12 from the HPV-16 C group and 13 from the HPV-16 P group were longitudinally tested at two to four separate time-points over a preceding period of 9–31 months (mean 17.7 months; SD 6.0). At the start of the follow-up period, the median time since HPV-16 DNA was last detected among the patients with cleared infections was 9 months (range 3 to 34 months).

Plasma samples were available from 54 of the 63 CIN patients and of all nine HPV-16+ women with normal cervical cytology. These samples

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**Table 1. Characteristics of patient groups from the CIN cohort study at the time of cross-sectional analysis at the end of follow-up**

Patients were allocated to one of four groups, as described in the text. Patient ages and the period of clinical follow-up are given as means ± SD. Disease outcomes, as histological diagnoses of biopsies taken at the end of follow up, were allocated to one of four classes as outlined in the text, and are given as numbers in each class with percentages of the total in that group in parentheses.

<table>
<thead>
<tr>
<th>Patient group (n)</th>
<th>Age (years)</th>
<th>Clinical follow-up (months)</th>
<th>Disease outcome (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CIN 0</td>
</tr>
<tr>
<td>HPV− (14)</td>
<td>38.3 ± 8.1</td>
<td>33.6 ± 11.1</td>
<td>5 (36)</td>
</tr>
<tr>
<td>HPV non-16 (13)</td>
<td>32.0 ± 5.8</td>
<td>34.0 ± 8.7</td>
<td>5 (38)</td>
</tr>
<tr>
<td>HPV-16 C (15)</td>
<td>35.7 ± 8.3</td>
<td>40.9 ± 13.5</td>
<td>6 (40)</td>
</tr>
<tr>
<td>HPV-16 P (21)</td>
<td>35.0 ± 9.1</td>
<td>34.3 ± 12.7</td>
<td>2 (10)</td>
</tr>
</tbody>
</table>
were tested for both IgG and IL-2 reactivity to HPV-16 L1-VLP. Of the patients tested in follow-up, six of 13 HPV-16 P women, six of 12 HPV-16 C women and four of five HPV⁻ women could be tested in parallel for both IL-2 and IgG responses.

■ HPV genotyping. HPV detection and genotyping were performed on cellular material from cervical smears as described elsewhere (de Roda Husman et al., 1994, 1995). Samples were tested for the following genital HPV types: 6, 11, 13, 16, 18, 30–33, 35, 39, 40, 42–45, 51, 52, 54–56, 58, 59 and 61 (de Roda Husman et al., 1994; Jacobs et al., 1995).

■ HLA-DRB1 and -DQB1 typing. DRB1 and DQB1 typing was performed on peripheral blood mononuclear cells (PBMC) as previously described by using the second British Society of Histocompatibility and Immunogenetics (BSHI) class II oligotyping kit (Duggan-Keen et al., 1996).

■ Synthetic peptides. Two 15-mer peptides with a five amino acid overlap were synthesized. These peptides were derived from the HPV-16 L1 sequence and were previously found to contain Th cell epitopes (P1, amino acids 311–325, NLASSNYTPGSAGM; P2, amino acids 321–335, PSGMVTSDAQFPNK) (Shepherd et al., 1997). The peptides were synthesized with a Millipore 9050 peptide synthesizer employing the Fmoc method, dissolved in DMSO to a stock concentration of 10 mM and further diluted in culture medium.

■ HPV-16 L1-VLP. Self-assembled HPV-16 L1-VLP were expressed in baculovirus-infected Spodoptera frugiperda (Spf)–21 insect cells, isolated and purified by sucrose gradient and caesium chloride gradient centrifugation, as described elsewhere (Le Cann et al., 1994). Gradient fractions were tested for the presence and density of VLP by electron microscopy and ELISA (Le Cann et al., 1994). For use in T cell in vitro cultures, the VLP fraction was further purified by a second round of sucrose and caesium chloride centrifugation, as previously reported (Dupuy et al., 1997). The same fractions derived from Spf–21 cells infected with wild-type (wt) baculoviruses were collected and used as control preparations in the T cell IL-2 production assay (Dupuy et al., 1997). The VLP were suspended in PBS and used in ELISA and T cell cultures.

■ Isolation of PBMC. PBMC were isolated from 40 ml heparinized blood by density centrifugation with Lymphoprep (Nycomed) and used at the same concentration as a negative control. All antibodies were dialysed against PBS before use in order to remove NaN₃.

■ IL-2 bioassay. Production of IL-2 in culture supernatants was measured in a bioassay with the IL-2-dependent cell line HT2 (Ertl et al., 1989). HT2 cells (1 × 10⁴ per well) in Iscove’s modified Dulbecco’s medium supplemented with 50 U/ml penicillin–streptomycin, 10⁻⁶ M t-glutamine, 0.01 mM β-mercaptoethanol and 10% FCS were cultured for 24 h with PBMC culture supernatants used at final dilutions of 1:2, 1:4 and 1:8. Triplicate wells were set up for each test condition and supernatant dilution. During the last 4 h, the cells were incubated with [³H]thymidine (0.4 μCi per well) (Amersham). The cells were harvested onto fibreglass filters and [³H]thymidine incorporation was determined with a flat-bed 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 and 0 IU/ml IL-2) (Cetus). Counts in the L1 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 (c.p.m.) in the test wells exceeded proliferation in the medium control wells by a factor of two (HT2 stimulation index, SI₄₅ > 2) for at least two of the tested culture supernatant dilutions, with a difference of at least 500 c.p.m. between the means of the triplicate sets of test and medium control wells.

■ VLP ELISA. HPV-16 L1-VLP-specific plasma IgG reactivity was determined by using 250 ng per well HPV-16 L1-VLP and 1:10-diluted plasma samples in an ELISA test system, as previously described for L1/L2-containing VLP (de Grujil et al., 1997). Duplicate tests were performed for all plasma samples.

IgG reactivities were expressed as the mean absorbance of the duplicate tests at 492 nm. To compensate for inter-assay variability, absorbance values were adjusted in relation to the absorbances of a set of internal control plasma samples (with both positive and negative IgG reactivities), which were included in each plate. The background reactivity found in the negative control wells coated with PBS and blocked with PBS–TWEEN–newborn calf serum was subtracted from the corresponding absorbances obtained for the test wells of each plasma sample. A cut-off level, above which absorbances were considered positive, was based on the distribution of absorbances found in the HPV-negative CIN patients included in this study. This cut-off value was obtained by adding 2 SD to the mean absorbance (cut-off absorbance = 0.443).

■ Statistical analysis. Frequencies of positive responses between the test groups were compared using 2 × 2 table analysis and Fisher’s exact test. Comparisons between sets of SI₄₅ were carried out using the Mann–Whitney U test. All tests were two-tailed and differences were considered significant when P < 0.05.

Results

IL-2 production in response to HPV-16 L1-VLP

Th responses were measured by the use of a sensitive IL-2 bioassay, which was described previously (de Grujil et al., 1996). To check whether the IL-2 detected was derived from CD4⁺ or CD8⁺ T cells, cultures were incubated under the conditions described above after CD4⁺ or CD8⁺ cells had been depleted. PBMC were first incubated consecutively with anti-CD4 or anti-CD8 mouse monoclonal antibodies (Becton Dickinson) and goat anti-mouse IgG antibodies conjugated to MACS magnetic microbeads (Miltenyi Biotec). CD4⁺ or CD8⁺ cells were then removed with the MiniMACS magnetic micro bead separation system (Miltenyi Biotec), according to the manufacturer’s instructions. To check the CD4⁺/CD8⁺ cell depletion, CD4 and CD8 FACS analyses were performed after double staining with fluorescein isothiocyanate (FITC-) and phycoerythrin-conjugated antibodies (Simulset, Becton Dickinson).
1998; Tsukui et al., 1996) and allows for the detection of Th-dependent IL-2 release after only 1 week of culture. The concentration of purified HPV-16 L1-VLP (1-5 µg/ml) that gave the optimal IL-2 response was determined in titration experiments with different PBMC samples. Thus, lymphocytes from umbilical cord blood (n = 4) and PBMC from patients in the CIN cohort study (two HPV−; four HPV-16 C; four HPV P) showed no IL-2 production to wt baculovirus fractionated as for VLP purification, while VLP-specific responses were observed in three HPV-16 C women (data not shown). As reactivities obtained with the wt fraction were comparable to reactivities found with medium alone, the latter was included as a control in all subsequent tests.

The VLP-specific IL-2 production observed (expressed as SIHT2) was found to depend on the presence of Th cells (Fig. 1). After depletion of the CD4+ T cells by magnetic microbead separation, VLP-specific IL-2 production was completely abolished in two HPV-16+ women without cytological evidence of cervical dysplasia, in two HPV-16 P patients with histologically confirmed CIN III and in one HPV-16 C patient with no histological evidence of cervical dysplasia at the end of follow-up (Fig. 1a). In line with this observation, VLP-specific IL-2 production could also be blocked by the addition to the T cell cultures of an antibody binding to HLA class II molecules (n = 3, Fig. 1b). In all patients, strong VLP-specific IL-2 production was still detectable after depletion of CD8+ cells, although in one case a reduction of approximately 50% was observed (Fig. 1a). These results indicate that the observed T-cell activation, as measured by IL-2 production, is principally dependent on CD4+ Th cells.

HPV-16 L1-VLP-specific IL-2 production, observed in all the control and CIN patient groups in a cross-sectional analysis at the end of follow-up, is presented in Fig. 2. Strong and comparable IL-2 production in response to PHA was found in all the test groups (data not shown). Responses to VLP were observed in both HPV-16+ women with normal cervical cytology and in all CIN patient groups, regardless of HPV status (Fig. 2b–f). It is important to note that exposure of patients to HPV-16 prior to follow-up cannot be excluded in the HPV− and HPV non-16 groups. The frequency of VLP-induced IL-2 production in the patients was significantly higher than in the umbilical controls (Fig. 2a, indicating that the responses resulted from in vivo priming (P < 0.0005, two-tailed Fisher’s exact test). Although they did not differ in response frequencies, the group of HPV-16+ patients with CIN (HPV-16 C and P, Fig. 2e, f) showed significantly stronger responses (higher SIHT2 at the 2 × T cell culture supernatant dilution) than either the HPV− group (Fig. 2c, P = 0.008) or the HPV non-16 group (Fig. 2d, P = 0.032, Mann–Whitney U test).

Ten of 12 HPV-16 C patients and 10 of 13 HPV-16 P patients, who were tested at two or more separate time-points (over a period of 9–31 months preceding the cross-sectional analysis), showed VLP-specific IL-2 production at least once during follow-up. Three of five longitudinally tested HPV− patients, who were negative for VLP-specific IL-2 production...
in the cross-sectional analysis, showed positive IL-2 responses at earlier time-points, possibly indicating the presence of HPV infections prior to follow-up. IL-2 follow-up data from the positively responding patients with either HPV-16 clearance or persistence did not reveal consistently increasing or decreasing IL-2 production over time in either of the two groups, and no consistent trends arose in relation to cervical disease development. This is illustrated in Fig. 3: increasing and decreasing VLP-specific IL-2 production was observed during follow-up, both in HPV-16 C patients with resolved CIN lesions (Fig. 3a, b) and in HPV-16 P patients with high-grade CIN III lesions (Fig. 3c, d).

IgG responses to HPV-16 L1-VLP in relation to IL-2 reactivity

Plasma samples were available to determine L1-VLP-specific IgG reactivity of 54 of 63 CIN patients tested and all HPV-16+ women with normal cervical cytology. IgG responses in the same group of patients against VLP containing both L1 and L2 were reported previously (de Gruijl et al., 1997). For comparison with Th cell activity, IgG responses in this study were measured against the same VLP preparation (containing only L1) as was used in the IL-2 release experiments. A good correlation was found between these IgG reactivities and IgG reactivities against L1/L2-VLP (de Gruijl et al., 1997) previously determined in the same plasma samples (data not shown: linear regression analysis; r = 0.782, P < 0.0001, two-tailed). By combining the cross-sectional and longitudinal analyses, L1-VLP IgG and IL-2 reactivity data were available from one to four time-points, depending on the patient. A patient was considered to respond positively when she showed a positive response at least once during the follow-up. The resulting IgG response frequencies in relation to IL-2 response frequencies from the same patients are shown in Table 2.

In contrast to the Th responses, IgG responses to L1-VLP were restricted to patients known to have been exposed to HPV-16 and were significantly more frequent among patients with persistent infections than in patients with cleared HPV-16 infections (Table 2).

When the HPV-16+ patients from the CIN cohort study were stratified according to disease status at the end of follow-up, no clear associations were found with Th IL-2 response rates (eight of 13 patients with CIN III compared to 12 of 23 patients with CIN I/II or resolved lesions), whereas positive IgG responses to L1-VLP were significantly more frequent among HPV-16+ women with CIN III (seven of 11) than among HPV-16+ women with lower grade or resolved lesions at the end of follow-up (two of 17; P = 0.01, two-tailed Fisher’s exact test).

Of the 63 women tested for both IL-2 production and IgG reactivity in response to L1-VLP, 28 showed only IL-2 production, 13 showed both IL-2 production and IgG reactivity and two showed only IgG reactivity (Table 2).

Th IL-2 responses to the L1-derived peptides P1 and P2: relation with IL-2 and IgG response to L1-VLP and HLA-DR/-DQ genotype

Whereas HPV-16 L1-VLP-specific IgG responses showed type specificity, as indicated by an association with known exposure to HPV-16, Th responses did not. The relevant specificity of the Th response may be restricted to a particular epitope. This was further investigated by using peptides covering L1 amino acids 311–335, which have previously been shown to elicit an HPV-16-specific proliferative response in patients with CIN (Shepherd et al., 1997). The overall response rates to the L1-derived 15-mer peptides P1 (amino acids 311–325) and P2 (amino acids 321–335) were lower than those found for L1-VLP (Table 3). The responses appeared to correlate with HPV exposure but it could not be concluded that they were HPV-16-specific (16 of 40 HPV+ versus one of...
Table 2. IL-2 and IgG response frequencies to HPV-16 L1-VLP among patients and controls: combined results from the cross-sectional and longitudinal analyses

<table>
<thead>
<tr>
<th>Disease state</th>
<th>HPV status</th>
<th>IL-2+ (%)</th>
<th>IgG+ (%)</th>
<th>IL-2+ and IgG+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>HPV-16+</td>
<td>8/9 (88.9)</td>
<td>5/9 (55.6)</td>
<td>4/9 (44.4)</td>
</tr>
<tr>
<td>CIN</td>
<td>HPV−</td>
<td>8/13 (61.5)</td>
<td>1/13 (7.7)</td>
<td>1/13 (7.7)</td>
</tr>
<tr>
<td></td>
<td>HPV-16 P+C</td>
<td>20/28 (71.4)</td>
<td>9/28 (32.1)*</td>
<td>8/28 (28.6)</td>
</tr>
<tr>
<td></td>
<td>HPV-16 P</td>
<td>12/16 (75.0)</td>
<td>8/16 (50.0)†</td>
<td>7/16 (43.8)</td>
</tr>
<tr>
<td></td>
<td>HPV-16 C</td>
<td>8/12 (66.7)</td>
<td>1/12 (8.3)</td>
<td>1/12 (8.3)</td>
</tr>
<tr>
<td></td>
<td>HPV non-16</td>
<td>5/13 (38.5)</td>
<td>0/13 (0)</td>
<td>0/13 (0)</td>
</tr>
</tbody>
</table>

* HPV-16 P+C versus HPV non-16, P = 0.038, two-tailed Fisher’s exact test.
† HPV-16 P versus HPV-16 C, P = 0.039, two-tailed Fisher’s exact test.

There was no absolute association between IL-2 responsiveness to P1 or P2 and L1-VLP-specific IgG reactivity, but all IgG-positive patients also showed IL-2 responses either to L1-VLP or to P1 or P2.

Of the 19 subjects with positive responses to amino acids 311–335, 15 also showed IL-2 responsiveness to L1-VLP.

12 HPV−, P = 0.076; 11 of 29 HPV-16+ versus five of 11 HPV non-16+, P = 0.733; two-tailed Fisher’s exact tests.

Fig. 3. Follow-up of Th cell-dependent IL-2 production in four CIN patients. IL-2 production is presented as SIHT2 for three dilutions (2×, 4×, 8×) of the T cell culture supernatants. The dashed line indicates the cut-off level (SIHT2 = 2). The time of testing is indicated in months since the start of clinical follow-up; the last indicated time-point is at the end of follow-up (i.e. when biopsies were taken). (a)–(b) CIN patients with a cleared HPV-16 infection and resolved lesions at the end of follow-up. IL-2 follow-up started 5 months (a) and 20 months (b) after HPV-16 DNA was last detected. (c)–(d) CIN patients with a persistent HPV-16 infection and CIN III lesions at the end of follow-up.
Of the 36 HPV-16+ CIN patients included in this study, 35 were tissue-typed for their HLA DR and DQ alleles (HPV-16 C, n = 15; HPV-16 P, n = 20). In these patients, recognition of the immunogenic region covering amino acids 311–335 was found to be associated with the HLA DRB1*11/DQB1*0301 haplotype (P = 0.05). This association appears to depend on the DRB1*11 allele, as eight of 11 patients that responded to P1/P2 carried this allele as compared to four of 17 that did not respond (P = 0.019), while no significant difference in frequency was found for the DQB1*0301 allele between these two groups.

Further examination of the Th IL-2 and IgG responsiveness in the HPV-16+ patient group in relation to the patient tissue type revealed an association between the HLA DRB1*0101/DQB1*0501 haplotype and the presence of IgG responses to HPV-16 L1-VLP (six of nine giving an IgG response versus one of 19 that did not respond, P = 0.0014). Again, this association was found to depend more on the DR allele (P = 0.0014) than on the DQ allele, which showed a slightly weaker association (P = 0.0068).

**Discussion**

This study has investigated whether Th and/or IgG responses to HPV-16 L1-VLP are correlated with clearance of the virus and associated CIN lesions. Differential patterns of Th and IgG responsiveness were observed in terms of HPV infection and CIN development.

Th-dependent, HPV-16 L1-VLP-specific IL-2 responses were significantly stronger in patients with abnormal cervical cytology and known HPV-16 infections compared with HPV− patients or HPV non-16 patients. This, together with the lack of responses in umbilical cord lymphocytes, is consistent with the detection of memory responses. Responses detected in HPV− and HPV non-16 patients may be due to exposure to HPV-16 prior to follow-up or by cross-reactivity with other HPV types. The possibility of prior HPV exposure in HPV− patients was supported by IL-2 responses observed early on during follow-up, but found to decline over time, in three out of five longitudinally tested HPV− patients.

Th IL-2 responses did not correlate with clinical outcome, defined by either virus clearance or persistence or CIN grade. In contrast, IgG responses to L1-VLP were associated with persistent HPV-16 infection and the development of high-grade CIN. Thirteen of 15 patients producing anti-VLP IgG also showed Th IL-2 release. This is consistent with Th activity preceding and required for the generation of L1-specific IgG responses.

Using the data from this study and other previously reported results, we can now attempt to reconstruct the temporal relationship between HPV-16 infection, the generation of immune responses to the viral capsid proteins and the development of cervical neoplasia.

A high frequency of Th cell-dependent IL-2 responses to L1 was found among women with HPV-16 infections but normal cervical cytology (eight of nine or 88.9%). This is consistent with L1-specific T-cell responses occurring upon virus exposure and preceding the development of any lesions. In addition, five of these nine women also showed positive IgG responses. However, as these were prevalent HPV-16 infections, there was no way of knowing how long these women had been infected by HPV-16 or within what time-period the subsequent T-cell activation and seroconversion occurred. Other longitudinal studies have shown that upon primary infection, HPV-16 VLP IgG seroconversion can occur within 6–8 months, preceding any overt lesions (Wikström et al., 1995; Carter et al., 1996). As the generation of IgG responses depends on Th activity, it is to be expected that some women will show Th responses without IgG seropositivity.

Two previous studies showed that cytologically normal women who harboured persistent HPV-16 infections and who seroconverted were more likely to develop dysplastic cervical

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**Table 3. IL-2 response frequencies to the HPV-16 L1-derived peptides P1 and P2 among patients and controls**

The number of patients in each group showing an IL-2 response to peptides P1 and/or P2 is shown as positive/no. tested, with the percentage within the group shown in parentheses.

<table>
<thead>
<tr>
<th>Disease state</th>
<th>HPV status</th>
<th>P1 (%)</th>
<th>P2 (%)</th>
<th>P1 or P2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>HPV-16+</td>
<td>1/7 (14.3)</td>
<td>1/7 (14.3)</td>
<td>1/7 (14.3)</td>
</tr>
<tr>
<td>CIN</td>
<td>HPV−</td>
<td>0/12 (0)</td>
<td>1/12 (8.3)</td>
<td>1/12 (8.3)</td>
</tr>
<tr>
<td>HPV-16 P + C</td>
<td>7/29 (24.1)</td>
<td>8/29 (27.6)</td>
<td>11/29 (37.9)*</td>
<td></td>
</tr>
<tr>
<td>HPV-16 P</td>
<td>4/18 (22.2)</td>
<td>6/18 (33.3)</td>
<td>7/18 (38.9)</td>
<td></td>
</tr>
<tr>
<td>HPV-16 C</td>
<td>3/11 (27.3)</td>
<td>2/11 (18.2)</td>
<td>4/11 (36.4)</td>
<td></td>
</tr>
<tr>
<td>HPV non-16</td>
<td>4/11 (36.4)</td>
<td>3/11 (27.3)</td>
<td>5/11 (45.5)</td>
<td></td>
</tr>
<tr>
<td>UC controls</td>
<td>–</td>
<td>0/7 (0)</td>
<td>0/7 (0)</td>
<td>0/7 (0)</td>
</tr>
</tbody>
</table>

* HPV-16 P + C versus HPV−, P = 0.073, two-tailed Fisher’s exact test.
lesions (Carter et al., 1996; Chua et al., 1996). Thus, serum IgG antibodies to L1 do not necessarily contribute to the clearance of HPV at an early stage of infection. Indeed, seropositivity to VLP is predictive for the development of CIN III and cervical carcinoma (Nonnenmacher et al., 1995; Lehtinen et al., 1996; de Gruijl et al., 1997).

In the longitudinal analysis, there were no consistent trends in Th IL-2 activity among patients with either virus clearance or persistence. This contrasts with IL-2 responses to the HPV-16 E7 oncoprotein, which show a significant decline after HPV clearance but increase in patients with persistentHPV infections and CIN III lesions (de Gruijl et al., 1998). Transient exposure to HPV virions may be sufficient to trigger memory Th cells specifically reactive to capsid proteins, influencing the detection of IL-2 release during follow-up but independent of overt disease or HPV DNA positivity.

The longitudinal analysis revealed VLP-specific IL-2 responses in a majority of patients with CIN, independent of HPV status and disease course. These results suggest that the T-cell activity detected does not necessarily influence disease outcome or virus clearance.

In addition, we have studied HPV-16 specificity in Th responsiveness in relation to IgG production. To this end, IL-2 release induced by peptides P1 and P2 covering L1 amino acids 311–335 was examined. This response showed considerable overlap with responses to L1-VLP but no significant correlation with IgG production, consistent with a recent report (Luxtton et al., 1997). A strong association among HPV-16+ patients between IL-2 responsiveness to amino acids 311–335 and the HLA DRB1*11 allele suggests that this epitope may be specifically recognized by DR11-restricted Th cells. However, responses to L1 amino acids 311–335 were shown to be more frequent in HPV+ patients with CIN than in HPV− patients with resolved or low-grade lesions or in HPV− women with normal cervical cytology, suggesting that recognition of this region does not protect from the development of premalignant cervical lesions. Indeed, the highest response rates reported by Shepherd et al. (1997) were in patients with CIN III.

The IgG response to HPV-16 L1-VLP showed a strong association with the DRB1*0101/DQB1*0501 haplotype. Although the small number of subjects included in this study and the high polymorphism of the HLA locus (for which the probabilities listed in the text were not corrected) necessitates caution, it is an interesting observation that particular HLA class II alleles may be involved in the generation of IgG responses. This is in line with recent studies showing that different peptide/MHC II combinations can give rise to qualitatively different Th responses (Th1 or Th2), in turn leading to the preferential generation of either cell-mediated or humoral immune responses (Chaturvedi et al., 1995).

It is apparent that neither the Th IL-2 nor the IgG responses measured in this study are sufficient to deliver natural control of HPV infection and CIN development. This may be due to the expression of HPV capsid proteins in the top layers of the squamous epithelium of the cervix. This relatively remote localization may hamper transport of L1 by migrating professional antigen-presenting cells (APC) to draining lymph nodes, which has been proposed as a crucial step in the generation of virus-specific immune responses (Zinkernagel, 1996). This in turn would account for the observed lag between HPV-16 infection and the generation of VLP-specific antibody responses at generally low serum titres (Carter et al., 1996). However, prophylactic vaccination with VLP may induce immediate and vigorous immune responses and high serum titres of neutralizing IgG antibodies. Such responses have proved to be protective against subsequent experimental challenge with papillomaviruses and to prevent the development of papillomavirus-induced lesions in various animal models (Breitbart et al., 1995; Suzich et al., 1995; Kirnbauer et al., 1996).

In terms of natural immunity against HPV capsids, local IgA responses in the cervix, with the capacity to neutralize virions at the actual site of infection, may be more relevant than peripherally recirculating T cells and serum IgG levels (Wang et al., 1996). Therefore, in the following paper, we have determined IgA reactivity to HPV-16 VLP in cervical mucus samples of women with CIN, to relate these responses to HPV and disease status over time (Bontkes et al., 1999). Identification of the most effective type of immunity will also have implications for immune intervention protocols, as the method of vaccine administration and routing will depend on the type of immune response to be targeted.

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