Human papillomavirus type 16 E2- and L1-specific serological and T-cell responses in women with vulval intraepithelial neoplasia

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Human papillomavirus type 16 (HPV-16)-associated vulval intraepithelial neoplasia (VIN) is frequently a chronic, multifocal high-grade condition with an appreciable risk of progression to vulval cancer. The requirement to treat women with VIN has recently stimulated the use of immunotherapy with E6/E7 oncogene vaccines. Animal models have shown that E2 may also be a useful vaccine target for HPV-associated disease; however, little is known about E2 immunity in humans. This study investigated the prevalence of HPV-16 E2-specific serological and T-cell responses in 18 women with HPV-16-associated VIN and 17 healthy volunteers. E2 responses were determined by full-length E2–GST ELISA with ELISPOT and proliferation assays using E2 C-terminal protein. As positive controls, HPV-16 L1 responses were measured using virus-like particles (VLPs) and L1–GST ELISA with ELISPOT and proliferation using VLPs as antigen. The VIN patients all showed a strong serological response to L1 compared with the healthy volunteers by VLP (15/18 vs 1/17, \(P < 0.001\)) and L1–GST ELISA (18/18 vs 1/17, \(P < 0.001\)). In contrast, L1-specific cellular immune responses were detected in a significant proportion of controls but were more prevalent in the VIN patients by proliferation assay (9/17 vs 17/18, \(P < 0.02\)) and interferon-γ ELISPOT (9/17 vs 13/18, \(P = \text{not significant}\)). Similar and low numbers of patients and controls were seropositive for E2-specific Ig (2/18 vs 1/17). In spite of previous studies showing the immunogenicity of E2 in eliciting primary T-cell responses \textit{in vitro}, there was a low prevalence of E2 responses in the VIN patients and controls (2/18 vs 0/17).

INTRODUCTION

Genital human papillomavirus (HPV) infections are extremely common in young, sexually active women (Ho et al., 1998). While the majority of infections are inconsequential and short-lived, virus persistence is associated with anogenital disease, especially cervical carcinogenesis (Rozendaal et al., 1996). Several observations have indicated that host immunity plays an important role in effecting viral clearance, and this has provided the impetus for the development of vaccines to prevent and/or treat cancer of the cervix and its precursor lesions. Most of these approaches have concentrated on eliciting specific immunity against the HPV E6 and E7 oncoproteins, which are expressed throughout the spectrum of HPV-associated disease development (Borysiewicz et al., 1996; Klencke et al., 2002; Muderspach et al., 2000; Santin et al., 2002).

E2 has recently been proposed as a potential immune target for vaccines aimed at treating pre-malignant disease (Schiller & Hildesheim, 2000). This has been supported by data from the cotton tail rabbit papillomavirus (CRPV) model, where vaccination of rabbits with DNA encoding CRPV E2 was found to protect against the development of papillomas following subsequent tumour challenge (Han et al., 2000a, b). There are limited data on responses to E2 in patients with HPV-associated disease. Serology studies have provided conflicting results with IgA and IgG responses to...
a cryptic peptide correlating with antigen exposure in cervical cancer patients (Dillner et al., 1994; Hamsikova et al., 1994; Lehtinen et al., 1992; Lenner et al., 1995), while IgA (but not IgG) responses detected using a baculovirus-produced E2 protein were associated with low- rather than high-grade cervical intraepithelial neoplasia CIN) and cervical cancer (Rocha-Zavaleta et al., 1997). These assays variously can detect linear or conformational epitopes, respectively, so their lack of concordance may not be surprising. A single study has investigated naturally occurring E2-specific T-cell responses in women with HPV-associated CIN where helper T-cell responses to the E2 C terminus, but not to the N terminus, occurred at the time of viral clearance (Bontkes et al., 1999). Clearly there is a need for further baseline investigations of naturally occurring E2-specific immune responses in patients with HPV-associated disease.

VIN is associated with high-risk HPV-16 infection in over 90% of cases and has shown a significant increase in incidence over recent decades, particularly in younger women (van Beurden et al., 1995). It is a chronic disease characterized by recurrence following standard treatment (Herod et al., 1996). It therefore provides an excellent opportunity for the study of naturally occurring HPV-specific immune responses in women with persistent high-risk HPV infections. This immunity is likely to have arisen as a consequence of chronic exposure to the virus but may be insufficient to effect disease regression and virus clearance. The relapsing and recurring nature of VIN is likely to represent an ongoing battle between viral and immune factors in the natural control of this disease. Thus, a comparison of HPV-specific immunity in women with chronic HPV infections and in those without may provide some insight into those immunological responses that play an important role in the natural history of HPV-associated disease.

We have previously described an interferon-γ (IFN-γ) ELISPOT assay, which facilitates the detection of HPV-16 E2-specific memory T-cell responses to E2 C terminus protein following its processing and presentation by autologous dendritic cells (DCs) (Davidson et al., 2001). The advantage of this system is that it is not limited by the use of HLA-restricted peptides but rather allows the study of the full range of T-cell responses that may have arisen as a consequence of natural antigen presentation in vivo. In the present study, we have measured the 86 amino acid C-terminal E2- and virus-like particle (VLP) L1-specific T-cell responses in women with HPV-16-associated VIN and healthy female volunteers using this methodology and proliferation. HPV-16 L1 VLP-specific T-cell responses were measured as a positive control, since previous studies have found high proportions of healthy volunteers and patients with HPV-16-associated high-grade CIN to respond to this antigen. In addition we measured full-length GST–E2- and both GST–L1 and VLP L1-specific serological responses in the VIN and control subjects.

**METHODS**

### Patient and volunteer materials

Eighteen women with HPV-16-positive VIN 3 were recruited from the Vulva Clinic at St Mary’s Hospital, Manchester, UK. Seventeen female, age-matched, healthy volunteers were recruited from the staff at the Paterson Institute for Cancer Research, Manchester, UK. These healthy volunteers were recruited on the basis that they were medically fit with a lifetime history of normal cervical cytology and no previous history of cervical or vulval pathology. The Central Manchester Local Research Ethics Committee approved the study and all subjects gave written informed consent. A vulval biopsy was taken from the patients for histological grading and HPV analysis. Histological grading of all tissue specimens was performed by two independent consultant histopathologists with a special interest in gynaecological oncology. HPV analysis was performed on DNA extracted from vulval biopsies by proteinase K digestion and amplified with β-globulin primers (Bauer et al., 1992). HPV DNA was detected using GP5+/-GP6+ consensus primers (De Roda Husman et al., 1995) and genotyped using type-specific primers (Soler et al., 1991). Amplification products were resolved on 2% agarose gels and visualized by ethidium bromide staining.

### Isolation of plasma/PBMCs

Forty ml of blood from each subject was heparinized (6000 iU heparin per 20 ml blood), layered on to Lymphoprep lymphocyte separation medium (LSM; Nycomed-Amersham) and centrifuged at 900 g for 20 min at 20 °C. Aliquots of plasma (250 μl) were stored at −20 °C until required. PBMCs were collected from the LSM interface and washed by centrifuging at 400 g in PBS three times for 10 min each. PBMCs were frozen at a concentration of 5 x 10⁶ cells ml⁻¹ in 10% DMSO in foetal calf serum and stored in liquid nitrogen until required.

### Serological assays

HPV-16 E2- and L1-specific Ig was detected in the patient plasma by ELISA. L1 antibodies were measured using both a recombinant GST-tagged L1 fusion protein and an HPV-16 VLP protein as antigen.

### HPV-16 L1 VLP preparation

The HPV-16 L1 gene was cloned into the baculovirus transfer vector pAcGHLT (Pharmingen) and co-transfected with baculovirus DNA into Sf9 cells (obtained from Wei Zhang, Dept of Pathology, University of Cambridge). The resultant supernatant was then titrated and used for infection. Flasks of 80–90% confluent Sf9 cells were infected with baculovirus expressing the HPV-16 L1 protein for 72 h under constant agitation at 27 °C. Cells were then pelleted by centrifugation at 200 g and processed in a similar manner to that previously described (Kirnbauer et al., 1993; Zhou et al., 1992). In summary, cells were homogenized using a Dounce homogenizer, and then centrifuged at 7500 r.p.m. in a JA20 rotor. The pellet was resuspended, and then sonicated at 22 μm using a probe sonicator. This was loaded on to 40% sucrose and centrifuged at high speed (45 000 r.p.m.) in an SW50 Ti rotor for 2 h. The pellet was resuspended and sonicated again briefly before mixing with caesium chloride solution to a final density of 1.33 g cm⁻³. This mixture was centrifuged overnight at 45 000 r.p.m. in an SW50 Ti rotor and the resultant VLP band at a density of 1.29 g cm⁻³ was collected. This was dialysed against PBS to remove the caesium chloride and the preparation quality was checked by Western blot, Coomassie staining and visualization by electron microscopy.

### HPV-16 VLP ELISA

Protein immobilizer microwell plates (Eixon) were coated with baculovirus HPV-16 VLP at 2 μg ml⁻¹ in PBS (antigen) or with PBS only (control) at 37 °C for 1 h. The plate was washed thoroughly with 0.05% Tween in PBS (PBST; Sigma) after this and each subsequent step. Plasma dilutions (1:100, 1:400 and 1:1600) were prepared in 3% BSA (highly purified >97%; Sigma)
and added to the plate in duplicate wells for 1 h at room temperature. One plasma sample with a high HPV-16 L1 VLP absorbance was included as a positive control on every plate. Bound antibodies were detected by incubation with goat anti-human IgG (heavy and light chain; Jackson ImmunoResearch Laboratories) (1:2400 in 1% BSA), followed by biotinylated rabbit anti-goat (1:5000 in 1% BSA; Dako) each for 1 h and streptavidin–HRP (1:5000 in 1% BSA; Dako) for 30 min. The plates were developed with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) and hydrogen peroxide (H₂O₂) in phosphate/citrate buffer (pH 5). The reaction was stopped by addition of 1 M H₂SO₄ and the plates read on an automatic plate reader. The sample total VLP-specific absorbance was determined by subtracting the mean absorbance of the control-coated wells at a plasma dilution of 1:400 from the mean absorbance of the antigen-coated wells at the same plasma dilution (1:400). This was normalized across the plates by dividing the sample total VLP-specific absorbance by the positive control total VLP-specific absorbance from that particular plate.

GST capture ELISA for HPV-16 L1 and E2. These ELISAs were performed as described by Sehr et al. (2001, 2002). Briefly, 96-well Polysorb plates (Nunc) were coated with gluthathione cross-linked to casein. Thereafter, wells were blocked with casein (0.2%, w/v, in PBST) (blocking buffer) followed by incubation with cleared lysates from E. coli overexpressing either L1 lacking the 10 N-terminal amino acids or full-length E2 from HPV-16 as GST–X–tag proteins. E. coli PBST) (blocking buffer) followed by incubation with cleared lysates normalized across the plates by dividing the sample total VLP-specific absorbance by the positive control total VLP-specific absorbance from that particular plate.

T-cell assays

E2 C-terminal protein. The plasmid pWEB-E2Ct expresses a truncated 86 amino acid E2 protein that lacks the N-terminal amino acids of E2 from 1 to 279 but dimerizes and binds DNA normally. Human sera were diluted 1:50 in blocking buffer containing 0.1% casein. Thereafter, wells were blocked with casein (0.1% w/v in PBST) (blocking buffer) and added to the plate in duplicate wells for 1 h at room temperature. One plasma sample with a high HPV-16 L1 VLP absorbance was included as a positive control on every plate. Bound antibodies were detected by incubation with goat anti-human IgG (heavy and light chain; Jackson ImmunoResearch Laboratories) (1:2400 in 1% BSA), followed by biotinylated rabbit anti-goat (1:5000 in 1% BSA; Dako) each for 1 h and streptavidin–HRP (1:5000 in 1% BSA; Dako) for 30 min. The plates were developed with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) and hydrogen peroxide (H₂O₂) in phosphate/citrate buffer (pH 5). The reaction was stopped by addition of 1 M H₂SO₄ and the plates read on an automatic plate reader. The sample total VLP-specific absorbance was determined by subtracting the mean absorbance of the control-coated wells at a plasma dilution of 1:400 from the mean absorbance of the antigen-coated wells at the same plasma dilution (1:400). This was normalized across the plates by dividing the sample total VLP-specific absorbance by the positive control total VLP-specific absorbance from that particular plate.

T-cell stimulation. Autologous PBLs at a concentration of 3×10⁶ ml⁻¹ in RPMI 1640 supplemented with 10% human AB serum (Quest Biomedical), 100 μg streptomycin ml⁻¹ and 100 iU penicillin (Life Technologies) ml⁻¹ were added to E2, VLP or PPD protein-loaded DCs and cultured for 4 days. DCs without added antigen were also cultured with autologous PBLs for 4 days (negative control).

ELISPOT assay. The PBL cultures were harvested, washed and seeded in three replicate wells at three doubling dilutions from 1×10⁵ cells per well of a multiscreen 96-well Immobilon-P filtration plate (Millipore) coated with anti-IFN-γ antibody [capture mAb (1-D1K), ELISPOT assay kit; Mabtech]. In addition, PBMCs were seeded in duplicate wells at a concentration of 2×10⁵ cells per well with medium alone (negative control), 10 μg E2 terminus protein ml⁻¹, 10 μg HPV-16 VLP ml⁻¹, 35,000 U PPD ml⁻¹ or 0.5 μg phytohaemagglutinin (PHA; Murex Diagnostics) ml⁻¹ (positive control) in the same plate. The plates were incubated overnight at 37°C and the assay developed according to the manufacturer's instructions. Spots were counted at each cell density using a dissecting microscope, scores were normalized and the mean and standard errors calculated. Results were plotted as the number of IFN-γ-producing cells per 2×10⁴ cells (for DC-stimulated T cells) or 2×10⁵ cells (for PBMCs) after subtracting the background (medium control) responses. A positive ELISPOT was defined as ≥20 spots per 2×10⁴ or 2×10⁵ cells, respectively.

Proliferation assay. PBMCs were seeded in triplicate wells of a 96-well round-bottomed microtitre plate (Alpha Laboratories) at 2×10⁵ cells per well in RPMI supplemented with 10% AB serum, 100 μg streptomycin ml⁻¹ and 100 iU penicillin ml⁻¹. PBMCs alone (medium control) or PBMCs with 5 μg E2 protein ml⁻¹, 2.5 μg HPV-16 VLP ml⁻¹, 35,000 U PPD ml⁻¹ or 0.5 μg PHA ml⁻¹ were incubated for 5 days at 37°C. During the final 18 h of culture, 1 μCi per well of [³H]thymidine (NEN Life Science Products) was added. The cells were harvested using a Packard 96-well vacuum cell harvester on to Unifilter plates (Packard Biosciences), left to dry overnight and 30 μl per well of Microscint 20 scintillation fluid (Packard Biosciences) was added. [³H]Thymidine incorporation was measured using a Topcount scintillation counter (Packard). Results were plotted as the mean number of counts for the antigen-stimulated PBMCs divided by the mean number of counts for the medium only (negative control) PBMCs to give the stimulation index. A stimulation index of 2 or more was scored as positive.

Statistical analysis. Cut-off values to define antibody-positive sera were calculated separately for each antigen and assay as the mean of the specific absorbance values of all control sera+3SD excluding positive outliers. The χ² test was used to compare serological and T-cell responses in the patient and control groups. Student’s t-test was used to compare the mean ages between the two groups. A value of P<0.05 was considered statistically significant and values of P>0.05 were considered not significant (NS).

RESULTS

Patient and control group characteristics

The mean age of the VIN patients and healthy volunteers was 41-1 years (range 28–61 years) and 39-5 years (range 27–58), respectively (P=NS). The healthy volunteers

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comprised women with a lifetime history of normal cervical cytology and no previous history of cervical or vulval pathology. The VIN patient group comprised women with HPV-16-positive high-grade VIN. A histological diagnosis of VIN 3 had been made on average 6.4 years (range 6 months–17 years) prior to recruitment into the study. Fourteen women had undergone at least one previous treatment and nine women had undergone more than three previous treatments for VIN 3 (surgical excision or laser therapy) before trial entry. Thirteen patients had also previously been treated for high-grade CIN either by laser cone biopsy, large loop excision of the transformation zone or hysterectomy.

**L1- and E2-specific serological responses**

Serological responses to HPV-16 L1 were measured in the VIN patients and controls by ELISA using both recombinant GST-tagged L1 fusion protein and HPV-16 VLP as antigen. L1-specific serological responses were more prevalent in the VIN group compared with the controls using both the L1 fusion protein (18/18 patients vs 1/17 controls, \( P < 0.0005 \)) and the VLP ELISA (15/18 patients vs 1/17 controls, \( P < 0.0005 \)) (Fig. 1). Linear regression analysis revealed a statistically significant correlation between the two ELISAs (\( r = 0.85, P < 0.001 \)). Small but similar numbers of women in both groups were seropositive for HPV-16 E2-specific Ig (2/18 VIN patients vs 1/17 controls) (Fig. 1).

**L1- and E2-specific T cells**

L1- and E2-specific T-cell responses were measured in the patient and control groups by proliferation assay and IFN-\( \gamma \) ELISPOT. PHA and tuberculin PPD were included as positive control antigens in both assays. All donors showed strong proliferative responses to PHA and PPD. Two different ELISPOT assays were used; in the first, PBMCs were incubated with the protein antigens for 24 h before testing for IFN-\( \gamma \) release. In the second, protein-loaded DCs were used to stimulate autologous T cells for 4 days before testing the latter for IFN-\( \gamma \) release. In both ELISPOT assays, strong PHA and PPD responses were observed, although the frequency of responding cells was much greater in the DC-stimulated T-cell assays than in the full PBMC assays. In contrast, L1- and E2-specific T-cell responses were only observed in the DC-stimulated T-cell assays and not in the PBMC assays (Fig. 2).

L1-specific T-cell responses were more prevalent in the VIN group than in the healthy volunteers, both by proliferation assay (17/18 patients vs 9/17 controls, \( P < 0.02 \)) and by IFN-\( \gamma \) ELISPOT (13/18 patients vs 9/17 controls, \( P = \text{NS} \)) (Table 1), although the latter did not reach statistical significance. Of the 35 women tested for L1-specific T-cell responses, seven showed proliferative responses only, 19 showed both proliferative responses and IFN-\( \gamma \) release, and three showed IFN-\( \gamma \) release only. All of the patients who showed L1-specific serological responses also demonstrated L1-specific T-cell responses, either by proliferation assay alone (5/17), by both proliferation assay and IFN-\( \gamma \) ELISPOT (12/17) or by IFN-\( \gamma \) ELISPOT alone (1/17). E2-specific T-cell responses were only detected in two VIN patients and in none of the controls. These responses were observed by both IFN-\( \gamma \) ELISPOT and proliferation assay; one of these patients was also seropositive for E2-specific
In addition, both showed L1-specific serological and T-cell responses by both T-cell assays.

**DISCUSSION**

In this study, we have investigated HPV-16 E2- and L1-specific serological and T-cell responses in women with HPV-16-positive high-grade VIN and female age-matched healthy volunteers with a negative history of previous or current cervical or vulval pathology. Studies in cervical neoplasia have shown that antibodies against the viral capsids are seen concomitantly with or within a few months following acquisition of HPV-16 DNA in up to 70% of those infected (Carter et al., 1996; Wikstrom et al., 1995) and are stable with time (Carter et al., 1996), even after 15 years of follow-up (Shah et al., 1997). There is therefore little evidence of exposure to HPV-16 in our cohort of healthy volunteers. This may be partly explained by their lifetime history of normal cervical cytology, although genital high-risk HPV infections are frequent and not always associated with abnormal smears. The cut-off values that were defined in this study for both assays were consistent with the absorbance values measured from sera from virgins (data not shown), suggesting that the normal sera were generally representative of a population not previously exposed to HPV-16. In contrast, VLP- and L1-specific antibodies were associated with the majority of HPV-16-positive high-grade VIN patients (15/18 and 17/18, respectively) compared with just one of the healthy volunteers demonstrating seropositivity.

A dominant antibody response in humans against HPV-16 capsids involves a type-specific epitope only present on assembled capsids and not on the individual pentavalent capsomeres (Heino et al., 1995; Wang et al., 1997). The L1 forms pentamers when its N terminus is fused to GST and this can allow detection of other L1-reactive antibodies. Thus, the GST–L1 assay may allow epitopes that are ordinarily hidden within the fully assembled capsid phenotype to become available for serological reactivity and this may explain why more responses were seen using this assay than with the VLP assay. Previous studies of HPV-16 VLP-specific antibodies in women with high-grade VIN found approximately 60% of patients to be seropositive, but these studies did not determine the HPV status of the lesions and therefore may not have identified HPV-16-negative disease (Hildesheim et al., 1997; Sun et al., 1996).

We found HPV-16 E2-specific Ig in one of the healthy volunteers, consistent with this population’s lack of exposure to HPV-16, but only 2/18 HPV-16-positive high-grade VIN patients were seropositive. From previous studies, serological responses to the E2 protein appear to correlate with antigen exposure (Lehtinen et al., 1992; Lenner et al., 1995). However, it is not clear how the different assays (baculoviral protein, peptide and GST fusion) for E2 antibodies can be directly compared. In particular, we do not know whether the GST–E2 fusion protein has any native conformation in the ELISA. Nevertheless, the relatively infrequent detection of E2-specific Ig responses in our VIN patient cohort may indicate a lack of E2 expression by
Table 1. E2- and L1-specific Ig and T-cell responses in VIN patients and healthy volunteers

Results of the serological assays are based on the E2 and L1 GST-tagged fusion protein ELISAs. A positive serological response to HPV-16 E2 and L1 GST-tagged fusion proteins was defined as a mean specific absorbance ≥ the mean of the control group + 3SD minus positive outliers. A positive ELISPOT response was defined as ≥ 20 spots per 10^6 cells, after subtracting the background (DCs + 0) responses. Note that background responses have not been subtracted in this table. A positive proliferative response was defined as a stimulation index ≥ 2. Positive responses are shown in bold.

(a) VIN patients

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high-grade VIN lesions. The E2 open reading frame is typically disrupted following virus integration into the host cell genome, a process that has been shown to occur during the malignant transformation of HPV-infected cells (Graham & Herrington, 2000; Peitsaro et al., 2002; Stevenson et al., 2000; Tonon et al., 2001).

Cell-mediated immunity is likely to play a pivotal role in eradicating established HPV infection and effecting disease regression. IFN-γ ELISPOT potentially allows the detection of low-frequency memory CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ T helper 1 (Th1) cells, since both release IFN-γ following re-exposure to specific antigens (Sallusto et al., 1999; van der Burg et al., 2001). Multiple restimulation of normal donor T-cell cultures with autologous E2 C terminus protein-pulsed DCs was shown to induce CTLs capable of E2-specific lysis of autologous lymphoblastoid cell lines infected with a recombinant vaccinia virus encoding HPV-16 E2 (Davidson et al., 2001). Thus, there is an E2-specific CTL repertoire available in normal individuals. Other studies using overlapping peptides from the HPV-16 E2 protein have revealed a broad E2-specific T helper cell repertoire in healthy volunteers (de Jong et al., 2002). DCs pulsed with HPV-16 VLP have also been shown to stimulate primarily type 1-dominated T helper cell responses (Lenz et al., 2001) and MHC class I-restricted CTL responses (Rudolf et al., 2001) *in vitro*. The specific release of IFN-γ by CD4+ Th1 cells and CD8+ CTLs following stimulation with E2 and VLP protein antigens may therefore provide a useful measure of those cell-mediated immune responses that are likely to be important in the natural clearance of HPV infections. In the present study, ELISPOT responses to C-terminal E2 and L1 required a 4 day stimulation of T cells with autologous protein-pulsed DCs; a 24 h stimulation of bulk PBMC cultures with antigen was not sufficient to reveal these responses. This suggests that appropriate antigen processing and presentation is necessary to demonstrate these low-frequency memory responses.

VLP L1-specific T-cell responses were more prevalent in the VIN group compared with the controls, both by IFN-γ ELISPOT (72% vs 53%, \( P = \text{NS} \)) and by proliferation assay (94% vs 53%, \( P < 0.02 \)). All but one of the patients who were seropositive for L1-specific Ig also demonstrated L1-specific proliferative T-cell responses. Twelve of these patients additionally showed L1-specific IFN-γ release by ELISPOT. All but one of the healthy volunteers were seronegative for HPV-16 L1-specific Ig, but a significant proportion of the group were L1-specific proliferative and IFN-γ-releasing T-cell responders. In the absence of HPV-16 L1-specific antibodies, the T cells measured by these assays most likely reflect cross reactivity with, or shared, epitopes following exposure to other HPV types. There is evidence for this in the studies of Williams et al. (2002) for HPV-11 and Steele et al. (2002) for HPV-1. Overall, HPV-16-positive VIN patients have high levels of antibodies to the viral capsid proteins and these are likely to be capable of neutralizing the virus. This antibody production is presumably also reflected in the high prevalence of T-helper responses measured by either proliferation or ELISPOT.

E2 seropositivity is not prevalent in the disease and, as with T-helper responses (de Jong et al., 2002), may reflect exposure to different HPV types with shared epitopes. However, there does appear to be a subgroup of patients with specific T-cell responses to E2, as measured by proliferation and ELISPOT. These two patients were clinical responders in a trial of an HPV vaccine (E. J. Davidson, C. M. Boswell, P. Sehr, M. Pawlita, A. E. Tomlinson, R. J. McVey, J. Dobson, J. St C. Roberts, J. Hickling, H. C. Kitchener & P. L. Stern, unpublished results). The clinical outcome may be a result of a shift in immune factors that clear the lesion(s) (which may be heterogeneous) and concomitant reduction in local virus immune suppression (Tindle, 2002). None of the healthy volunteers showed evidence of E2-specific T-cell responses either by proliferation assay or by ELISPOT. This is consistent with the results of Bontkes et al. (1999), who found no E2-specific T-helper cell responses in healthy control subjects using an IL-2 release assay (Bontkes et al., 1999), but contradictory to the findings of de Jong et al. (2002), who found frequent strong T-helper cell responses to HPV-16 E2 peptides in four out of eight normal donors.

There are several explanations for the discrepancies noted in these studies. Firstly, there was only evidence of exposure to HPV-16 in one of the healthy volunteers recruited into this study. By comparison, de Jong et al. (2002) tested anonymous donors for E2-specific T-helper cell responses and had no details regarding the age, sex, cervical smear history or HPV-16 VLP antibody status of their subjects. Secondly, the methodology used was very different. De Jong et al. used 30-mer overlapping peptides from the full HPV-16 E2 sequence. Significant cross-reactivity was seen between particular HPV-16 E2-specific T-cell responses and homologous peptides from other HPV types. Thus, the measured responses may reflect priming with HPV types other than HPV-16 itself. Furthermore, it is not clear whether all of the peptides investigated in this study are naturally processed by antigen-presenting cells *in vivo*. In contrast, since the intact E2 C terminus protein was used to measure T-cell responses in this study, any measured E2-specific immunity must reflect stimulation with naturally processed peptides. This is also true for the study by Bontkes et al. (1999), where E2 C terminus or N terminus proteins were used to stimulate PBMCs for 7 days before testing the supernatant for IL-2 concentration. However, the assay used in the present study should also test responses to the HPV-16 E2 N terminus, since it is possible that we are missing a significant response not evident in the IL-2 release assays (Bontkes et al., 1999).

It appears that exposure to HPV-16-positive VIN stimulates both antibody and T-cell responses to L1 protein but these may not always be sufficient to clear the lesions. In contrast, E2-specific T-cell responses are rare, although a repertoire exists, so it is possible that their absence may
facilitate the persistence of VIN lesions. Without clear proof of E2-specific T-cell responses associated with lesion resolution, it is unclear whether a lack of responsiveness indicates the inappropriateness of the assay or a true absence of E2-specific T cells. Nevertheless, these studies of immunity to an early and late HPV-16 protein in VIN provide a baseline for comparison of responses to vaccines such as chimeric VLPs including L1–E7–E2 (Schiller & Hildesheim, 2000). The challenge is to demonstrate such immune responses in patients at the time of virus clearance (systemic and local analyses), and for this, long term follow-up studies of large numbers of young women are required. This will resolve whether E2 is simply non-immunogenic or whether absence of immunogenicity is specifically associated with persistence of HPV-16 infection.

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