Human papillomavirus type 16-specific T cell responses and their association with recurrence of cervical disease following treatment

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Human papillomavirus type 16 (HPV-16) L1- and E7-specific T cell responses were measured in 58 women with abnormal cervical cytology in a prospective study. On recruitment, patients responded most frequently and with the highest numbers of responding cells to the L1 region aa 311–345 and this response was significantly associated with the presence of cervical disease ($P = 0.041$). Responses to the L1 peptide aa 281–295 were significantly higher in patients with CIN III than in those with HPV/CIN I or CIN II lesions ($P = 0.027$). The E7 region aa 70–98 was the most immunogenic in patients with squamous intraepithelial lesions of the cervix (SIL) but the responses detected were not significantly higher than in patients without SIL. Following treatment, the T cell response profiles of patient groups did not change significantly. However, on analysis of the responses of individual patients with and without recurrent disease on follow-up, significant differences were found. Recurrence of disease was associated with T cell responses to the E7 region aa 70–98 at the patient’s first clinic visit ($P = 0.017$). Recurrence of disease was also accompanied by an increase in the total number of L1-specific short-term T cell lines (STLs) at follow-up, whereas absence of disease was accompanied by a decrease in L1-specific STLs. The data also suggested a possible link between E7 70–98-specific responses and acquisition of disease by patients who were previously disease-free. Further studies are warranted to determine whether this response could be useful as a marker of recurrent disease in some patients.

INTRODUCTION

Previous cross-sectional studies have demonstrated that healthy, asymptomatic individuals are capable of making peripheral blood T cell responses to the HPV-16 L1, E6 and E7 proteins (Altmann et al., 1992; Strang et al., 1990). In the case of HPV-16 E7, we have shown that the N- and C-terminal regions are particularly immunogenic in healthy controls, whereas in women with cervical disease, these responses were found to be less frequent (Luxton et al., 1996). There is also evidence for the impairment of HPV-16 E5-, E6- and E7-specific T cell responses in patients with cervical disease (Gill et al., 1998; Nakagawa et al., 1996; Tsukui et al., 1996), while others have found that responses to the HPV-16 E7 peptide aa 72–97 are more predominant in women with cervical disease than in controls (Kadish et al., 1994).

A better understanding of the role of HPV-specific cell-mediated immune responses in the natural history of cervical disease requires prospective studies to be carried out whereby the immune responses of patients and controls are closely monitored over a period of time and correlated with HPV infection and disease status. This should also result in better-defined and more easily comparable patient and control groups. Relatively few such studies have been conducted to date and no clear pattern has emerged as to which T cell responses (if any) might be associated with regression or progression of disease. De Gruijl et al. (1996, 1998) showed that patients with virus persistence and disease progression were more likely to respond to HPV-16 E7 peptides. Kadish et al. (1997), however, showed that responses to an N-terminal peptide of E6 and a C-terminal peptide of E7 were more strongly associated with clearance of virus infection on follow-up. A prospective study of T cell responses to L1 found no association with virus persistence or clearance (De Gruijl et al., 1999), although the authors reported evidence of an immunogenic region at aa 311–335, which is within the region previously identified by our group in cross-sectional studies (Shepherd et al., 1994, 1996). An association between responses to...
this region and the HLA DRB1*11/DQB1*0301 haplotype was also demonstrated.

We have conducted a prospective study in order to investigate further the down-regulation of HPV-16 E7-specific T cell responses, which was previously observed in women with squamous intraepithelial lesions of the cervix (SIL). The main objective of the study was to compare T cell responses of patients to HPV-16 L1 and E7 at different time points in order to determine whether T cell down-regulation might be linked to recurrence of cervical disease in patients following treatment, or progression of disease in untreated patients. The effects of treatment on both HPV-16 L1- and E7-specific T cell responses were also investigated, including whether or not effective treatment could result in a restoration of the E7 responses previously found to be absent. Other questions addressed were whether the specificity of the initial response or any changes in response observed during the follow-up period reflected the outcome of disease following treatment.

**METHODS**

**Patient recruitment and sample collection.** Ethical permission was obtained to recruit patients referred to Guy’s and St Thomas’ Hospital Trust for investigation of abnormal cervical smears into the study. On their first visit to the clinic (day 0), ectocervical and endocervical smears were taken, colposcopy was performed and routine biopsies were obtained (for histological diagnosis) by the investigating clinician. Patients were assigned to one of three main histological categories following diagnosis: (i) no dysplasia (patients who were referred for colposcopy due to an abnormal cervical smear but who subsequently showed no evidence of CIN on histology); (ii) HPV/CIN I (where either koilocytosis was observed suggesting HPV infection or CIN I disease was present); or (iii) CIN II/III (where evidence of high-grade disease was found). For comparison of T cell responses, the control group comprised patients who presented with no dysplasia. A 50 ml venous blood sample was obtained for studies of HPV-16-specific proliferative T cell responses. T cell assays were performed on 58 patients at day 0. A cervical brush swab sample was also taken for HPV DNA typing by PCR. Patients with cervical disease were treated according to colposcopy clinic guidelines, most often on a ‘see and treat’ basis on day 0, either by cold coagulation, loop excision or knife cone biopsy. Patients were asked to attend a follow-up appointment 6 months later when the same procedure was repeated. The mean time to follow-up for 33 patients who had CIN I (where either koilocytosis was observed suggesting HPV infection or CIN I disease was present) and 25, 281 and 295, and 311 and 345. The amino acid numbering was from the classical L1 start codon and peptides used were as described in Shepherd et al. (1996). E7-specific STLs were assayed against phytohaemagglutinin (1 μg ml–1) as a positive control, GST–E7 fusion protein and GST protein alone (10 μg ml–1), and three pools of 15-mer peptides overlapping by five amino acids, representing HPV-16 E7 aa 1–34, 30–74 and 70–98 (10 μM). All cell lines were tested against culture medium only as a negative control. In proliferation assays, a positive STL response was required to have a c.p.m. of >500 above the medium-only control value and a stimulation index of >2.5. In addition, a positive patient response was required to have ≥2/20 STLs responding to the same peptide.

**HPV DNA detection in cervical biopsy tissue and cervical brush swab samples.** The methods used for extraction of DNA from cervical biopsy tissue and HPV DNA typing by PCR were as previously described (Shepherd et al., 1996). The method for processing of cervical brush swabs prior to HPV DNA typing was as follows. Swabs were collected into 5 ml sterile PBS and stored at −20°C prior to processing. The sample was thawed and vortexed vigorously for 30 s in order to free cellular material from the brush. The brush was then carefully discarded and the cell suspension transferred to a sterile centrifuge tube. The cells were pelleted by centrifugation at 1000 g for 5 min. The pellet was then resuspended in 100–200 μl proteinase K buffer (0.01 M Tris/HCl, pH 7.8, 0.005 M EDTA, 0.5% w/v SDS) containing 1 mg ml–1 proteinase K (Qiagen) and the solution was incubated at 56°C overnight. The enzyme was inactivated by heating at 95°C for 10 min. The digest was then centrifuged at 13 000 g for 30 s before storing at −20°C. PCR was performed on undiluted proteinase K digests.

**RESULTS**

**Cross-sectional analysis of HPV-16-specific T cell responses at recruitment**

**Histology and HPV DNA typing.** Histological diagnosis was performed on cervical biopsy tissue obtained from each patient on recruitment into the study. The results showed that 15/58 patients (26%) had no dysplasia, 26/58 (45%) had HPV/CIN I, 10/58 (17%) had CIN II, 6/58 (10%) had CIN III and one patient had carcinoma of the cervix. HPV DNA typing by PCR was performed on DNA extracts obtained from either cervical brush swabs and/or cervical biopsy tissue samples from 42/58 patients. Of those patients tested who had no dysplasia (n = 8), four (50%) were HPV-negative and four were HPV-16-positive, including one individual with a dual infection with HPV type 18. Of those patients tested with HPV/CIN I lesions (n = 23), the numbers infected with each HPV type were as follows: nine (39%) had HPV-16; two (9%) had HPV-16 + 18; one (4%) had HPV-16 + 31; two had HPV31; two had HPV33; six (26%) had HPVX and one was HPV-negative. Of those patients tested with CIN II lesions (n = 6), one individual (17%) was positive for each of the HPV types 16, 18, X and 6, and two (33%) were HPV-negative, whereas of the four patients with CIN III lesions, two (50%) were HPV-16-positive, one was HPV31-positive and one was HPV-negative. The single patient with cervical carcinoma was HPV-16-positive.

**HPV-16-specific T cell responses in patients and controls.** In Fig. 1, the magnitude and peptide specificity of

**T cell culture and proliferation assays.** Short-term T cell lines (STLs; 20 per antigen per patient) were established using either virus-like particles (VLPs) of HPV-16 L1 or an HPV-16 GST–E7 fusion protein as the stimulating antigen. Details of the methods involved have been described previously (Luxton et al., 1996, 1997; Shepherd et al., 1996). It was previously established that the majority of HPV-16-specific cell lines generated by this technique are CD4-positive (Shepherd et al., 1996).

In standard 3-day proliferation assays, L1-specific STLs were tested for specificity against VLPs of L1, HPV-16 β-gal–L1 fusion protein and β-galactosidase protein alone, each at 1 μg ml–1, and 15-mer peptides or pools of peptides (10 μM) representing previously identified immunogenic regions of the L1 molecule between aa 191
HPV-16 L1- and E7-specific T cell responses of patients with \((n=42)\) and without \((n=15)\) SIL on their first visit to the clinic were compared. The percentage of responders to each peptide or peptide pool is summarized in the inset figure. Patients with SIL responded to each of the HPV-16 L1 regions tested, where the most immunogenic region was between aa 311 and 345 with 23/42 (55%) patients responding, whereas 11/42 patients (26%) responded to peptide pool of aa 191–225 and 9/42 (21%) responded to the single peptide aa 281–295. The peptide pool representing aa 311–345 also gave the highest numbers of responding STLs per patient of all the peptide pools tested (range 0–18, mean 3.4). On comparison with the HPV-16 L1-specific responses of the 15 patients who presented without SIL (Fig. 1b), a statistically significant reduction in the response to the L1 peptide pool of aa 311–345 was observed \((P=0.041)\), whereas changes in the responses to other peptides observed were not statistically significant \((P>0.05)\). The range of responses to the L1 peptide pool of aa 311–345 was also reduced in patients without SIL (range 0–7, mean 1.33) reflecting lower peptide-specific T cell precursor frequencies in this patient group. Overall, 28/42 (67%) patients with SIL responded to one or more L1 peptides compared with 5/15 (33%) patients without SIL \((P=0.027)\).

The most immunogenic region of E7 in patients with SIL was the C terminus at aa 70–98 to which 15/42 (36%)

Fig. 1. HPV-16 L1- and E7-specific T cell responses of patients at baseline. (a, b) Peptide-specific T cell responses of 20 L1- and 20 E7-stimulated STLs per individual for patients with \((n=42)\) (a) and patients without \((n=15)\) SIL (b). The x-axis figures are amino acid residues designating regions of L1 or E7 spanned by a single 15-mer peptides (marked with an asterisk) or peptide tri-pools; solid black bars represent mean numbers of STLs responding to each peptide; inset figures represent the percentage of the group who responded to each peptide or peptide pool. (c) The percentage of responders to each peptide pool according to histological group. The definition of a positive STL response is given in Methods.
patients responded (Fig. 1a), whereas 8/42 (19%) responded to the N-terminal region at aa 1–34 and 1/42 (2%) to the central portion of the molecule at aa 30–74. Fewer patients responded to any one or more E7 peptide than to L1 peptides (19/42 or 45% compared with 28/42 or 67%). Also, the percentage of responders to the E7 pool of aa 70–98 was reduced in patients without SIL (2/10 or 20% responders, Fig. 1b) compared with those with SIL (15/42 or 36%), but neither of these findings was statistically significant (P>0.05). The single patient with cervical cancer responded to the L1 regions of aa 281–295 and 311–345 and did not respond to E7.

#### HPV-16-specific T cell responses and relationship to severity of cervical lesion and infecting HPV type.

The T cell responses of patients with different grades of cervical lesion were compared with those of patients who presented with no dysplasia (Fig. 1c). Patients from all histological groups responded to all peptides tested except the E7 pool of aa 30–74, which failed to induce a response in patients with HPV/CIN I or CIN II lesions. Responses to most peptide pools were greater in patients with CIN III lesions than in those with HPV/CIN I or CIN II lesions, although the only statistically significant difference was between responses to the L1 peptide aa 281–295 (P=0.027). On the other hand, patients responded equally well to the peptide pool of aa 311–345, no matter what grade of lesion they had.

The responses detected, however, were not significantly associated with current cervical HPV-16 DNA infection (P>0.05), as 9/15 (60%) individuals responded to L1 and 6/15 (40%) to E7 in the HPV-16 DNA-positive group, compared with 12/18 (67%) who responded to L1 and 10/18 (56%) to E7 in the HPV-16 DNA-negative group. It should be noted that four individuals who responded to L1 and/or E7 were HPV31-positive, hence responses detected in the absence of HPV-16 infection may result from cross-reactivity to closely related HPV types such as HPV 31 or from previous HPV-16 infections.

#### HPV-16 L1- and E7-specific T cell responses in patients following treatment for SIL of the cervix

**Patient follow-up, changes in histology and HPV DNA status.** Thirty-three of the 58 patients who entered the study were followed up with a mean time to first follow-up of 14±11 months. Twenty-three patients were followed up after 3–11 months (mean ± SD = 7 ± 1.9 months) and the remaining 10 patients after 17–39 months (mean ± SD = 28 ± 8.1 months). On their first visit to the clinic, 10 patients (30%) had no dysplasia, 17 (52%) had HPV/CIN I lesions, four (12%) had CIN II lesions and two (6%) had CIN III lesions. All patients with disease were treated on or shortly after their first visit to the clinic. Fig. 2 illustrates the outcome of treatment for each patient, comparing cervical histology at first and follow-up visits. To summarize these data, at their first follow-up visit, most patients had no dysplasia (20/33 or 61%), 12/33 (36%) had low-grade (HPV/CIN I) disease and one patient (3%) had a recurrent CIN III lesion. Of those patients with no SIL at follow-up who were typed (n = 7), three were HPV-16-positive (30% of the whole group) and four were HPV-negative. Those patients with low-grade lesions that were typed (n = 5) were all HPV-16-positive.

![Fig. 2. Comparison of cervical histology before and after treatment showing the number of patients with different grades of cervical lesion at first visit (i), the outcome of their treatment in terms of cervical histology at follow-up (ii) and the total number of patients with different grades of lesion at follow-up (iii).](image)
(29% of the whole group) and the single CIN III lesion was HPV-negative.

Comparison of patient T cell responses before and after treatment. The response profiles of patients with and without SIL, before and after treatment, did not change greatly and the same L1 and E7 peptide-specific responses remained immunodominant after treatment (data not shown). Although small increases in the percentage of responders to the L1 peptide aa 281–295 and the E7 peptide pool of aa 70–98 were observed after treatment, they were not statistically significant \( (P>0.05) \). At follow-up, as at first visit, patients without SIL showed a decrease in the range and frequency of many responses compared with those with SIL, but none of these changes was statistically significant.

HPV-16-specific T cell responses and recurrence of cervical disease following treatment. In order to investigate the relationship between the T cell responses of individual patients and the outcome of their disease following treatment, patients with SIL at first visit were grouped according to whether or not they had recurrent disease at follow-up and those without SIL at first visit according to whether or not they had acquired disease by the time of follow-up. By analysis of HPV-16-specific T cell responses before and after treatment, we could then determine whether the peptide specificities of responses measured at the outset or any changes in the specificities of responses occurring during the period of follow-up might reflect the outcome of disease following treatment.

Of the 23 patients with SIL at first visit, nine had recurrent disease (this was recurrent high-grade disease in one case only) and 14 were disease-free at follow-up (Fig. 2). Of the 10 patients with no SIL at first visit, four had acquired low-grade lesions by the time of follow-up and six remained disease-free.

On comparison of the magnitude of responses made by patients (data not shown), 2/14 patients without recurrent lesions made particularly strong responses to the L1 region of aa 311–345. However, the mean STL response to this region in patients without recurrent lesions was not significantly higher than that of patients with recurrent lesions \( (P>0.05) \). On comparison of the total percentage of patients responding to each peptide pool (Fig. 3), patients with and without recurrent lesions were found to respond equally well to all peptide pools, with the exception of the E7 pool of aa 70–98 to which 67% (6/9) of patients with recurrent disease responded compared with 14% (2/14) of patients without recurrent disease. Therefore, T cell responses to the HPV-16 E7 region of aa 70–98 was significantly associated with recurrence of disease following treatment \( (P<0.05) \).

Five healthy, asymptomatic individuals with no previous history of cervical disease were demonstrated to respond to the HPV-16 E7 region of aa 70–98 in an earlier cross-sectional study (Luxton et al., 1996), but were subsequently shown to have a different clinical outcome to that of women with cervical disease. Thus, 4/5 individuals reported that they had been screened regularly for cervical cancer during the intervening period of 6–8 years and that no cervical abnormalities had been detected during this time.

Patient T cell responses were compared before and after treatment to determine whether any change (loss or gain) in response to either L1 or E7 was associated with the recurrence or absence of disease on follow-up (data not shown). However, none of the changes observed were significantly associated with recurrent disease \( (P>0.05) \).

Interestingly, on comparison of the total number of STLs per patient group responding to each L1 peptide pool before and after treatment, increases were observed in patients with recurrent disease following treatment (aa 191–225, 54%; aa 291–295, 36%; aa 311–345, 48%). In patients without recurrent disease on follow-up, there were corresponding decreases in the total numbers of L1-specific STLs of 42, 8 and 58%, respectively.

HPV-16-specific T cell responses and acquisition of cervical disease. Fig. 4 compares the T cell responses of 10 patients who were disease-free at recruitment according to the outcome of their disease at follow-up. Three out of four (75%) of those patients who acquired disease during the study period also gained responses to E7, including responses to the C-terminal region of aa 70–98 in all cases, whereas only 1/6 (17%) of those individuals who did not acquire disease during the study period gained responses to E7. Although this difference was not statistically significant \( (P=0.119) \) and the numbers involved
DISCUSSION

Cross-sectional analysis of HPV-16-specific T cell responses of patients at recruitment

The first part of this study presented a cross-sectional analysis of the HPV-16-specific T cell responses to previously identified immunogenic regions of L1 and to the whole of E7. Interestingly, responses to L1 were similar whether VLPs of HPV-16 L1 were used or L1 fusion proteins as in previous studies. Hence, the most immunogenic region of L1 was aa 311–345 (responses detected in 55% of patients), followed by aa 191–225 (26% of patients) and aa 281–295 (21% of patients). As in previous studies, responses to E7 were reduced in comparison with responses to L1 (45% versus 67%, respectively), although in this study the C terminus (aa 70–98) was the most immunogenic (36% of patients), with some response to the N terminus (aa 1–34) and very little to the central region (aa 30–74). Responses to L1 aa 311–345 were significantly associated with disease ($P<0.041$) but not with current HPV-16 DNA positivity, as determined by PCR ($P>0.05$). This might be expected because memory T cell responses will be detected by this technique, irrespective of whether a current HPV-16 infection is present or not. HPV-16 L1- and E7-specific T cell responses were detected in the absence of SIL, although generally at lower levels; these may also be memory responses resulting from previous HPV-16 infections or may be due to current subclinical infections. In support of this theory, PCR data showed that HPV-16 DNA was present in the cervix of 29% of patients without SIL at their first visit to the clinic.

On comparison of the peptide-specific responses of patients with different histological grades of lesion at first visit, the levels of response to the L1 peptide aa 281–295 and E7 peptide pools of aa 1–34 and 70–98 were clearly raised in patients with CIN III compared with those with lower-grade lesions. This difference was statistically significant for responses to peptide aa 281–295 only ($P=0.027$). The differences observed were not directly linked to the levels of current HPV-16 positivity observed in different histological groups ($P>0.05$); it is therefore assumed that they resulted from recent or perhaps repeated previous exposure to HPV-16 infection.

As found in previous studies, the ability of patients with SIL to respond to any one or more HPV-16 E7 peptides differed from their ability to respond to L1 peptides. Whilst T cell responses to L1 peptides increased with increasing histological grade of lesion, responses to E7 were reduced in comparison in all grades of lesion, although none of these differences was statistically significant. It is not clear why the levels of T cell response to the L1 and E7 proteins are different. It is possible that the higher levels of E7 expression that occur in high-grade lesions might be necessary to stimulate a detectable E7-specific response, whereas in low-grade lesions, many of which involve productive virus infections, T cell responses to capsid proteins such as L1 may predominate.

Fig. 4. HPV-16-specific T cell responses and development of cervical disease. The figure shows the T cell responses of 10 patients without SIL at first visit (a) and the T cell responses at follow-up of four of these patients who acquired L-SIL (b) and six patients who remained disease-free during the follow-up period (c). For comparison of responses between (a), (b) and (c), refer to patient number.
HPV-16-specific T cell responses and treatment for SIL of the cervix

The HPV-16-specific T cell responses of patients with SIL before and after treatment did not change greatly, in terms of peptide specificity, the range of response observed, or the numbers of patients responding, suggesting that treatment itself had no significant positive or negative effect on these responses. Small increases in the level of T cell response to certain peptides/peptide pools were observed (the L1 peptide aa 281–295, the pool of aa 311–345 and the E7 pool of aa 70–98), although these were not statistically significant. On comparison of patients with the same histological grade of lesion, HPV/CIN I, before and after treatment, responses to L1 peptide 281–295 and the E7 pool of aa 70–98 were found to be increased on follow-up. The increases in responses observed in patients with SIL at follow-up could be due to memory responses, which are boosted by reinfection with HPV-16, or the result of a ‘new’ HPV-16 infection, both of which occurred in our patient group. Overall, the ability of patients with SIL to respond to any one or more E7 peptides was only slightly increased at follow-up, whereas the ability to respond to L1 peptides did not change.

Comparison of the HPV-16-specific T cell responses of patients without SIL at first and follow-up visits showed little change in the response of the group as a whole, even though 3/7 of these patients had evidence of ‘new’ HPV-16 infections at follow-up by PCR (four were HPV-negative). These new infections were accompanied by the acquisition of an L1-specific response in one case and an E7-specific response in another.

HPV-16-specific T cell responses and recurrence of SIL

To investigate the relationship between HPV-16-specific T cell responses and SIL of the cervix, patients were grouped according to the outcome of their treatment, that is, whether or not they had recurrent disease, whether they remained disease-free throughout the study period or whether they acquired disease during this period. By studying the T cell responses of these groups of patients, it was found that changes in the HPV-16 L1 and E7 epitope specificity of T cells were not significantly associated with the outcome of cervical disease following treatment. Therefore, neither the absence of E7-specific responses at the outset nor the loss of any E7-specific response during the study period were associated with recurrence of disease. Conversely, successful treatment did not lead to the restoration of E7 responses in individuals where they were previously absent. Therefore, the down-regulation of HPV-16 E7-specific responses in patients with cervical disease, which was observed in this and previous studies (Luxton et al., 1997), was not associated with a worse prognosis for the patient.

Although recurrence of disease was accompanied by an increase in the frequency of HPV-16 L1-specific T cells and absence of disease on follow-up by a decrease in the frequency of L1-specific T cells, these findings were not statistically significant.

However, T cell responses to the E7 peptide pool of aa 70–98, measured at the patient’s first visit to the clinic, were significantly associated with the recurrence of disease following treatment (P<0.017). This does not appear to be true for healthy controls who respond to the same region of E7. The acquisition of this response also accompanied the acquisition of disease in the majority of patients (75 %) who were disease-free at the start of the study. Although the patient numbers involved were small, the data suggest that E7 aa 70–98-specific responses do not protect against reinfection by HPV and recurrence of cervical disease. It is not clear why this particular T cell response should be linked with a worse prognosis for the patient at a later date; perhaps repeated or persistent infection by HPV-16 is required to achieve the levels of antigen expression necessary to stimulate E7-specific T cells and therefore these patients are those in whom disease is more likely to reoccur following treatment or progress in those who are untreated.

We propose that larger prospective studies are conducted to confirm the link between E7 aa 70–98-specific T cell responses and recurrence of cervical disease. If these findings are substantiated, this T cell response might be useful in identifying a subset of patients who are at greater risk of developing recurrent or persistent disease.

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