Proliferative T cell responses to human papillomavirus type 16 L1 peptides in patients with cervical dysplasia

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Human papillomavirus type 16 (HPV-16) can cause genital warts, cervical dysplasias and carcinoma of the cervix. Cell-mediated immunity is thought to be important in protection against the virus and in its elimination, but little is known about the mechanisms involved. In a cross-sectional study we have demonstrated proliferative T cell responses to peptides representing the HPV-16 L1 capsid protein (aa 199-409) in the peripheral blood of 63% of patients (n = 41) with histological evidence of cervical dysplasia and in 45% of healthy age-matched controls (n = 11). This was achieved by generating short-term T cell lines (STLs) from each individual in vitro against a β-galactosidase-HPV-16 L1 (aa 199-409) fusion protein for 2 weeks, and then identifying the HPV epitopes they recognized with overlapping synthetic peptides (15-mers) spanning this region in 3 day specificity assays. Histological grading and HPV typing by PCR were performed on patients' cervical biopsies taken at the same clinical visit as the peripheral blood samples. An immunogenic region was identified between aa 311-345 in 73% of patients (18% in controls) who responded to HPV-16 L1 (aa 199-409). The number of responders to this region was significantly higher in patients with HPV-16-positive biopsies when compared to those with HPV-16-negative biopsies (P = 0.006), as was the number of responders to individual peptides 311-325 (NLASSNYFPTPSGSM; P = 0.04) and 321-335 (PSGSMVTSDAQIFNK; P = 0.004) representing this region. The mean level of response to each individual peptide was also higher in the patient group than the controls (P < 0.05). The most significant finding was that all patients with evidence of a current HPV-16 infection responded to one or more L1 peptides (P = 0.0004) and 92% had high grade cervical intraepithelial neoplasia (CIN III). We also found that the CIN III group was more likely to respond to any L1 peptide than either the atypical group (P = 0.04) or the controls (P = 0.05). Data from four individuals showed that the majority of peptide-specific STLs were CD4+ but some CD8+ STLs were also detected.

Introduction

Human papillomaviruses (HPVs) are involved in a variety of epithelial lesions of the skin and genital tract. Specific HPV types are associated with lesions at different anatomical sites. In the skin the most common types are HPV-1, -2, -3, -5 and -8, whereas in the genital tract HPV-6, -11, -16, -18 and -33 are associated with HPV-related diseases. The latter have been subdivided into 'high-risk' and 'low-risk' groups based on their association with cervical carcinoma (Hausen, 1991). Hence, HPV-6 and -11 are predominantly found in benign genital warts (condyloma acuminata) and in low grade cervical intraepithelial neoplasia (CIN), whereas in high grade CIN and invasive carcinoma of the cervix, HPV-16, -18 and -33 are predominant (Koutsky et al., 1988).

The role of cell-mediated immunity in the control of HPV infection has been inferred from various clinical observations concerning HPV-related lesions of the skin and genital tract. These include the spontaneous regression of skin warts after a mononuclear cell infiltrate of the dermis and epidermis (Tagami et al., 1993) and an increased incidence of HPV-related lesions of the skin and genital tract in patients with impaired cell-mediated immunity, as in the genetically determined disorder epidermodysplasia verruciformis (Orth, 1987). Immunosuppressed allograft recipients have an increased incidence of skin warts (Rudlinger et al., 1986), genital warts and CIN (Porreco et al., 1975). In addition, in immunocompromised individuals infected with human immunodeficiency virus, HPV-16 and -18 have been isolated more frequently (Melbye et al., 1990; Johnson et al., 1992). Studies which directly describe cellular
immunity to genital HPVs have been limited by the lack of an effective in vitro culture system capable of producing sufficient high quality antigens. Most have used peptides or fusion proteins produced by bacterial expression systems as antigens and have focused on the immune response to HPV-16. Using this type of approach in mice, T cell epitopes in the HPV-16 L1 and E7 proteins have been described (Davies et al., 1990; Tindle et al., 1991; Comerford et al., 1991; Shepherd et al., 1992). In humans, epitopes in the HPV-16 E6, E7 and L1 proteins have been described (Strang et al., 1990; Altmann et al., 1992).

The main aim of this study is to describe the proliferative T cell response to HPV-16 L1 in women with HPV-related cervical disease and in healthy individuals. We selected three immunogenic regions to be mapped in these patients following preliminary data obtained from a pilot study by our group identifying human T cell immunogenic regions within the HPV-16 L1 protein (aa region 199-409) and data demonstrating their immunogenicity in mice. Our approach has been to generate short-term T cell lines (STLs) in vitro (Ota et al., 1990) from the peripheral blood of patients and healthy control subjects and use them to map T cell epitopes on the molecule with overlapping synthetic peptides. In addition, we have described patients’ cervical lesions in terms of their histological appearance and the type of HPV DNA present at the site of the lesion, as determined by type-specific PCR. This enabled us to determine firstly whether HPV-16-specific T cell responses of patients with cervical dysplasia differed significantly from those with atypical histology or from those of healthy controls and secondly whether this response correlated with the presence of HPV-16 DNA in the lesion.

**Methods**

**Tissue culture medium.** The standard medium used was RPMI-1640 with l-glutamine (Life Technologies), supplemented with 1 mm-sodium pyruvate (Flow Laboratories), 2 mm-l-glutamine, 10 mm-HEPES, 100 U/ml penicillin, 25 mg/ml gentamicin (Flow Laboratories) and 50 mm-2-mercaptoethanol (Sigma). This medium was supplemented with autologous human sera for all tissue culture procedures because pooled human sera produced unacceptably high background levels of thymidine uptake in the 3 day peptide specificity assays. Fetal calf serum was not used for the same reasons.

**HPV antigens and fusion proteins.** The HPV-16 L1-β-galactosidase (β-gal) fusion protein was constructed from a 637 bp fragment of DNA (BamHI and PstI) from the L1 gene of HPV-16 cloned into the pEX1,-2 and -3 vectors (Stanley & Luzio, 1984) and used to transform Escherichia coli strain pop.2316 (Patel et al., 1989). The resulting fusion protein (141 kDa) was induced by raising the temperature of the bacterial cultures from 28 °C to 42 °C for 90 min. Isolation of the protein from pelleted bacteria was performed according to the method of Doorbar et al. (1986). Removal of SDS and pyrogens from the final protein preparation was achieved by passing it through Extracti-Gel D and Detoxigel columns, respectively (Pierce). The purified protein gave a single band on SDS gels and Western blots using laboratory-prepared MAb to HPV-16 L1 as previously described (Patel et al., 1989). After testing for mitogenicity and cytotoxicity against normal peripheral blood mononuclear cells (PBMCs) the protein was stored at -20 °C and used at 1 μg/ml.

**Synthetic peptides.** A set of overlapping peptides (15-mers overlapping by 5 amino acids) representing the HPV-16 L1 fragment between aa 199-409 was synthesized by standard Fmoc chemistry, HPLC-purified and their sequences verified (commercially prepared by M. Munns, Peptide & Protein Research Cons, Devon, UK and H. Davies, King’s College, London, UK).

**Patient selection, collection of peripheral blood and biopsy material.** Ethical Committee approval was obtained to collect peripheral blood samples from patients referred by GPs in the SE London area to the colposcopy clinic at Guy’s Hospital Trust for investigation of abnormal cervical smears. Patients’ details were recorded by the investigating physician who was also responsible for clinical investigation and performing cervical biopsies. Clinical diagnosis was made on histological evidence from cervical biopsy material and patients were assigned to one of three categories; atypical (patients referred for colposcopy who subsequently showed atypical changes in cytology only and no evidence of CIN), CIN I/II or CIN III. The age distribution of these groups was similar; atypical group (n = 10), age range 23-49, mean ± SD 31 ± 8.2; CIN I/II group (n = 14), age range 20-57, mean ± SD 32 ± 9.8; CIN III group (n = 17), age range 21-69, mean ± SD 34 ± 10.4. For all patients, sections were taken from selected biopsy blocks with evidence of abnormal cytology and this material was used to prepare DNA extracts for PCR analysis as described below. A control group of women (n = 11) was selected from the same population area of London who had normal routine cervical smears, no previous history of genital warts and a similar age distribution to patients (age range 24-50, mean ± SD 32 ± 8.6). On ethical grounds it was not possible to obtain cervical biopsy material from this control group and therefore their HPV status was unknown; the group was designed to be representative of a normal healthy population where HPV-16 is known to be present.

PBMCs were obtained from each patient by taking 50 ml of venous blood which was then centrifuged by rotation in a sterile centrifuge tube containing glass beads for 10 min at room temperature. Serum was collected after centrifugation (780 g for 10 min) and the cells were separated by layering onto 15 ml Lymphoprep (Nycomed) and spinning at 942 g for 20 min. After washing, each patient’s cells were resuspended in autologous serum containing 10% (v/v) DMSO and stored as three 1.5 ml aliquots in liquid nitrogen until required.

**HPV typing by PCR.** DNA was obtained from paraffin-embedded biopsy material as described by Wright & Manos (1990). HPV typing was performed by the method of van den Brule et al. (1992), using consensus primers from a highly homologous region of L1 to detect HPV DNA from a wide range of HPV types. Consensus primer-positive samples were then subjected to type-specific PCR (TS-PCR) analysis using a mixture of HPV-6, -11, -16, -18, -31 and -33 type-specific primers. The primers used for the consensus PCR were GP6 (Snijders et al., 1990) and MY11 (Resnick et al., 1990) which amplified a 184 bp fragment of L1, and the type-specific primers were those described by van den Brule et al. (1992).

Consensus PCR was performed in 50 μl of reaction mix containing 50 mm-KCl, 1 mm-MgCl2, 0.01 % gelatine, 200 mm of each dNTP, 0.5 μM of each primer, 1 U of thermostable DNA polymerase (Advanced Biotechnologies) and 10 μl DNA extract. Forty cycles of amplification were performed on a Biometra Trio-thermoblock T1 (Gottingen) as previously described (van den Brule et al., 1992) except for the final cycle which had an elongation time of 5-5 min. Consensus PCR-negative samples were checked for amplifiable DNA using...
primers (1 μM) which amplify a 355 bp segment of the β-globin gene (British Biotechnology) and similar reaction conditions except the MgCl₂ concentration, which was 2 mM, and the annealing temperature, which was 55 °C.

A ‘hot start’ method was employed for TS-PCR whereby template DNA and thermostable polymerase was added during the initial denaturation step in order to separate DNA from primers and thus minimize non-specific annealing, which occurs at lower temperatures. This separation was achieved by means of a wax layer which melts at 75 °C. PCR products were analysed by electrophoresis on a 1:5 agarose gel.

For ease of interpretation, TS-PCR was performed in two separate reaction mixtures containing three primer pairs in each (set 1, HPV-6, -16 and -33; set 2, HPV-11, -18 and -31). An extract containing HPV-16 L1 fusion protein (aa 199-409) was diluted in medium and medium containing 10 % autologous serum and adjusted to 3 x 10⁶/ml.

**STLs.** These were set up using one of the three vials of stored patients’ cells. The cells were thawed rapidly at 37 °C, washed in culture medium containing 10% autologous serum and adjusted to 3 x 10⁶/ml. HPV-16 L1 fusion protein (aa 199-409) was diluted in medium and 100 μl (1 μg/ml) was dispensed into 25 wells of a 96-well round-bottomed microtitre plate (Greiner). Then, 100 ml of cell suspension in a humidified 5 % CO₂ atmosphere for 14 days. The cell cultures were harvested wells in a fl-spectrometer (Hewlett Packard). The data was plotted as the response in c.p.m. for each cell line to all the antigens and labelled thymidine was measured by counting the radioactivity in harvest wells.

**Stimulation Index (SI).** The proportion of CD4⁺ and CD8⁺ cells in STLs cultured with HPV-16 L1 was determined by indirect immunofluorescent staining and FACS analysis (Coulter cell analyzer). The phenotype of responding T cells from patients and control was done using unseparated PBMCs (20 lines), CD8-depleted T cells (20 lines) and CD4-depleted T cells (20 lines). Cultures were set up using approximately the same cell numbers as found in PBMC cultures (60 % CD4⁺ and 30 % CD8⁺ cells). Irradiated APCs were added to produce a total cell number of 3 x 10⁶/well. The percentage of contaminating CD4⁻ and 30 % CD8⁺ cells). Irradiated APCs were added to produce a total cell number of 3 x 10⁶/well. The percentage of contaminating

**Phenotypic assays on STLs.** Each of the 20 STLs was tested on day 14 against HPV-16 L1 fusion protein (1 μg/ml), HPV-16 L1 synthetic peptides and culture medium. In preliminary experiments the peptides were used in 3 day specificity assays to define responses to regions within HPV-16 L1. The composition of the peptide pools is shown in Fig. 1. Later studies used individual peptides to map more accurately the T cell epitopes recognized and peptides 305–319 and 315–329 were replaced by peptides 301–315 and 311–325. The peptide concentration was 10 μM in both pooled and single peptide assays. Cell counts were performed on five pooled cell lines and the mean counts used to estimate the numbers of cells per line (this number varied between 1–3 x 10⁶ cells). Each cell line was set up in duplicate in 96-well round-bottomed microtitre plates against the 11 antigens and culture medium on its own as a background control in the presence of autologous antigen-presenting cells (APCs). The latter were obtained from two vials of the patient’s cells stored in liquid nitrogen, thawed, washed and irradiated (40 Gy). The peptides were dispersed in 100 μl/well together with the irradiated APCs (2.5 x 10⁶/ml) and STL cells (105/ml) in a further 100 μl of medium. The plates were incubated for 3 days at 37 °C in 5 % CO₂ before adding 20 μl of [methyl-³H]thymidine (Amersham; 9.25 kBq/well) and incubated for a further 4 h. Incorporation of labelled thymidine was measured by counting the radioactivity in harvested wells in a β-spectrometer (Hewlett Packard). The data was plotted as the response in c.p.m. for each cell line to all the antigens and medium controls. A positive proliferative response was defined by a stimulation index (SI) of > 2.5 and a Δ c.p.m. of > 500 above the medium control value. A positive patient response was when two or more of the 20 STLs (≥ 10 %) responded to the same peptide.

**Phenotype of responding T cells.** The proportion of CD4⁺ and CD8⁺ cells in STLs cultured with HPV-16 L1 was determined by indirect immunofluorescent staining and FACS analysis (Coulter cell analyzer). Phenotyping of responding T cells from patients and control was done on PBMCs deleted of CD4/CD8 cells using OKT4 and OKT8 antibodies, respectively, and sheep anti-mouse IgG-coated magnetic beads (Dynabeads). Sixty STLs were set up as previously described using unseparated PBMCs (20 lines), CD8-depleted T cells (20 lines) and CD4-depleted T cells (20 lines). Cultures were set up using approximately the same cell numbers as found in PBMC cultures (60 % CD4⁺ and 30 % CD8⁺ cells). Irradiated APCs were added to produce a total cell number of 3 x 10⁶/well. The percentage of contaminating

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**Fig. 1. Overlapping peptide pools representing HPV-16 L1 (aa 199–409).** A panel of 26 overlapping peptides (with two gaps of 5 amino acids at the points indicated by an asterisk) were grouped into eight pools of three and a di-pool containing peptides 305–319 and 315–329 and used in 3 day proliferation assays at a final concentration of 10 μM per peptide. The HPV-16 L1 fusion protein (aa 199–409) was used at 1 μg/ml.
cells present in the CD4+ and CD8+ cell populations was determined by indirect immunofluorescent staining and FACS analysis using the antibodies UCHT1 (anti-CD3), OKT4 and OKT8.

Statistical analyses. Chi-squared tests were performed on contingency tables of responders and non-responders to peptides in the patient and control groups, and on grades of cervical biopsy histology and HPV DNA status. Also, 2 × 2 tables were analysed using the Fisher’s exact test when sample numbers were small. A P value of less than 0.05 was taken as statistically significant. The Mann–Whitney test was used to test for variance between the patient and control groups in their responses to peptides, and analyses of variance for within groups.

Results
Patient peripheral blood responses to HPV-16 L1 antigens

A small pilot study was conducted to determine whether peripheral blood T lymphocytes from patients (n = 17) with cervical disease and control subjects (n = 3) responded to HPV-16 L1 (aa 199–409) and to identify the immunogenic regions recognized within the molecule in those who responded. This was done by setting up 20 STLs per patient in culture in the presence of an HPV-16 L1 fusion protein. Each cell line was tested for specific proliferation after 14 days in a 3 day proliferation assay using HPV-16 L1 antigen as a positive control and nine pools of synthetic HPV-16 peptides spanning this region.

In this study, 11/17 patients (65%) responded to HPV-16 L1 fusion protein and 10/17 patients (59%) to one or more of the peptide pools (Fig. 2). An immunodominant region at aa 305–345 was identified in 8/10 patients (80%) who responded to HPV-16 L1 peptides with 6/17 patients (35%) responding to peptide pool 305 and 4/17 (24%) to peptide pool 321. Positive responses were also made to four other sites by 4/17 patients (24%) and to β-gal as a positive control antigen by 6/17 patients (35%). In a small control group (n = 3), one individual responded to HPV-16 L1 fusion protein but there were no responses to specific peptides.

Mapping of the T cell response to HPV-16 L1 with synthetic peptides

To confirm the findings of the pilot study and to define more accurately proliferative responses to this region of HPV-16 L1 a further 41 patients with cervical disease and 11 healthy control subjects were investigated. Three regions of interest defined by aa 191–225, 281–315 and 301–345 were investigated, based on the preliminary peptide pool data. The same STL technique was used, but the specificity of responses was determined in the 3 day proliferation assays with one pool of three peptides spanning region 191–225 and single peptides (15-mers overlapping by 5 aa) spanning the remaining two regions. Patients and controls were considered to be responders if two or more of their 20 STLs responded to the same single peptide or peptide pool. An example of the types of T cell responses made to HPV-16 L1 peptides by one patient and one control subject are shown in Fig. 3. The patient responded to the fusion protein and to five of the peptides, with different numbers of cell lines responding to each peptide (191, 281, 311, 321 and 331). The control individual responded to the protein and peptide 281 only.

A summary of all the responses made by the patients and controls is given in Fig. 4, which illustrates the numbers of cell lines from each individual that responded to the fusion protein and the peptides. All of the patients and controls responded to HPV-16 L1 fusion protein (patients’ STLs, range 10–20, mean 18.3; controls’ STLs, range 18–20, mean 19.2). Previous experiments have shown that part of this response is to the β-gal portion of the protein. L1 specificity was demonstrated in 26/41 patients (63%) and 5/11 controls (45%) who responded to one or more of the L1 peptides. The number of patients responding to the majority of peptides tested (191 pool, 281, 311, 321 and 331) was similar, varying between 9 (22%) and 14 (34%) with the exception of peptide 301 to which only two patients (5%) responded. The greatest number of responding cell lines per patient was directed against peptide 331 (range 0–20, mean 1.5). Considering the response to region 311–345 as a whole, 19/41 patients (46%) as compared to only 2/11 controls (18%) responded to this region; however, this was not a significant increase. In the control group only four of the six peptides tested were recognized, with pool 191 and peptide 281 equally the most predominantly recognized (three individuals or 27%) and to a lesser extent peptides 311 and 331 (both one individual or 9%). The mean levels of responses to these four peptides were significantly lower than in the patient group (P = < 0.05). This suggests that the precursor frequencies of HPV-16 L1-specific T cells was lower in the control group than in the patient group.

A comparison of the CD4 and CD8 phenotypes of STLs from six dysplasia patients before and after the 2 weeks culture with HPV-16 L1 fusion protein showed an increase of 3–16% in CD4+ cells in four of the patients and no change in the remaining two. A decrease in CD8+ cells of between 4 and 16% occurred in three of these patients. In the remaining patients the percentage change in CD4 or CD8 cells was ≤ 1% (data not shown). To determine the T cell phenotypes of responding cells to specific peptides, we prepared CD4- and CD8-depleted cell populations from responding patients and controls and set them up in parallel with unseparated peripheral blood in STL culture assays. The majority of peptide-
specific responding cell lines (28 in total) were found in the CD4+ population, although one of the controls and one patient each had two CD8+ cell lines responding to either the same or different peptides as those recognized by their CD4 cells (Table 1).

Analysis of cervical biopsy tissue

Cervical biopsy tissue was obtained for clinical diagnostic purposes from the 41 patients at the same colposcopy clinic visit as the samples of blood were collected. These biopsies were histologically graded independently and without prior knowledge of the T cell data. Additional biopsy sections were cut at a later time from selected blocks which had evidence of disease activity to detect and type any HPV DNA present by PCR. In Fig. 5 the patients have been grouped according to the histological grading of their cervical biopsies and to the presence or absence of different HPV types. PCR typing of the 41 biopsies identified 13 (31.7%) cases with no detectable HPV DNA, 12 (29.3%) with an unidentifiable HPV DNA type (type X); HPV DNA-positive but not HPV-6, -11, -16, -18, -31 or -33) and a further 12 patients (29.3%) were HPV-16 positive (one had a mixed infection with HPV-16 and -18). Of the remaining four patients, three (7.3%) were HPV-18-positive and one (2.4%) was HPV-33-positive. The patients’ biopsies have been grouped into one of three histological categories, atypical, CIN I/II or CIN III. In the atypical group, the most prevalent HPV type identified was type X in 6/10 biopsies (60%), HPV-18 was found in 1/10 biopsies (10%) and the remaining 3/10 (30%) were HPV-negative. A similar pattern was found with CIN I/II biopsies where 5/14 (35%) were HPV X, 1/14 (7%) each were positive for HPV-16, -18 and -33 and 6/14 (42%) were HPV-negative. In the high grade CIN III group, the majority of patients (11/17 or 65%) had HPV-16 with 1/17 (6%) each having type X or 18, and 4/17 (24%) HPV-negative. There is therefore a strong association between HPV-16 DNA-positive patients and CIN III grade lesions (P = 0.0001) although there were four patients in this group in whom no HPV DNA could be found. No relationship was found between those patients who were HPV DNA-positive (irrespective of HPV type) and histological grading. All three histological grades were represented in patients with HPV-18 and the single patient with HPV-33 had a CIN II grade lesion, but the patient numbers were too small (n = 4) for statistical analysis.

Correlation of patients' T cell responses with histology and HPV DNA status of cervical biopsy material

Also illustrated in Fig. 5 are the patients’ ability to generate HPV-16 L1-specific proliferative T cell responses. The most striking finding was that all 12 patients who had evidence of a current HPV-16 infection, as indicated by the presence of HPV-16 DNA, responded to one or more peptides and 11/12 (92%) of them had CIN III lesions. The remaining responder had a CIN I/II lesion and no patients were found in the atypical group. Statistical analysis revealed a highly significant association between responders and the presence of HPV-16 DNA (P = 0.0004). T cell responses to HPV-16 peptides were also found in two other HPV type categories; HPV X (n = 5) and HPV-33 (n = 1). No proliferative T cell
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Fig. 3. Individual T cell responses to HPV-16 L1 peptides. Peptides used were single 15-mers denoted by the first amino acid residue of each peptide (see Methods) and overlapping by 5 aa except for 191*, which was a tri-pool of peptides 191–205, 201–215 and 211–225. Positive and negative controls were as for Fig. 2. (a) Responses of a 33-year-old patient with an HPV-16-positive CIN III lesion. Positive response range to HPV-16 L1 was 1286 c.p.m. (SI = 11.4) to 14542 c.p.m. (SI = 27.5); to individual peptides it was 808 c.p.m. (SI = 2.9) to 9434 c.p.m. (SI = 17.9). (b) Responses of a healthy 27-year-old control subject. Positive response range to HPV-16 L1 was 2642 c.p.m. (SI = 5.7) to 8371 c.p.m. (SI = 10.5); to individual peptides it was 2713 c.p.m. (SI = 4.5) to 5491 c.p.m. (SI = 5.2).

responses were detected in the HPV-18-positive group. In the HPV DNA-negative group there were responders found in the atypical group (n = 3), the CIN I/II group (n = 4) and the CIN III group (n = 1). There are a number of reasons why T cell responses may be detected in apparently HPV-16-negative individuals. These T cell responses may have been the result of a recent or prior HPV-16 infection which had resolved and was therefore not detected at this sampling time point or of a current infection of the cervix or elsewhere in the genital tract other than the area which was biopsied. Cross-reactive T cell responses may also be detected as a result of the high degree of homology between the L1 molecule of different HPV types. Additionally, the detection of HPV DNA by this method is subject to sampling errors (as in any biopsy technique) due to the small fraction of the biopsy material being analysed. We found that there was a significant association between T cell responses of individuals who were HPV DNA-positive and had CIN lesions when compared with those with atypical histology (P = 0.003).

Further statistical analysis of our data enabled us to determine whether there were significant differences in T cell responses to individual peptides between women with different grades of cervical disease and those with lesions containing different HPV types (data not shown). These revealed a significant increase in the number of HPV-16-positive patients who responded to peptides 311 (NLASSNYFPTPSGSM, P = 0.04) and 321 (PSGSMVTSDAQIFNK, P = 0.004) when compared to those who were HPV-16-negative or had another HPV type.

Peptide-specific T cell responses elicited by patients and controls were plotted according to the histological findings (Fig. 6). The data showed that the number of responders was significantly higher in the CIN III group when compared to the atypical group (P = 0.04) or to the controls (P = 0.05). Considering T cell responses to the region 311–345 as a whole, i.e. the immunogenic region...
Table 1. Number of STLs responding to HPV-16 L1 fusion protein and peptides

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* Peptides tested were as follows: control subject 1, peptides 281 and 311; control subject 2, peptides 281, 331 and 335; patients 1 and 2, peptides 281, 311 and 321.
† CD4- and CD8-depleted cell populations contained ≤ 4% contaminating cells.
‡ Twenty cell lines were set up for each STL type (PBMCs, CD8-depleted PBMCs and CD4-depleted PBMCs) except in patient 1 where 16 cell lines were set up in the CD8-depleted part of the experiment.

Fig. 5. Patient T cell responses, HPV types and cervical biopsy histology. HPV typing was performed by PCR on DNA extracted from formalin fixed, paraffin embedded sections cut from selected blocks of cervical biopsies with histological evidence of cervical disease. Shaded areas of boxes represent the numbers of patients responding to HPV-16 L1 peptides. Asterisk indicates statistical significance for HPV-16 DNA and CIN III (P = 0.0001) and for HPV-16 DNA and responses to HPV-16 peptides (P = 0.0004).

Fig. 6. Patients' and controls' T cell responses to HPV-16 L1 peptides according to cervical histology. Single peptides were used in T cell assays except for pool 191 (see Fig. 1). For further details of peptides used see Fig. 3. The patient response to two peptides was significantly associated with the presence of HPV-16 DNA; peptide 311, P = 0.004; peptide 321, P = 0.04.

defined by our preliminary studies using pooled peptides, we found there was a significant association between responses and the presence of HPV-16 DNA (P = 0.006), but not with the presence of HPV DNA (P = 0.74) or cervical histology (P = 0.94), which is in agreement with the data previously described.
Discussion

Recently, serological studies on patients with HPV-16 infections have confirmed the presence of IgG antibodies to capsid proteins in 59% of cases using synthetic virus-like particles of HPV-16 in an ELISA assay system (Krnabauer et al., 1994; Galloway, 1994). T cell help will have been necessary for the production of these IgG antibodies and some indirect evidence for its existence is presented in this report. To identify low numbers of recirculating HPV-16-specific T cells in the peripheral blood we had to amplify them by generating STLs in vitro for 2 weeks in the presence of an HPV-16 L1 fusion protein and cytokines (rIL-2 and rIL-4) and then showed their specificity in 3 day proliferation assays using synthetic peptides of HPV-16 L1. Preliminary studies (data not shown) using synthetic peptides in 7 day proliferation assays to detect these T cells failed to produce significant reproducible T cell responses. This type of problem has been reported in other studies mapping T cell epitopes in multiple sclerosis (Ota et al., 1990; Weiner et al., 1993) and was resolved by using the STL technique. As might be expected for a predominantly sexually transmitted virus that is widely detected in all populations, the number of responders was higher in people with active disease (63%) than in our control group of women (45%). The mean level of response to each individual peptide (P < 0.05) was also higher, where the control group had no previous history of HPV-related disease and normal cervical smears when examined as part of routine screening. We feel that our control group of women of a comparable age range represents a fair reflection of those exposed to HPV in the population as a whole but cannot be regarded as either naive or virus-free for the following reasons. Firstly, HPV infections can also be transmitted from mother to child at birth or in early infancy (Sedlacek et al., 1989) and secondly, women tested for the virus by PCR on cervical swabs taken at regular 5 weekly intervals over a 12 month period could change from positive to negative and back to positive again (Schneider et al., 1992). Also, a biopsy from the cervix of a ‘normal healthy individual’ might fail to detect the presence of latent virus by PCR due to the focal nature of the disease, its low copy number and the limitations of taking small punch biopsies.

The most likely explanation for these T cell responses to HPV capsid antigens detectable in the systemic immune system of patients and controls is that they represent secondary expansions of T cells following an earlier primary exposure to the virus at an epithelial surface; the genital mucosa is the most likely site. They are unlikely to be due to in vitro priming with peptides as we did not stimulate our cultures with peptides and no single peptide was consistently detected either within a patient’s 20 cell lines or in the patient and control groups collectively. Furthermore, in vitro priming usually takes more than 2 weeks to develop. In addition we have shown that all patients with HPV-16 DNA in their cervical lesions responded significantly to one or more L1 peptides (P = 0.0004), and that they are more likely to respond to the immunogenic region aa 311–345 (P = 0.006) or to individual peptides (aa 311–325 and 321–335) within this region (P = 0.04 and P = 0.004, respectively). Thus individuals from whom HPV-16 DNA can be isolated from their cervical lesions have a higher probability of demonstrating a T cell proliferative response to HPV-16 L1 peptides in vitro.

However, some individuals who had no detectable HPV-16 DNA in their cervical biopsies and others who had different HPV types (five with HPV X and one with HPV-33) responded to HPV-16 L1 peptides. The latter may have detected cross-reactive T cell epitopes due to the high degree of homology between L1 molecules of HPV-16 and other HPV types. Alternatively the 'HPV DNA-negative' individuals may have had no detectable HPV at the site of the dysplasia when assayed, but have had previous HPV-16-positive lesions or have been exposed to infection by other HPV types at the same or other sites. It is also possible that biopsies were HPV-positive but had no HPV DNA present in the sections extracted for PCR, or that PCR did not detect the HPV DNA because it was present at too low a level or was damaged by the fixation process in paraffin. We have also shown that proliferative T cell responses to HPV-16 L1 can be positively linked to histological grading. Hence patients with CIN III histology are more likely to respond to any peptide than either those with atypical histology (P = 0.04) or healthy controls (P = 0.05). As shown in other HPV studies (Koutsky et al., 1988) we have also demonstrated a significant association between high grade CIN III lesions and the presence of HPV-16 DNA, and all HPV-16-positive patients responded to one or more peptides. Whether these T cell responses provide the right kind of help to the immune system to protect against HPV infections in individuals with different grades of cervical dysplasias is unknown and awaits longitudinal studies, which should also include serology and cytotoxic responses to the early region virus proteins to give a more complete picture. The vast majority of HPV-related dysplastic lesions do not progress to malignancy and given time they eventually regress. So our T cell findings in women with these types of lesions may indicate part of the normal response that will eventually lead to regression unless other, as yet unknown, factor(s) tip the balance towards progression. Furthermore, epitope mapping has defined three immunogenic regions; aa 191–225, 281–295 and 311–345 on
HPV-16 L1 (aa 199–409) with significant differences in patient and control responses found to region 311–345. Individuals’ T cell responses to different areas within this region are variable. This is to be expected from individuals of different HLA backgrounds, expressing different peptides from within these L1 regions on their MHC class II molecules following antigen processing and presentation by their APCs. Also, the T cell repertoire of an individual will further influence how these regions are seen immunologically. One of the two epitopes most frequently seen within this region shares a high degree of homology with other HPV types. The closest fit is with peptide 321–335, which has 11/15 identical residues in HPV-6; the remaining four are comparable substitutions (leucine → methionine, serine → threonine, glutamic acid → aspartic acid and leucine → isoleucine). For peptide 311–325 the equivalent HPV-6 sequence has only 8/15 identical residues, 2/15 (valine → leucine and leucine → methionine) possible equivalents and five unmatched. It is therefore possible that some of the responses to peptide 321–335 may include cross reactions with other HPV types and thus make this a type common epitope. There is no evidence based on peptide sequence homologies between any of the HPV peptides and β-gal to account for the responses detected.

In the four individuals studied the phenotype of peptide-specific T cells was predominantly CD4+ which would be expected in this type of assay system, but there were also some CD8+ cells that responded to peptides. Recently, it has been shown that non-specific CD8+ CDw60+ T cells can help pokeweed mitogen-stimulated B cells to produce IgG in vitro (Rieber & Rank, 1994). We are now investigating whether our peptide-specific CD8+ T cells are CDw60+ and whether they can help autologous B cells in our STL system. Taken together these findings would indicate that T cell responses which are predominantly CD4+ are made to HPV-16 L1 in women with cervical disease and to a lesser extent in normal control subjects with a similar age range. It is probably also true that people with genital warts caused by HPV-6, -11 and other low risk types mount responses to L1 and possibly L2 in the same way as shown here for HPV-16. Prevention of HPV infections and consequently genital warts, cervical dysplasias and ultimately HPV-related carcinomas of the cervix by immunization with virus-like particles or combinations of smaller immunogenic peptides is a real possibility in the future. Animal studies have already given credence to this idea with the use of L1 or L2 proteins either singly or together in cattle (Jarrett et al., 1991) and in rabbits (Lin et al., 1992) to give protection on challenge with live virus. In beagle dogs immunized intradermally with a formalin-inactivated canine oral papillomavirus (COPV) homogenate and then challenged in the oral mucosa with infectious COPV, all the animals exhibited long-term protection and no side effects (Bell et al., 1994). Although the types of lesions induced in these experimental systems are not directly comparable to genital HPV lesions, they do indicate that papillomavirus type-specific capsid proteins are potentially good immunogens and they can induce immunophrophylaxis. We are also investigating the use of immunogenic determinants of L1 as multiple antigenic peptides in immunization studies.

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References


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