Cross-typic specificity and immunotherapeutic potential of a human HPV16 E7-specific CTL line

Sarah J. Youde, Corinna M. McCarthy, Karen J. Thomas, Kelly L. Smith and Stephen Man*

Section of Infection and Immunity, Henry Wellcome Building, Wales College of Medicine, Cardiff University, Heath Park, Cardiff, United Kingdom

Cervical cancer (CaCx) is strongly associated with human papillomavirus (HPV) infection, particularly HPV types 16 and 18. The constitutive expression of HPV E6 and E7 proteins in CaCx makes them attractive targets for CTL based immunotherapy. However cervical carcinomas may have features, e.g., antigen processing defects, that limit the effectiveness of HPV specific CTL. Furthermore most vaccine development has concentrated on HPV type 16, and it is not clear whether such vaccines could induce CTL able to cross-react on related oncogenic HPV types, e.g., HPV31 and 52. To investigate these potentially important parameters in vitro, we used a CTL (D4) specific for HPV16 E711–20. D4 was able to kill a variety of HPV16+ CaCx cell lines including those with suspected (CaSki) or known antigen processing defects (C33A), and with low HPV DNA copy number (SiHa). D4 was also able to cross react on a related peptide from HPV52 E7 but not HPV31 E7. Further analysis suggested that D4 cross reactivity against related peptides was influenced both by TCR contact residues and a certain threshold for peptide binding. The HPV cross-reactivity was confirmed at the whole protein level as D4 was also able to recognize the endogenously processed forms of HPV16 and 52 E7 but not 31 E7. These results suggest that HPV16 E711–20 would be a useful epitope for immunotherapy in both HPV 16 and 52 tumours. Despite this, it is difficult to generate these CTL in response to vaccination, emphasizing the need for definition of novel epitopes and more efficient vaccination strategies.

Key words: HPV; CTL; cervical cancer; immunotherapy; cross-reactivity

Cervical cancer (CaCx) is the second most common cause of cancer in women world wide and with premalignant cervical intraepithelial neoplasia (CIN3) is associated with HPV infection. In developing countries where 80% of cases occur, this is the principal female cancer. The DNA of HPVs, particularly 16 and 18, are found in >99% of CaCx patients. It is the E6 and E7 proteins, which are consistently retained and expressed in cervical tumour cells, that give the virus its transforming properties.

HPVs are defined as low and high risk, high-risk types being associated with invasive cervical cancer, while low risk types are associated with warts. There are 11 HPV types that are consistently classified as high-risk types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58. These types are further divided into classes: type A (16, 31, 33, 35, 52 and 58), C (18, 39 and 45) and D (51 and 56).

HPV type-specific prevalence varies geographically. Worldwide, types 16 and 18 dominate but in other non-Caucasian populations, 31, 52 and 58 dominate. The infection rate with each HPV type varies from country to country, i.e., HPV16 is found in 43.9% of cases in the Philippines and 72.4% in Morocco. According to Muñoz et al., 95% of all infections were caused by 8 types: 16, 18, 31, 33, 35, 52, 58 and 35. They suggest that vaccination against the 5 most common types could prevent 90% of cases of CaCx.

The geographical variation of HPV prevalence shows that the target population, i.e., the population bearing the highest burden of CaCx, requires the incorporation of other HPV types, other than the commonly occurring 16 and 18, into vaccine design.

Little work has been done to examine cross reactivity between oncogenic HPV types at the T-cell level, although extensive cross-reactivity of HPV11 L1-specific CD4+ T cells has been demonstrated. We have previously shown that it is possible to detect CTL specific for HPV16 E711–20 in the blood of patients with both CaCx and CIN3 and in the blood of healthy donors. These CTL can mediate the lysis of B cells endogenously expressing the E7 protein and the HPV 16 transformed cervical cancer cell line CaSki. Since the E711–20 peptide from HPV16 has homology to the E7 peptide of the phylogenetically related types 31 and 52, we used these CTL to investigate in vitro the potential for cross-recognition between these HPV types.

Methods

Media

RPMI1640 (Invitrogen Corporation, Paisley, Scotland, UK) was always used with the following additions: 0.02 M HEPES (Sigma-Aldrich Co. Ltd., Poole, UK), 2 mM L-glutamine (Invitrogen Corporation) 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen Corporation) and known as TCM. For the culture of T cells, this media was supplemented with 10% pooled human AB serum (National Blood Transfusion Service, Pontychurch, Wales) and known as RAB. DMEM with 4,500 mg/l glucose and pyridoxine HCL (Invitrogen Corporation) was always used with the following additions: 0.02M HEPES, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

Cell lines

The C1R-A2 cell line, a B lymphoblastoid cell line transfectant expressing the HLA-A*0201 allele (provided by Professor A.J. McMichael, Institute of Molecular Medicine, Oxford, UK) was maintained in RPMI1640 containing 10% fetal calf serum (FCS, Invitrogen Corporation) and 400 µg/ml G418 (Invitrogen Corporation). RPMI1640 containing 10% FCS was used to maintain the following cell lines: 174CEM.T2 (T2,11) cells, which contain an antigen processing defect due to a homozygous deletion of the MHC class II region located on chromosome 6, which results in a low density of HLA-A*0201 on the cell surface; CaSki, an HLA-A*0201 positive cervical carcinoma cell line expressing HPV16 E6 and E7 proteins (ECACC 87020501,12) and MDA-231, an HLA-A*0201 positive breast epithelial carcinoma cell line (kind gift from L. Sherman, Scripps Clinic, La Jolla, CA (ECACC 92020424,12). The C33A-HPV16 cell line was created by transfecting an epithelial cell line free from any known papillomavirus sequences C33A (ATCC HTB-31,13) with a plasmid containing the whole HPV16 genome.14 Both cell lines were maintained in

Abbreviations: B-LCL, B-lymphoblastoid cell line; CaCx, cervical cancer; CIN, cervical intraepithelial neoplasia; CTL, cytotoxic T lymphocyte; DC, dendritic cell; HLA, human leukocyte antigen; HPV, human papillomavirus; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; VLP, virus-like particles.

Grant sponsor: CR UK; Grant sponsor: MRC

Dr. McCarthy’s current address is: Tumour Immunology Group, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DS, UK

*Correspondence to: Section of Infection and Immunity, Henry Wellcome Trust Research Institute, University of Wales College of Medicine, Heath Park, Cardiff, CF14 4XW, United Kingdom. Fax: +44-029-20745003 or +44-029-20743858. E-mail: mans@cf.ac.uk

Received 11 June 2004; Accepted after revision 22 September 2004

DOI 10.1002/ijc.20779

Published online 17 December 2004 in Wiley InterScience (www.interscience.wiley.com).
DMEM containing 10% FCS. SiHa (ATCC HTB-35), an adherent squamous cell cervical carcinoma cell line with 1–2 copies of HPV16 integrated into the cell genome, was also maintained in this media.

Peptides
HPV peptides and analogues were synthesized by Immune Systems, Ltd., Paignton, UK and purified by reverse phase HPLC to between 80–95% purity. The identity of the sequences (Table I) for each peptide was confirmed by mass spectrometry.

**Generation of HPV16 E711–20 monospecific polyclonal CTL line D4**

D4, a monospecific polyclonal CTL line was generated as described previously. Briefly, this HPV16 E711–20-specific CTL line was generated from a patient with stage IIIB cervical cancer. Single cell sorting based on CD8 (tricolor-anti CD8, Caltag, Burlingame, CA)/HPV16 E711–20 tetramer (PE labeled) double staining was then carried out using a FACSVantage (Becton-Dickinson, Mountain View, CA).

**Generation of HPV16 E629–38 clone 7E7**

A T-cell clone against the HPV16 E629–38 peptide was generated as described previously. Briefly, memory CTL to this peptide were identified in a patient with invasive carcinoma following in vitro restimulation with the peptide. The CTL line was then cloned by limiting dilution in 96-well plates using irradiated allogeneic peripheral blood mononuclear cells (PBMC) feeders, phytohaemagglutinin (PHA, Bio Stat, Ltd., Stockport, UK) and lymphocult T (Biotest Ag, Dreieich, Germany). The clone was expanded in T75 flasks using irradiated allogeneic PBMC, PHA and IL-2 (Chiron UK, Ltd., Harefield, and Middlesex, UK).

**Generation and maintenance of C33A transfectants**

Transfectants of the C33A cell line were established to investigate recognition of the full length E7 protein from various HPV types. The C33A cell line was chosen for transfection experiments for a variety of reasons. It is an HPV negative cervical carcinoma cell line having a point mutation in p53; it has previously been transfected to express HPV16 E6 and E7 and work from our cell line having a point mutation in p53; it has previously been described previously. Briefly, cells were plated in 6-well plates with 2.5 µg of DNA and 15 µl DOTAP per well. Cells were selected with 400 µg/ml of G418 48 hr post transfection and when abundant, cells were sorted on a Dako Cytomation MoFlo for HPV-A*0201 expression.

**Cytotoxicity assays**

Cytotoxicity was measured in a standard 4 hr 51Cr release assay as described previously. CIR-A2 target cells were pulsed with varying doses of the peptides and analogues for 1 hr after labeling for 2 hr with 51Cr (Na251CrO4; Amersham Biosciences, Little Chalfont, UK). Previously, cytotoxicity against HPV 16 and 18 E6 and E7 was measured using a recombinant vaccinia virus, TA-HPV (gift of Xenova Plc., Cambridge, UK17). TA-HPV has been shown to express HPV antigens by both Western blot17 and by HPV-specific CTL. CIR-A2 cells were infected with the vaccinia viruses (MOI of 15) for a maximum of 12 hr before 51Cr labeling. This method was also used to infect SiHa cells with a recombinant vaccinia virus encoding HLA A*0201. The recognition of naturally, endogenously, processed antigens was assessed using the cell lines CaSki and C33A-HPV16, which were labeled for 2 hr with 51Cr. The cell lines MDA231 and C33A, which contain no HPV antigens, were used as controls. After 4 hr incubation, radioactive counts were obtained by beta plate liquid scintillation counting on a 1450 microbeta Trilux liquid scintillation and luminescence counter (Wallac, Milton-Keynes, UK).

**ELISPOT assays**

The clone, D4, was seeded in triplicate wells at a density of 2×10^5 cells/well of a Multiscreen 96-well plate (Millipore Corporation, Bedford, MA) that had been coated overnight with an IFN-γ catching antibody (Mabtech AB, SE-131, Sweden). Wild-type C33A or the transfectants were added at a ratio of 1 D4:1 transfectant and cells incubated for 18 hr before ELISPOT analysis was performed according to the manufacturer’s instructions. As positive controls, the HPV16 E711–20 peptide was added at a concentration of 10 µg/ml with CIR-A2 as antigen presenting cells. A cocktail was also created containing concavalin A (20 µg/ml, Sigma-Aldrich, St. Louis, MO), ionomycin (1,500 ng/ml; Merck Biosciences, Ltd., Beeston, Nottingham, UK), PHA (2 µg/ml, Bio Stat, Ltd.) and pokeweed mitogen (PMA,1,000 ng/ml, Sigma-Aldrich) to show optimal IFN-γ secretion by D4 under the same conditions. Analysis of the spots was carried out using a dissecting stereomicroscope at a magnification of 8 times by 2 independent observers.

**T2 binding and stability assays**

Peptide binding studies were carried out as described previously by Nijman et al. Briefly T2 cells were washed before incubating with the peptide overnight (37°C, 5% CO2). After washing, the T2 cells were then stained with MA2.1, a hybridoma supernatant specific for HLA-A*0201, at 4°C for 20 min. The T2 cells were washed twice in cold 0.1% FCS/PBS before FITC-labeled rabbit anti-mouse IgG (Dakocytomation, Ltd., Denmark House, Ely, Cambridgeshire, UK) at a 1:20 dilution was added for a further 20 min at 4°C. The

| Peptide             | Sequence       | Table I – Names and sequences of peptides and analogues used in our study |
|---------------------|----------------|--------------------------------------------------------------------------|
| HPV16 E7            | YMLDLQPETT     |                                                                          |
| HPV31 E711–20       | YDIHQVETT      |                                                                          |
| HPV52 E7           | YLDLQPETAT     |                                                                          |
| Analogue 31/52      | YLDLQPETT      |                                                                          |
| Analogue 52/31      | YLDLQPETT      |                                                                          |
| HPV16 E7          | YMLDLQPETT     |                                                                          |
| HPV16 E711–20      | YMLDLQPETT     |                                                                          |
| Fluorescein        | GILGFVFTL      |                                                                          |

1. All peptides were reconstituted in DMSO to a concentration of 10 mg/ml and stored at −20°C. For peptides related to HPV16 E711–20 amino acid sequences are underlined.
and 2.15 Once these cells have been transfected to express HPV 16 the C33A cells. Western blot analysis has shown these cells to generation of this epitope is not limited by low level expression of TAP 1 and 2. Killing of CaSki cells by D4 therefore suggests the antigen processing and presenting molecules LMP 2 and 7, and E6 and E7 they are recognized and lysed by D4. This is in contrast to the activity of 7E7. This clone is unable to kill CaSki cells in levels of HLA-A*0201 expression over time.

Results

CTL against HPV16 E7 11–20 can kill several cervical carcinoma cell lines including those with antigen processing defects unlike CTL against HPV16 E6 29–38.

Two different CTL clones were used to investigate the effect of antigen processing defects and HPV DNA copy number on the susceptibility of carcinoma cells to be lysed. D4 was specific for HPV16 E7 11–20 while 7E7 was specific for HPV16 E6 29–38.

The ability of D4 to recognize the HLA-A*0201 E7 11–20 peptide is shown by its ability to lyse C1R-A2 transfecants pulsed with the peptide (Fig. 1a). D4 can also recognize the peptide in the endogenous form when presented by the HLA-A*0201 +, HPV16 transformed cell line CaSki (Fig. 1a). Western blot analysis carried out in our laboratory has shown that CaSki has low levels of the antigen processing and presenting molecules LMP 2 and 7, and TAP 1 and 2. Killing of CaSki cells by D4 therefore suggests the generation of this epitope is not limited by low level expression of these proteins. This is further supported by the data observed with the C33A cells. Western blot analysis has shown these cells to have almost undetectably low levels of LMP 2 and 7 and TAP 1 and 2. Once these cells have been transfected to express HPV16 E6 and E7 they are recognized and lysed by D4. This is in contrast to the activity of 7E7. This clone is unable to kill CaSki cells in release assays, it can be seen that D4 has the ability to kill C1R-A2 targets pulsed with a range of doses of HPV16 E7 11–20.

The peptide sequences of the E7 11–20 peptides of the related HPV types 16, 31 and 52 are very similar (Table I). We therefore investigated the ability of D4 CTL to recognize targets pulsed with these peptides as an indicator of cross reactivity. In chromium release assays, it can be seen that D4 has the ability to kill C1R-A2 transfecants pulsed with the HPV16 E7 11–20 peptide (10 μg/ml, closed diamond), the HPV16 transformed cervical carcinoma cell line CaSki (closed triangle), the cervical epithelial cell line C33A (open circle), C33A transfected with HPV16 E6 and E7 (closed circle) and SiHa that had been infected with a recombinant vaccinia virus to express HLA A*0201 (open triangle). The specificity of 7E7 for CaSki cells (closed triangle), CaSki cells pulsed with the HPV16 E6 29–38 peptide (10 μg/ml, - - - closed triangle - -), C33A HPV16 cells (closed circle), SiHa cells transfected to express HLA A*0201 and pulsed with the HPV16 E6 29–38 (10 μg/ml) ( - - - - - - - - ) Percentage specific lysis was calculated as follows: 100 × [(specific release – spontaneous release)/(maximal release – spontaneous release)].

CTL against HPV16 E7 11–20 (D4) can kill cervical carcinoma cell lines with antigen processing defects and low levels of HPV1 unlike CTL against HPV16 E6 29–38 (7E7). The 2 CTL lines D4 (a) and 7E7 (b) were assessed for their ability to kill a variety of carcinoma cell lines in a 4 hr chromium release assay. (a) The specificity of D4 for the following targets: C1R-A2 transfecants (closed square), C1R-A2 transfecants pulsed with the HPV16 E7 11–20 peptide (10 μg/ml, closed diamond), the HPV16 transformed cervical carcinoma cell line CaSki (closed triangle), the cervical epithelial cell line C33A (open circle), C33A transfected with HPV16 E6 and E7 (closed circle) and SiHa that had been infected with a recombinant vaccinia virus to express HLA A*0201 (open triangle). (b) The specificity of 7E7 for CaSki cells (closed triangle), CaSki cells pulsed with the HPV16 E6 29–38 peptide (10 μg/ml, - - - closed triangle - -), C33A HPV16 cells (closed circle), SiHa cells transfected to express HLA A*0201 and pulsed with the HPV16 E6 29–38 (10 μg/ml) ( - - - - - - - - ) Percentage specific lysis was calculated as follows: 100 × [(specific release – spontaneous release)/(maximal release – spontaneous release)].

The effect of low HPV DNA copy number on the susceptibility of cervical carcinoma cells to be killed was assessed using the cell line SiHa that had been infected with a recombinant vaccinia virus expressing the same protein. SiHa cells express a maximum of 2 copies of HPV and therefore expression of the antigens of interest is predicted to be low. As with other cervical carcinoma cell lines, SiHa-A2 was killed by D4 but not 7E7 CTL. These results suggest that E7 11–20 may be a better epitope for CTL-based immunotherapy than E6 29–38.

D4 CTL can discriminate between the E7 peptides from related HPV types

The peptide sequences of the E7 11–20 peptides of the related HPV types 16, 31 and 52 are very similar (Table I). We therefore investigated the ability of D4 CTL to recognize targets pulsed with these peptides as an indicator of cross reactivity. In chromium release assays, it can be seen that D4 has the ability to kill C1R-A2 targets pulsed with a range of doses of HPV16 E7 11–20.
LQPETT, 0.1–100 μg/ml, Fig. 2). D4 was also able to kill targets pulsed with a range of doses of the HPV52 E7 11–20 peptide (YILDLQPETT, Fig. 2). The level of lysis at high peptide doses, 100 and 10 μg/ml were comparable to that seen with the HPV16 peptide. At concentrations below 10 μg/ml however the level of lysis was greatly reduced, although significant lysis was still observed. However C1R-A2 targets pulsed with the HPV31 E7 11–20 peptide (YVLDLQPEAT, Fig. 2), were not killed regardless of the peptide dose. Therefore D4 shows cross reactivity between the E7 11–20 peptides of HPV types 16 and 52 but not between 16 and 31.

**Position 9 is important for the recognition of the E7 11–20 peptide**

To investigate the important amino acid substitutions in the peptide sequences that were affecting their recognition by D4 CTL, a series of analogue peptides were synthesized to map specificity.

C1R-A2 BLCL targets pulsed with an HPV31/52 analogue (YVLDLQPEAT, Fig. 3), were significantly lysed at all doses of the peptide, albeit at a lower level than the HPV16 peptide. However the analogue HPV52/31 (YILDLQPEAT) was not recognized at any peptide concentration tested (Fig. 3).

These data suggested that a conservative amino acid substitution at position 2 of the peptides had little effect on recognition by D4. Therefore, the non-recognition of HPV31 E7 11–20 was probably due to the non-conservative amino acid change at P9. This was tested using an analogue of the HPV16 E7 11–20 peptide (YMLDLQPEAT) that contained the same amino acid at P9 as the HPV31 peptide. This altered HPV16 P9 peptide (YMLDLQPEAT) was not recognized by D4 at any dose when pulsed onto C1R-A2 cells in a chromium release assay (Fig. 3). This suggests that P9 in the HPV31 peptide is having a negative effect on recognition by D4.

**Variation in the binding to HLA-A*0201 among the HPV E7 peptides and analogues**

Data described here suggested that the nonconservative amino acid substitution (threonine to alanine) at position 9 resulted in a lack of peptide recognition. In an attempt to assess if this was due to a lack of binding or due to interaction with the TCR, a crude measurement of peptide binding was carried out (Fig. 4).

The influenza matrix peptide (M1 5 8–6 6) was used as a positive control as it is known to bind strongly to HLA-A*0201. As can be seen in Figure 4, the HPV16 E7 peptide binds equally well. Peptides with only conservative amino acid substitutions at position 2 (HPV52 E7, YILDLQPEAT and analogue 31/52, YVLDLQPEAT), showed measurable binding when compared to the HPV16 E7 peptide but the level of binding was reduced. Analogue peptides with both conservative amino acid substitutions at position 2 and nonconservative substitutions at position 9 (HPV31 E7 (YVLDLQPEAT) and analogue 52/31 (YILDLQPEAT) showed greatly reduced levels of binding to the HLA-A*0201 molecule when compared to the HPV16 peptide (Fig. 4). In most cases the bond between the HLA-A*0201 molecule and the peptide was not.
very stable. Apart from the M1 peptide only 2 HPV peptides, HPV16 E7 and HPV16 E711–20 P9 (YMLDLQPE\Delta\text{T}), showed a binding stability greater than 4 hr (Fig. 4).

The HPV16 E711–20 P9 peptide bound as well, if not slightly better than peptides with substitutions at position 2 (Fig. 4). Therefore, nonrecognition of this peptide is likely to be due to interaction of the amino acid at position 9 with the TCR.

Overall, the results demonstrated that conservative amino acid substitutions at position 2 did not dramatically affect recognition by D4 CTL (HPV52 and analogue 52/52), despite a reduction in peptide binding. Peptides with simultaneous substitutions at positions 2 and 9 abolished CTL recognition (HPV31, analogue 52/31) and further reduced peptide binding. The results with HPV16 E711–20 P9, where there is no CTL recognition despite peptide binding, suggest that position 9 acts primarily as a TCR contact residue; there is no CTL recognition of any of the peptides with changes at this residue (HPV31, analogue 52/31 and HPV16 E711–20 P9). Therefore, the lack of recognition of HPV31 E711–20 by HPV16 E711–20 CTL is due to effects on both TCR contact residues and peptide binding. However the conservative amino acid difference at position 2 in HPV52 allows cross-reactivity.

**D4 CTL can recognize transfectants expressing the full length E7 of HPV types 16 and 52.**

We have shown that D4 CTL has the ability to recognize the E711–20 peptide of HPV types 16 and 52. At the peptide level, this is of limited importance and we decided to investigate if any cross-reactivity existed at the level of full length proteins, i.e., were the cross reactive epitopes generated endogenously. The HPV negative cervical carcinoma cell line C33A was transfected using DOTAP to express the full length E7 of HPV types 16, 31, 45 and 52 and antigen specific T-cell responses measured in an IFNγ ELISpot assay.

As can be seen, both the C33A HPV16 (full HPV16 genome\textsuperscript{[44]} cell line and the C33A transfectant generated in our laboratory (C33A16A, E7 only) stimulated a greater proportion of D4 cells to secrete IFNγ than when D4 was cocultured with wild-type C33A cells (Fig. 5). This augmented response was seen when D4 was cocultured with the C33A HPV52 transfectant.

However incubating D4 with the HPV31 and 45 positive transfectants failed to result in a substantial increase in IFNγ secreting cells. This data confirms that the HPV16 E711–20 peptide is endogenously processed and presented by HPV16 E7 transfectant C33A (Fig. 5). It also suggests that HPV52 E711–20 is endogenously processed and presented in the C33A cell line.

**Discussion.**

Our study demonstrates the epitope specificities of 2 different CTL lines, D4 and 7E7. The CTL D4, specific for HPV16 E711–20 is able to kill the HPV transformed cervical carcinoma cell line CaSkI, the cell line C33A that has been transfected to express HPV16 and has antigen processing defect\textsuperscript{[15,16]} and the cell line SiHa (once transfected to express HLA-A\textsuperscript{*}0201) that express few HPV16 DNA copies. In contrast, 7E7, which was generated against HPV16 E629–38, was unable to kill any cell line unless pulsed exogenously with peptide or infected with TA-HPV to increase levels of endogenous antigen. Although precise levels of endogenous antigen could not be quantitated in the CaSkI and SiHa lines, it is likely to be low for both. A recent study has suggested that despite difference in DNA copy number, HPV gene expression is similarly low in both these cell lines.\textsuperscript{[22]} Nevertheless these results suggest the D4 epitope would be more useful in a vaccine as it generates CTL capable of killing targets expressing endogenous HPV antigens.

We used the D4 CTL as a tool to investigate the potential cross-reactivity between the E711–20 peptide of phylogenetically related HPV types. This peptide from HPV 16, 31 and 52 is very similar with changes only at anchor positions 2 and 9. The change of M to I at position 2 seen in the 52 sequence did not result in a loss of recognition at high peptide doses (>10 µg/ml). Reduced, but still significant recognition was seen at peptide doses lower than this. However altering the amino acids at position 2 and 9 as seen in the HPV31 peptide resulted in an abolition of recognition. By generating a synthetic analogue of the HPV16 peptide (YMLDLQPEAT), we were able to demonstrate that the amino acid at position 9 is vital to peptide recognition by D4. Using a crude measurement of a peptide binding to HLA-A\textsuperscript{*}0201, we were able to show that the lack of recognition is due to a loss in the ability to bind to the HLA molecule and be presented but is due to conformational change affecting recognition by the TCR.

Cross reactivity at the peptide level is not of importance unless the cross reactive epitopes are produced endogenously. We demonstrate that this is the case following transfection of the cervical epithelial cell line C33A to express full length E7 of HPV types 16 and 52.

There are however some limitations to our study. The method we have used to show the ability of peptide to bind to HLA-A\textsuperscript{*}0201, the T2 binding assay, is very crude. The results could therefore only be said to be an indication of the true interaction.\textsuperscript{[23]} Also during the course of this work, D4 became increasingly difficult to expand and maintain as it aged, limiting the number of available CTL. We therefore used ELISPOT to measure the interaction of D4 with the C33A transfectants rather than a chromium release assay as we had for the previous experiments.

We have investigated the potential of the HPV16 E7 epitope for immunotherapy as few other T-cell epitopes have been identified from HPV proteins. Non-HLA-A\textsuperscript{*}0201 restricted epitopes have been defined in the E6 and E7 proteins of HPV16.\textsuperscript{[24]} Two epitopes, E6\textsubscript{62–70} and E7\textsubscript{44–52}, were found to be restricted by HLA-B18. The E6 epitope was shown to be produced endogenously in BLCL infected with vaccinia virus encoding E6. This E6 epitope may have therapeutic potential, however HLA B18 is a rare HLA allele...
 CTL immunotherapy is a promising strategy to fight disease as has been shown in studies in both mice and humans. In cancer patients, the E6 and E7 proteins are constitutively expressed, with HPV16 E7 being highly expressed in the majority of people. Post vaccination responses to a derivative of the HPV16 E7 peptide, 11–20, have been demonstrated by Muderspach and co-workers. A study by Murakami and colleagues used an E6/E7 fusion protein in vitro to pulse autologous DC and stimulate T cells, an in vitro response was seen to the E7 11–20 peptide pulsed onto target cells or produced endogenously.

Although the HPV16 E7 11–20 epitope can be used to generate CTL that are able to lyse targets expressing endogenous antigens and shows specificity for the E7 peptide of a related HPV type, the lack of E7 11–20 responses seen in clinical trials with patients immunized with full length E7 suggests immunization with the peptide directly would be more beneficial. This only emphasizes the need for additional epitopes to generate CTL restricted by multiple MHC molecules. Once identified, CTL against these epitopes could be induced and amplified in vivo using prime boost techniques.

Acknowledgments

This work was supported by a project grant from CR UK (S.Y., K.L.S., and S.M.) and the MRC (K.T.). S.M. was supported by a Royal Society University Research Fellowship. We thank E.M. de Villiers and A.T. Lorincz for supplying plasmids used in our study. We are grateful to R. Dunbar and V. Cerundolo (Oxford University) for helpful discussions at the start of this project and Mr. T. Hoyer and Mrs. J Fisher for carrying out cell sorting.

References

1. Parkin DM. The global burden of cancer. Semin Cancer Biol 1998; 8:219–35.
2. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munro N. Human papillomavirus is a necessary cause of invasive cervical carcinoma worldwide. J Pathol 1999;189:12–19.
3. Crooke T, Morgenstern JP, Crawford L, Banks L. Continued expression of HPV-16 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV-16 plus Ras. EMBO J 1989;8:513–21.
4. Vousden K. Interactions between papillomavirus proteins and tumor suppressor gene products. Adv Cancer Res 1994;64:1–24.
5. Munoz N, Bosch X, Sanjosé Sd, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ. Epidemiological classification of human papillomavirus types associated with cervical cancer. New Engl J Med 2003;348:518–27.
6. Clifford G, Smith J, Pummer M, Munoz N, Franceschi S. Human Papillomavirus types in invasive cervical cancer worldwide: a meta analysis. Br J Cancer 2003;88:63–73.
7. Williams OM, Hart KW, Wang EC, Gelder CM. Analysis of CD4(+)/T-cell responses to human papillomavirus (HPV) type 11 L1 in healthy adults reveals a high degree of responsiveness and cross-reactivity with other HPV types. J Virol 2002;76:7418–29.
8. Evans EM, Man S, Evans AS, Borysiewicz LK. Infiltration of cervical tissue with human papillomavirus-specific cytotoxic T-lymphocytes. Cancer Res 1997;57:2943–50.
9. Youde SJ, Dunbar PR, Evans EM, Fiander AN, Borysiewicz LK, Cerundolo V, Man S. Use of fluorogenic histocompatibility leukocyte antigen-A*0201 positive cells. EMBO J 1986;5:943–9.
10. Cailleau R, Young M, Olive M, Reeves WJ. Breast tumour cell lines immortalized with Epstein Barr virus protein for CD8(+)/HLA-A*0201 positive individuals.

References
CJ. Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. J Immunol 1996;156:3308–14.

24. Muderspach L, Wileczynski S, Roman L, Bade L, Felix J, Small L, Kast WM, Fasico G, Marty V, Weber J. A phase I trial of human papillomavirus (HPV) peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV16 positive. Clin Cancer Res 2000;6:3406–3416.

25. Marsh SG, Parham P, Barber LD. The HLA facts book. London: Academic Press, 2000.

26. Nilges K, Hohn H, Pilch H, Neukirch C, Freitag K, Talbot PJ, Mauerer MJ. Human papillomavirus type 16 E7 peptide-directed CD8+ T cells from patients with cervical cancer are cross-reactive with the coronavirus NS2 protein. J Virol 2003;77:5464–74.

27. Feltkamp M, Smits HL, Vierboom M, Minnaar R, de Jongh B, Drijfhout J, ter Schegget J, Melief C, Kast W. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. Eur J Immunol 1993;23:2242–9.

28. Yee C, Thompson J, Byrd D, Riddell S, Roche P, Celis E, Greenberg P. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci U S A 2002;99:16168–73.

29. Schreurs MW, Scholten KB, Kueter EW, Ruizendaal JJ, Meijer CJ, Hooijschuur E. In vitro generation and life span extension of human papillomavirus type 16-specific, healthy donor-derived CTL clones. J Immunol 2003;171:2912–21.

30. van der Burg S, Kwappenberg K, O’Neill T, Brandt R, Melief C, Hickling J, Offringa R. Pre-clinical safety and efficacy of TA-CIN, a recombinant HPV16 L2E6E7 fusion protein vaccine, in homologous and heterologous prime-boost regimens. Vaccine 2001;19:3652–60.

31. Davidson EJ, Boswell CM, Sehr P, Pawlita M, Tomlison AE, McVey RJ, Dobson J, Roberts JS, Hickling J, Kitchener HC, Stern PL. Immunological and clinical responses in women with vulval intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. Cancer Res 2003;63:6032–41.

32. Baldwin PJ, van der Burg SH, Boswell CM, Offringa R, Hickling JK, Dobson J, Roberts JS, Latimer JA, Moseley RF, Coleman N, Stanley MA, Sterling JC. Vaccinia-expressed human papillomavirus 16 and 18 e6 and e7 as a therapeutic vaccination for vulval and vaginal intraepithelial neoplasia. Clin Cancer Res 2003;9:5205–13.

33. Murakami M, Gurski KJ, Marincola FM, Ackland J, Steller MA. Induction of specific CD8+ T-lymphocyte responses using a human papillomavirus-16 E6/E7 fusion protein and autologous dendritic cells. Cancer Res 1999;59:1184–7.

34. Palmowski MJ, Choi EM, Hermans IF, Gilbert SC, Chen JL, Gileadi U, Salio M, Van Pel A, Man S, Bonin E, Liljestrom P, Dunbar PR, et al. Competition between CTL narrows the immune response induced by prime-boost vaccination protocols. J Immunol 2002;168:4391–8.

35. Seedorf K, Krammer G, Durst M, Suhai S, Rowe Kemp WG. Human papillomavirus type 16 DNA sequence. Virology 1985;145:181–5.

36. Lorincz AT, Lancaster WD, Temple GF. Cloning and characterisation of the DNA of a new human papillomavirus from a woman with dysplasia of the uterine cervix. J Virol 1986;58:225–9.

37. Egawa K, Delia H, Matsukura T, Kawashima M, Villiers EMd. Two novel types of human papillomavirus, HPV 63 and 65: comparisons of their clinical and histological features and DNA sequences to other types. Virology 1993;194:789–99.

38. Shimoda K, Lorincz AT, Temple GF, Lancaster WD. Human papillomavirus type 52: a new virus associated with cervical neoplasia. J Gen Virol 1988;69:2925–8.