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

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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 E7$_{11-20}$. 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 E7$_{11-20}$ 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.
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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.[1] The DNA of HPVs, particularly 16 and 18, are found in >99% of CaCx patients.[2] It is the E6 and E7 proteins, which are consistently retained and expressed in cervical tumor cells, that give the virus its transforming properties.[3,4]

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 Mûnoz *et al.,* 95% of all infections were caused by 8 types: 16, 18, 45, 31, 33, 52, 58 and 35. They suggest that vaccination against the 5 most common types could prevent 90% of cases of CaCx.[5]

The geographical variation of HPV prevalence[6] 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.[7] We have previously shown that it is possible to detect CTL specific for HPV16 E7$_{11-20}$ in the blood of patients with both CaCx[8] and CIN3[9] and in the blood of healthy donors.[9] These CTL can mediate the lysis of B cells endogenously expressing the E7 protein[8] and the HPV 16 transformed cervical cancer cell line CaSki.[9] Since the E7$_{11-20}$ 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, Pontyclun, 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[10]) was maintained in RPMI 1640 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

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 E7$_{11-20}$ monospecific polyclonal CTL line D4

D4, a monospecific polyclonal CTL line was generated as described previously.[9] Briefly, this HPV16 E7$_{11}$ $_{20}$ specific CTL line was generated from a patient with stage IIB cervical cancer.[8] Single cell sorting based on CD8 (tricolor-anti CD8, Caltag, Burlingame, CA)/HPV16 E7$_{11-20}$ tetramer (PE labeled) double staining was then carried out using a FACSVantage (Becton-Dickinson, Mountain View, CA).

Generation of HPV16 E6$_{29-38}$ clone 7E7

A T-cell clone against the HPV16 E6$_{29-28}$ peptide was generated as described previously.[15] 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[14] and work from our laboratory[15] and others[16] has shown that this cell line has low level expression of molecules involved in antigen processing and presentation, *e.g.*, TAP 1 and 2, LPM2 and 7, and HLA-A*0201. E7 was amplified from the cDNA of HPV16, 31, 45 and 52, kindly supplied as described in Table II, by polymerase chain reaction (PCR) using E7 specific primers detailed in Table III obtained from Invitrogen Corporation.

PCR products were cloned into pCR2.1 TOPO PCR cloning vector according to TOPO TA cloning kit manufacturer's instructions (Invitrogen Corporation). E7 inserts were cut out of the pCR2.1 TOPO PCR cloning vector using restriction endonucleases BamHI and EcoR1 and ligated into a pcDNA6 expression vector (Invitrogen Corporation). Ligations were transformed into bacteria and the resulting clones screened by restriction digest and sequenc-

ing to isolate clones that correctly expressed E7. Sequencing confirmed all inserts were identical to published data.

To carry out transfection, C33A cells were plated out at 2×10^5/ well in 6-well plates (Greiner Bio-One, Ltd., Stonehouse, Gloucestershire, UK) for 18 hr before the addition of DOTAP liposomal transfection reagent (Roche Diagnostics, Ltd., Lewes, East Sussex, UK, according to the manufacturer's instructions) and 5 μg DNA. Forty-eight hours posttransfection E7 expressing cells were selected in 2.5 μg/ml blasticidin (Invitrogen Corporation). Cultures were maintained in selection media and cells used in assays were all less than passage 20.

Transfection of SiHa with HLA-A'0201

SiHa cells were transfected using DOTAP lyposomal transfection reagent with the A2/pRSV5neo plasmid conferring neomycin resistance (Invitrogen Corporation) cloned with HLA-A*0201. 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 HLA-A*0201 expression.

Cytotoxicity assays

Cytotoxicity was measured in a standard 4 hr ^{51}Cr release assay as described previously.[9] C1R-A2 target cells were pulsed with varying doses of the peptides and analogues for 1 hr after labeling for 2 hr with ^{51}Cr (Na$_2^{51}$CrO$_4$; 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, UK[17]). TA-HPV has been shown to express HPV antigens by both Western blot[17] and by recognition by HPV-specific CTL.[8] C1R-A2 cells were infected with the vaccinia viruses (MOI of 15) for a maximum of 12 hr before ^{51}Cr 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 ^{51}Cr. The cell lines MDA231 and C33A, which contain no HPV antigens, were used as controls. After 4 hr incubation, radioactive counts were obtained by βeta 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 2×10^4 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 E7$_{11-20}$ peptide was added at a concentration of 10 μg/ml with C1R-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.[18] Briefly T2 cells were washed before incubating with peptide overnight in TCM at 37°C in 5% CO$_2$. The cells were then stained with MA2.1, a hybridoma supernatant specific for HLA-A*0201, at 4°C for 20 min. The 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

TABLE I – NAMES AND SEQUENCES OF PEPTIDES AND ANALOGUES USED IN OUR STUDY[1]

Peptide	Sequence
HPV16 E7$_{11-20}$	YMLDLQPETT
HPV31 E7$_{11-20}$	YVLDLQPEAT
HPV52 E7$_{11-20}$	YILDLQPETT
Analogue 31/52	YVLDLQPETT
Analogue 52/31	YILDLQPEAT
HPV16 E7$_{11-20}$ P9	YMLDLQPEAT
HPV16 E6$_{29-38}$	TIHDIILECV
Influenza A matrix protein M1$_{58-66}$	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 E7$_{11-20}$, amino acid differences are underlined.

TABLE II – PLASMIDS USED IN THIS STUDY AND THEIR SOURCES

Virus type	Vector	Source	Institution	Reference
HPV16	pBR322	E-M de Villiers	DKFZ, Heidelberg	Seedorf et al.[35]
HPV31	pT713	AT Lorincz	Digene, MD	Lorincz et al.[36]
HPV45	pGEM4	E-M de Villiers		Egawa et al.[37]
HPV52	pUC19	AT Lorincz		Shimoda et al.[38]

TABLE III – THE SEQUENCE OF PRIMERS USED IN OUR STUDY

HPV type and protein	Primer sequence 5′–3′	Primer length (bp)
16E7	Sense CGGGATCCCGACACGTAGAGAAACCCAGCTG	31
	Antisense GGAATTCCCTATTAGTGATGGTGATGGTGATG CGATCCTCTTGGTTTCTGAGAACAGATG	60
31E7	Sense CGGGATCCCGCCTCGTACTGAAACCCAAGTG	31
	Antisense GGAATTCCCTATTAGTGATGGTGATGGTGATG CGATCCTCTCAGTCTAGTAGAACAGTTGGGGC	64
45E7	Sense CGGGATCCCGCGCAGACGTAGGGAAACACAA	31
	Antisense GGAATTCCCTATTAGTGATGGTGATGGTGATG CGATCCTCTTTGGTTAGTTGCACACCACGG	62
52E7	Sense CGGGATCCCGACCCCGACCTGTGACCCAAGT	31
	Antisense GGAATTCCCTATTAGTGATGGTGATGGTGATG CGATCCTCTTAGCCGTGCACAGCCGGGGC	61

cells were washed and fixed and the fluorescence analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

The fluorescence ratio was calculated as follows: Mean fluorescence experimental sample/Mean fluorescence of background. To measure the stability of the peptide/MHC interaction we used a modification of the method described by Feltkamp et al.[19] Briefly, 1×10^6 T2 cells were incubated with 100 µg/ml of peptide and 5 µg/ml B_2 microglobulin (Sigma-Aldrich) overnight at 37°C, 5% CO_2. Cells were then washed in TCM, resuspended in 1 ml TCM and returned to the incubator. An aliquot was taken at this point and then subsequently after 2, 4 and 6 hr and stained as above for 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$_{11-20}$[9] while 7E7 was specific for HPV16 E6$_{29-38}$.[15]

The ability of D4 to recognize the HLA-A*0201 E7$_{11-20}$ peptide is shown by its ability to lyse C1R-A2 transfectants pulsed with the peptide (Fig. 1*a*). D4 can also recognize the peptide in the endogenous form when presented by the HLA-A*0201+, HPV16 transformed cell line CaSki (Fig. 1*a*). Western blot analysis carried out in our laboratory[15] 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.[15] Once these cells have been transfected to express HPV 16 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
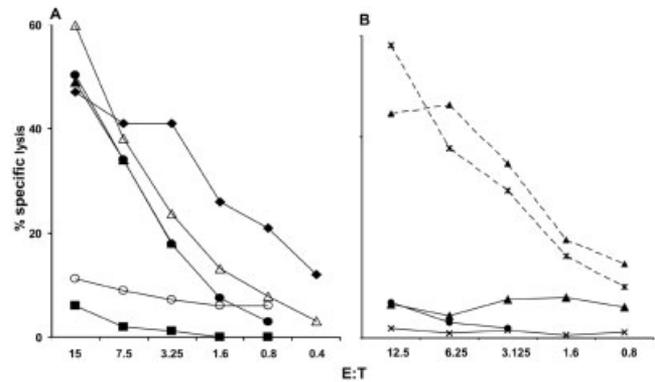

FIGURE 1 – CTL against HPV16 E7$_{11-20}$ (D4) can kill cervical carcinoma cell lines with antigen processing defects and low levels of HPV16 unlike CTL against HPV16 E6$_{29-38}$ (7E7). The 2 CTL lines D4 (*a*) and 7E7 (*b*) were assessed for their ability to kill a variety of carcinoma cell lines in a 4 hr chromium release assay. (*a*) The specificity of D4 for the following targets: C1R-A2 transfectants (closed square), C1R-A2 transfectants 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 (×), SiHa cells transfected to express HLA A*0201and 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)].

^{51}Cr release assays unless the cells have been pulsed with exogenous peptide (Fig. 1*b*). This suggests that generation of this epitope may be limited by antigen processing defects in CaSki cells, despite the report that CaSki has 200 copies of the HPV genome.[20] This is supported by the results seen with the C33A HPV16 cells. These cells, which have low levels of the molecules involved in antigen processing and presentation, are not killed by 7E7.

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 to express HLA-A*0201 or transfected to express 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$_{11-20}$ (YMLD-
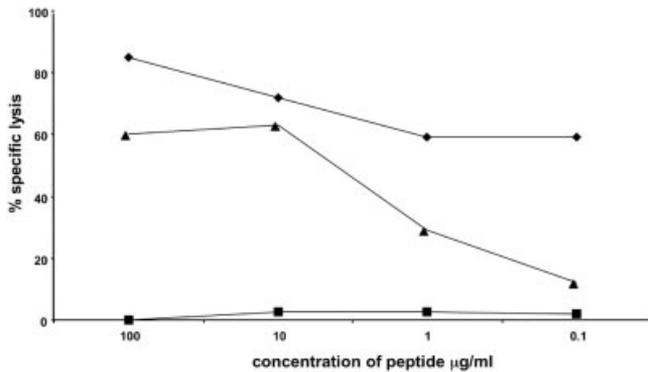
FIGURE 2 – D4 CTL discriminates between the E7 peptides of related HPV types. A 4 hr chromium release assay was used to asses the ability of D4 to kill C1R-A2 cells pulsed for 2 hr with varying doses of the 11–20 peptide from the following HPV types: HPV16 (YMLDLQPETT) closed diamond, HPV31 (YVLDLQPEAT) closed square or HPV52 (YIDLQPETT) closed triangle. In all cases an E:T of 10:1 was used.

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 (YVLDLQPETT, 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 ($M1_{58-66}$) was used as a positive control as it is known to bind strongly to HLA-A*0201.[21] As can be seen in Figure 4, the HPV16 E7 peptide binds equally well.
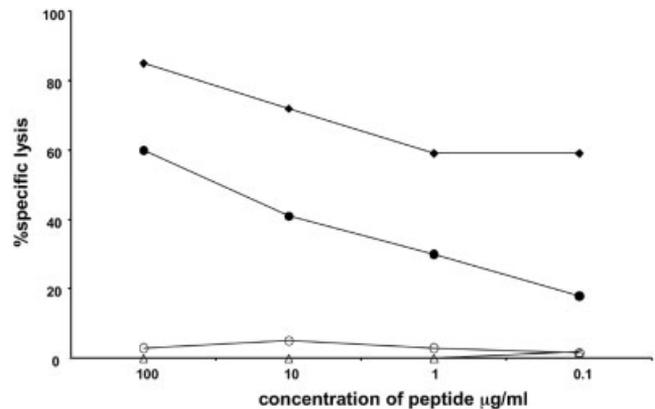

FIGURE 3 – Position 9 is important for the recognition of the $E7_{11-20}$ peptide. A 4 hr chromium release assay was used to assess the importance of the amino acid at position 9 in the peptide sequence of $E7_{11-20}$ peptides. D4 was used to kill C1R-A2 cells pulsed for 2 hr with varying concentrations of the analogue peptides comprising of the HPV31 and 52 $E7_{11-20}$ sequences. The peptides and sequences were HPV31/52 analogue, YVLDLQPETT (closed circle); HPV52/31 analogue, YILDLQPEAT (open circle) and an analogue of the HPV16 E7 peptide containing an alanine at position 9, YMLDLQPEAT (open triangle). In all cases and E:T of 10:1 was used. The killing of C1R-A2 cells pulsed with the HPV16 $E7_{11-20}$ peptide (YMLDLQPETT) at the same concentration is also shown (closed diamond).
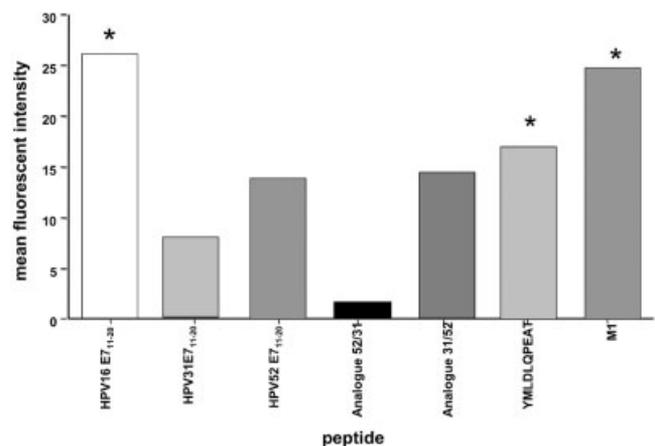

FIGURE 4 – Alterations in the amino acid sequence of the E7 peptide can affect binding to HLA-A*0201. The affect of amino acid substitutions in the E7 peptides on binding to HLA-A*0201 and the stability of this bond was assessed in a peptide binding assay using the antigen processing defective cell line T2. These cells have low levels of surface HLA-A*0201, which increases when a peptide binds stably. Briefly cells were incubated with the various peptides at 10 µg/ml before staining with MA2.1, a monoclonal antibody specific to HLA-A*0201. The influenza matrix peptide ($M1_{58-66}$), which is known to bind strongly to this HLA allele, was used as a positive control. *Peptides that bind to HLA-A*0201 stably for greater than 4 hr.

Peptides with only conservative amino acid substitutions at position 2 (HPV52 E7, YILDLQPETT and analogue 31/52, YVLDLQPETT), 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 E7$_{11-20}$ P9 (YMLDLQPE$\underline{A}$T), showed a binding stability greater than 4 hr (Fig. 4).

The HPV16 E7$_{11-20}$ 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 31/52), 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 E7$_{11-20}$ 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 E7$_{11-20}$ P9). Therefore the lack of recognition of HPV31 E7$_{11-20}$ by HPV16 E7$_{11-20}$ 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 E7$_{11-20}$ 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[14]) 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 E7$_{11-20}$ peptide is endogenously processed and presented by HPV16 E7 transfected C33A (Fig. 5). It also suggests that HPV52 E7$_{11-20}$ 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 E7$_{11-20}$ 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 defects[15,16] and the cell line SiHa (once transfected to express HLA-A*0201) that express few HPV16 DNA copies . In contrast, 7E7, which was generated against HPV16 E6$_{29-38}$, 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.[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 E7$_{11-20}$ 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
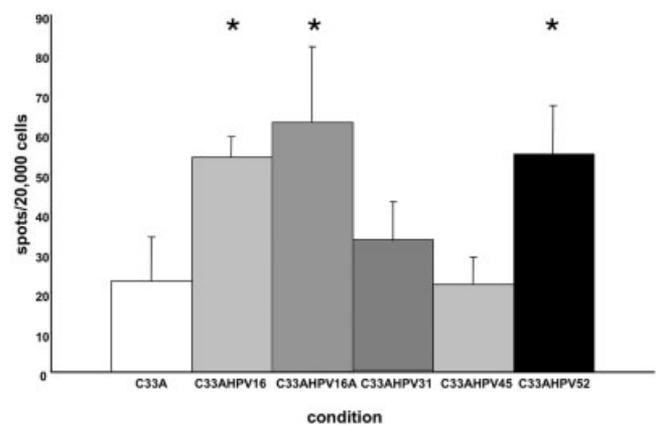

FIGURE 5 – D4 can kill transfectants expressing the full-length E7 of HPV types 16 and 52. The CTL D4 was assessed for its ability to kill transfectants expressing full length E7 of HPV types 16, 31, 45 and 52. C33A cells were transfected using the liposomal transfection reagent DOTAP and selected by growing in media containing 2.5 μg/ml blasticidin. The number of antigen specific T cells was analyzed by IFNγ ELISPOT. Responder cells were incubated with the following stimulators: C33A; C33A HPV16, C33A transfected in the laboratory to express HPV16 (C33AHPV16A), HPV31, HPV45 and HPV52. The ELISPOT was developed after 18 hr and cells were mixed at ratio of 1:1 with a total number of 20,000 responders/well. *Transfectants that stimulated CTL to produce a significantly higher amount of IFNγ compared to wild-type C33A cells incubated with D4. Under the same culture conditions, incubation of D4 with the HPV16 E7$_{11-20}$ peptide at 10 μg/ml produced 404 spots and with a positive control cocktail containing PHA, PMA, concavalin A and ionomycin more than 500 spots was seen (data not shown).

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*0201, we were able to show that the lack of recognition is not 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*0201, the T2 binding assay, is very crude. The results could therefore only be said to be an indication of the true interaction.[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*0201 restricted epitopes have been defined in the E6 and E7 proteins of HPV16.[24] Two epitopes, E6$_{68-88}$ and E7$_{44-52}$, 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

(7.4% in Caucasian population,[25]) and no recognition of cancer cells has been documented.

Cross reactivity has also been demonstrated between CD8+ T cells specific for HPV and other non-HPV peptides.[26] T cells from healthy individuals stimulated with dendritic cells (DC) pulsed with HPV16 E7$_{11-19}$ were able to recognize both the stimulating peptide and unrelated peptides from coronavirus. These cross-reactive epitopes were also produced endogenously. These authors argue that this epitope may therefore not be ideal for assessing anti HPV responses and may explain the detection of anti HPV T cells seen in healthy individuals. It would be of interest to test whether CTL clones against HPV16 E7$_{11-20}$ could cross react on both coronavirus and HPV52.

CTL immunotherapy is a promising strategy to fight disease as has been shown in studies in both mice[27] and humans.[28] In cancer patients, the E6 and E7 proteins are constitutively expressed making them attractive targets for immunotherapy. Our previous work has shown the presence (at low frequency) of CTL to HPV16 E7$_{11-20}$ in the blood of both patients with cervical cancer[8] and CIN3.[9] Our current results suggest that CTL to the E7$_{11-20}$ epitope would be useful for immunotherapy as CTL produced by in vitro stimulation protocols are effective against targets expressing endogenous antigen. This has been confirmed independently in a recent study by Schruers et al.[29] who used DC pulsed with a modified HPV16 E7$_{11-20}$ peptide (YMLDLQPETV) to generate CD8+ CTL clones from healthy donors. The clones killed targets expressing exogenous and endogenously processed E7 epitope presented either by CaSki cells or a cell line derived from an HPV16 positive biopsy of an HLA-A*0201 positive individual.

However, despite employing a variety of vaccines and vaccination strategies, the generation of CTL to the HPV16 E7$_{11-20}$ epitope has not been seen in clinical trials.[30] For example in multiple studies using TA-HPV, a live recombinant vaccinia virus encoding HPV 16 and 18 E6 and E7, responsiveness against E7$_{11-20}$ has not been seen even when using sensitive detection techniques such as ELISpot.[31,32] Despite a number of vaccines and vaccination regimes, HPV16 E7$_{11-20}$ responses are not seen in the majority of people. Post vaccination responses to a derivative of the E7$_{11-20}$ peptide, 12–20, have been demonstrated by Muderspach and co-workers.[24] A study by Murakami and colleagues[33] 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 HLA molecules. Once identified, CTL against these epitopes could be induced and amplified in vivo using prime boost techniques.[34]

Acknowledgments

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