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Use of Fluorogenic Histocompatibility Leukocyte Antigen-A*0201/HPV 16 E7 Peptide Complexes to Isolate Rare Human Cytotoxic T-Lymphocyte-recognizing Endogenous Human Papillomavirus Antigens[1]

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ABSTRACT

Cervical cancer (CaCx) is the second most common female malignancy worldwide and remains a clinical problem despite improvements in early detection and therapy. CaCx and preinvasive cervical intraepithelial neoplasia (CIN3) are strongly associated with infection by human papillomavirus (HPV), particularly types 16 and 18. Two nonstructural viral proteins, E6 and E7, are constitutively expressed in cervical tumors and are crucial for the maintenance of the transformed phenotype. These proteins thus provide attractive targets for immunotherapy of CaCx mediated by CD8+ CTLs. However, reliable detection and generation of HPV-specific CTLs in humans has been difficult. Recently, soluble fluorogenic MHC-peptide complexes (tetramers) have greatly increased the sensitivity of antiviral and antitumor CTL detection. To examine the feasibility of this approach for detecting HPV-specific CTLs, we constructed a tetramer consisting of HLA-A*0201 and the best studied HPV CTL peptide epitope, HPV 16 $E7_{11–20}$. Between 2 and 12% of short-term HPV 16 $E7_{11–20}$ CTL lines derived from CaCx patients stained highly with the tetramer. Direct *ex vivo* staining of peripheral blood mononuclear cells revealed $CD8^+$ $tetramer^+$ high cells at low frequencies in both CIN3 patients (1 of 1,260 to 1 of 19,073) and normal controls (1 of 1,855 to 1 of 42,004). However, short-term *in vitro* stimulation with the HPV 16 $E7_{11–20}$ peptide expanded $CD8^+tetramer^+$ cells to a greater extent in the peripheral blood mononuclear cells from CIN3 patients. Furthermore, the tetramer provided a powerful tool to isolate polyclonal and clonal peptide-specific CTLs from an established HPV 16 $E7_{11–20}$-specific CTL line. These purified CTLs were able to lyse both peptide-pulsed targets and targets expressing endogenously processed HPV antigens. This tetramer may therefore be useful for selecting rare high-affinity HPV-specific CTLs for the immunotherapy of CaCx.

INTRODUCTION

CaCx[6] is the second most common cause of cancer in women world wide (1), and with premalignant CIN3 is associated with HPV infection (2). The DNA of HPVs, particularly types 16 and 18, are found in >95% of CaCx patients (3). The E6 and E7 proteins of the virus can immortalize cells *in vitro*, and these proteins are consistently retained and expressed in cervical tumor cells (4, 5). Animal models suggest that HPV-specific CD8+ CTLs may be therapeutic against HPV-transformed tumor cells (6–9). There is also circumstantial evidence in humans that cell-mediated immunity, including $CD8^+$ CTLs, are important in controlling papillomavirus infection (10–13). However, it has been difficult to detect human CTLs against HPV (14–16).

Recently several groups, using peptides (17, 18), tumor cells (19), soluble proteins (20), or recombinant adenoviruses (21, 22), have demonstrated that human CTLs against HPV 16 restimulate PBMCs from patients *in vitro*. The use of different patient groups (CaCx or CIN3) and different techniques for *in vitro* restimulation of HPV-specific CTLs have led to detection rates of 0–80%. Even when the same HLA-A*0201-restricted peptide epitope (HPV 16 $E7_{11–20}$) has been used to restimulate CTLs, there has been considerable variation in detection of CTLs among patients (15, 17, 18). Despite this variation, HPV-specific CTLs cannot be detected in the PBMCs of normal controls unless dendritic cells are used as antigen-presenting cells *in vitro* (18). Collectively, these studies suggest that memory CTLs against HPV can be detected in patients but that they are at low frequencies compared with systemic CTLs against viruses such as influenza A. The variation in CTL responses detected may reflect either an individual variation in response against HPV or the efficiency of *in vitro* restimulation/detection methods (23). There is a need to increase the sensitivity of HPV-specific CTL detection both in blood and at sites of disease for immune monitoring in epidemiological and clinical trial studies.

Novel technologies such as ELISPOT assays, intracellular cytokine staining, and fluorogenic MHC-peptide complexes (tetramers) have been used to greatly increase the sensitivity of antigen-specific $CD8^+$ T-cell detection (24–28). The $CD8^+$ T-cell frequencies obtained with these techniques suggest that conventional techniques may greatly underrepresent the actual number of antigen-specific $CD8^+$ T-cell effectors (23, 25). The use of tetramers is particularly attractive because it allows direct quantitation of antigen-specific T cells from blood or sites of disease without the need for *in vitro* restimulation. Furthermore, the $CD8^+$ T cells detected by tetramers are largely cytotoxic T-cell effectors (25, 29). To investigate whether tetramers could be used to identify HPV-specific CTLs, we constructed a tetramer containing HLA-A*0201 complexed to the best studied HPV CTL epitope, HPV 16 $E7_{11–20}$. In this report, we describe the use of this HPV tetramer to quantify HPV-specific CTLs in peripheral blood and as a tool to isolate high-affinity CTLs capable of killing HPV 16^+ tumor cells.

MATERIALS AND METHODS

Media. RPMI 1640 (Life Technologies Inc., Gaithersburg, MD) was always used with the following additions: 0.02 M HEPES (Sigma-Aldrich Co. Ltd., Irvine, United Kingdom), 2 mM L-glutamine (Life Technologies), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies). For the culture of T cells, this medium was supplemented with 10% pooled human AB serum (National Blood Transfusion Service, Pontyclun, Wales) and known as RAB.

Cell Lines. The C1R-A2 cell line, a transfectant expressing the HLA *A*0201* allele (30) was maintained in RPMI 1640 containing 10% FCS (Life

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[6] The abbreviations used are: CaCx, cervical cancer; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; CD, cluster of differentiation; PBMC, peripheral blood mononuclear cell; RAB, RPMI 1640 supplemented with 10% pooled human AB serum; PE, phycoerythrin; IL-2, interleukin 2; FACS, fluorescence-activated cell sorting; TCR, T-cell receptor; CTL, cytotoxic T lymphocyte.

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Technologies) and 400 μg/ml G418 (Life Technologies). CaSki, an HLA-*A*0201* cervical carcinoma cell line expressing HPV-16 E6 and E7 proteins (obtained from ATCC) was maintained in RPMI containing 10% FCS (31). MDA-231, an HLA-A**0201* breast epithelial carcinoma cell line (kind gift from Linda Sherman, Scripps Clinic, La Jolla, CA) was maintained in RPMI containing 10% FCS (32).

Patients and Controls. CaCx patients were recruited (with informed consent) from patients presenting for surgical treatment at the University Hospital of Wales, Cardiff (18). All CIN3 patients had histologically confirmed CIN3 and were recruited when they attended colposcopy clinics at the University Hospital of Wales or presented for surgery at Llandough Hospital (22). For tetramer analyses, PBMCs from 10 HLA-A**0201*$^{+}$ patients were used, with samples from nine CIN3 patients and one CaCx patient. For controls, nine HLA-A**0201*$^{+}$ laboratory volunteers (ages, 26–50 years) were used: four males and five females. None of the females had any history of abnormal cervical cytology, and none of the controls were in high-risk groups for acquisition of sexually transmitted diseases.

PBMC Isolation. PBMCs were separated from heparinized blood samples (8.5–50 ml) by centrifugation on a Histopaque density gradients (Sigma) and washed three times with RPMI 1640 before use. Patient PBMCs were frozen in aliquots containing 5–10 $\times 10^6$ cells, stored in liquid nitrogen, and thawed before use for CTL generation (18) and tetramer staining.

Peptides. HPV 16 $E7_{11–20}$ (YMLDLQPETT; Ref. (33) was synthesized by Immune Systems Ltd., Paignton, United Kingdom. As controls, two other HLA-A**0201*-presented peptides were used; TP (GLLGFVGTL), derived from the TAP2 protein (34), synthesized by Immune Systems Ltd.; and CP36 (YLKTIQNSL) from *Plasmodium falciparum* (35), synthesized by the Peptide and Protein Facility of the University of Wales College of Medicine.

Tetramer Synthesis. MHC class I/peptide complexes were synthesized and tetramerized as described previously (24). Briefly, purified HLA-*A2.1* heavy chain and human β2-microglobulin were synthesized using a prokaryotic expression system (pET; R+D Systems, Abingdon, United Kingdom). The heavy chain was modified by deletion of the transmembrane region and cytosolic tail and addition at the COOH terminus of a sequence containing the biotinylation site recognized by the enzyme *Bir*A. Heavy chain and β2-microglobulin (Sigma) were refolded by dilution around peptide 11–20 from the E7 protein of HPV-16 (YMLDLQPETT). Refolded complexes (45 kDa) were purified by gel filtration and biotinylated using *Bir*A in the presence of biotin (Sigma), ATP (Sigma), and Mg^{2+}(Sigma). Biotinylated complexes were purified by gel filtration and ion exchange, using fast protein liquid chromatography, and then PE-conjugated streptavidin (Sigma) was added at a 1:4 molar ratio to form fluorogenic HPV-16 $E7_{11–20}$ tetramer.

***In Vitro* CTL Induction Using Peptide Stimulation.** Thawed PBMCs from patients were stimulated as described previously (18). Briefly, 2×10^6/ml PBMCs in RAB were cultured with peptide at a concentration of 10 μg/ml. On day 4, 1 ml of RAB containing 25 units/ml IL-2 (Chiron UK Ltd, Harefield, Middlesex, United Kingdom) was added. On day 6, 1 ml of medium was aspirated from each well and replaced with 1 ml of fresh medium containing 10 units/ml IL-2. On day 7, fresh or thawed irradiated autologous PBMCs were resuspended at 3×10^6/ml in RAB containing 10 μg/ml peptide and 3 μg/ml β_2-microglobulin. Antigen-presenting cells were allowed to adhere for 2 h, and then were washed before the addition of 1–2 $\times 10^6$/ml effectors. On day 9, 1 ml of RAB containing 25 units/ml IL-2 was added to each well. On day 13, the contents of the wells were split and topped up with medium containing 10 units/ml IL-2. The cells were used in a cytotoxicity assay on day 14.

FACS Staining, Sorting, and Generation of Monoclonal and Polyclonal CTL Lines. Thawed PBMC samples were stained with PE-labeled HPV-$E7_{11–20}$ tetramer for 15 min at 37° before the addition of Tricolour-anti-CD8 (Caltag, Burlingame, CA) or FITC-anti-CD8 (DAKO AS, Glostrup, Denmark) for 15 min on ice, followed by extensive washes with PBS containing 1% FCS. The cells were fixed in PBS containing 2% paraformaldehyde and 1% FCS before analysis on a FACScan (Becton Dickinson, Mountain View, CA). Small lymphocytes were gated by forward and side scatter profiling, with up to 1×10^6 cells being collected for analysis of PBMC samples. For some individuals, tetramer staining was performed after 1 week of *in vitro* culture with the HPV 16 $E7_{11–20}$ peptide.

The line from the patient JJ, stimulated as described (18), was stained with PE-labeled HPV-$E7_{11–20}$ tetramer for 15 min at 37° before the addition of Tricolour-anti-CD8 (Caltag) for 15 min on ice, followed by extensive washes, and then was sorted using a FACSVantage (Becton Dickinson). Small lymphocytes were gated by forward and side scatter profiling and then sorted according to tetramer/CD8 double staining. Single cells, or 500 cells for the monospecific, polyclonal line D4, were sorted directly into a U-bottomed 96-well plate. Each well had been coated overnight at 4°C with anti-CD3 and anti-CD28, both at 100 ng/ml in PBS, and contained 10^5 irradiated B cells (LG2) in CTL medium [Iscove's medium (Sigma) with 5% human serum containing 100 units/ml IL-2]. Plates were incubated at 37° in 5% CO_2 for 7–14 days without any manipulation, and then proliferating blasts were expanded in CTL medium, followed by restimulation using 5 μg/ml phytohemagglutinin with irradiated allogeneic peripheral blood lymphocytes and B cells as feeders (36).

Cytotoxicity Assays. Cytotoxicity was measured in a standard 4-h ^{51}Cr-release assay as described previously (18). C1R-A2 target cells were pulsed with 10 μg/ml peptide for 2 h after labeling with ^{51}Cr ($Na_2{}^{51}CrO_4$; Amersham International, Little Chalfont, United Kingdom). Cytotoxicity against HPV 16 and 18 E6 and E7 was measured using a recombinant vaccinia virus, TA-HPV (gift of Cantab Pharmaceuticals, Cambridge, United Kingdom; Ref. 37). TA-HPV has been shown to express HPV antigens by both Western blot (37) and by recognition by HPV-specific CTLs (18, 22). C1R-A2 cells were infected with the vaccinia viruses (multiplicity of infection, 15) for a maximum of 12 h before ^{51}Cr labeling. After a 4-h incubation, radioactive counts were obtained by βeta plate liquid scintillation counting (Wallac, Turku, Finland).

RESULTS

Characterization of HLA-A*A201/HPV 16 $E7_{11–20}$ Tetramer. Previously, we have shown that HPV 16 $E7_{11–20}$-specific CTL lines could be generated from four of five CaCx patients but not from control subjects unless dendritic cells were used as antigen-presenting cells (18). We used some of these CTL lines to assess the specificity of an HLA-A**0201* tetramer incorporating the peptide HPV 16 $E7_{11–20}$. These CTL lines all demonstrated specific HLA*0201-restricted recognition of the HPV 16 $E7_{11–20}$ peptide and were able to lyse targets expressing endogenous HPV antigens after infection with recombinant vaccinia virus (Fig. 1, *A–E*; Ref. 18). Clear populations of CD8^{+} tetramer^{+} cells could be distinguished in all CTL lines tested (Fig. 1, *F–J*), and this varied between 2 and 12% of the T cells analyzed. No CD8^{+} tetramer^{+} cells could be detected in an HLA-A**0201*-restricted CTL line recognizing influenza $M1_{58-66}$ peptide (data not shown).

Enumeration of HPV 16 $E7_{11–20}$-specific CD8 T Cells in Peripheral Blood of CIN3 Patients and Healthy Controls. Tetramers have been used in other viral and tumor systems to directly quantify antigen-specific CD8^{+} T cells in human peripheral blood without *in vitro* antigen-specific stimulation (27, 29, 38). PBMC samples from 10 HLA-A**0201*$^{+}$ patients with cervical neoplasia (9 CIN3 and 1 CaCx) and 9 HLA-A**0201*$^{+}$ healthy controls were stained using the HPV 16 $E7_{11–20}$ tetramer (Table 1). Previous work has shown that tetramers can be used directly to visualize and clone human T cells constituting at least 0.01% of CD8^{+} T cells (36, 39). Using this criterion, CD8^{+}tetramer^{+} T cells could be detected in PBMCs of both the patient and control groups. However, there was variation in the frequencies of CD8^{+}tetramer^{+} cells detected (Table 1). A range of 0.079–0.005% (1 of 1,260 to 1 of 19,073) was found in patients, with a similar range, 0.054–0.002% (1 of 1,855 to 1 of 42,004) in the normal control group (Table 1). These low frequencies are similar to those found for melanoma-specific CTLs in PBMCs (36, 39, 40) but are an order of magnitude lower than the frequencies detected for systemic antiviral CD8 T cells (24, 29, 41). In some patients and controls, *e.g.,* P6–P10 and C5–C9 in Table 1, the number of CD8^{+}tetramer^{+} cells was close to the limits of detection.

To enhance detection of rare tetramer^{+} cells, PBMCs from patients

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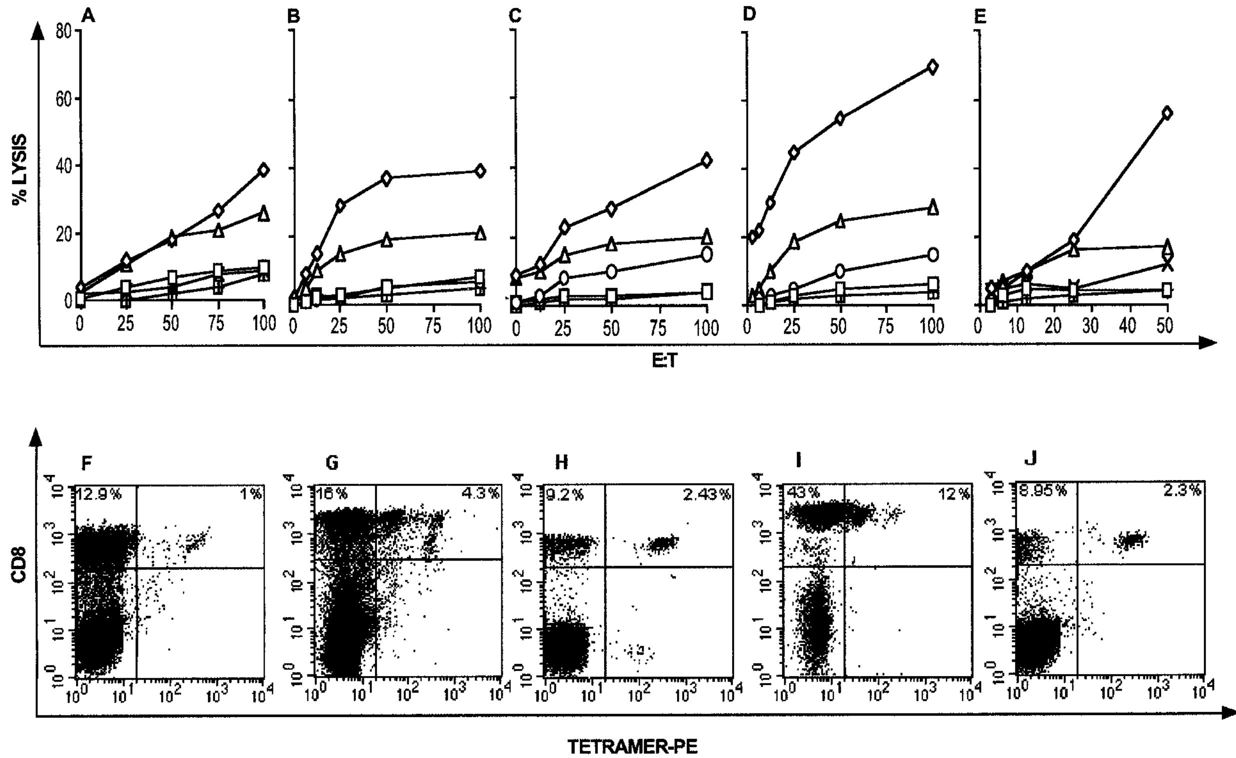

Fig. 1. Tetramer staining of short-term HPV 16 $E7_{11-20}$ peptide-specific CTL lines. These were generated from four individuals as reported previously (18). *A* and *B*, CTL lines derived from normal individuals using peptide-pulsed dendritic cells. *C–E*, CTL lines derived from patients with stage IIB CaCx. The CTL lines in *C* and *E* are derived from the same patient, with the CTL line in *C* derived from PBMCs, and the CTL line in *E* derived from lymph node-draining lymphocytes, respectively. Specificity was assayed on chromium-labeled C1R-A2 (□) cells sensitized with either $E7_{11-20}$ (◇) or as controls, TP from TAP_2 (○), or CP38 from *P. falciparum* (×). The recognition of endogenously processed antigens was measured after infection of C1R-A2 cells with the recombinant vaccinia virus, TA-HPV (△), or the wild-type vaccinia virus Wy-vac (⊠). These CTL lines were then double-stained with the HPV 16 $E7_{11-20}$ tetramer (*X axis*) and CD8-tricolour or CD8-FITC (*Y axis*). Each flow cytometric plot (*F–J*) corresponds to the CTL line above (*A–E*). The percentages of $CD8^+tetramer^+$ cells in the gated T-cell populations are shown in the *upper corners* of *panels F–J*.

and controls were stimulated with the HPV 16 $E7_{11-20}$ peptide for 1 week *in vitro* before staining with tetramer (40). This peptide stimulation increased the numbers of $CD8^+tetramer^+$ cells in six of nine of the patient PBMC samples tested, with approximately 4-fold increases seen for four of the patients. (Table 1 and Fig. 2). In contrast, no expansions of similar magnitude were seen for the eight control PBMC samples (Table 1). These results are consistent with previous reports that document a failure to generate HPV 16 $E7_{11-20}$-specific CTL in normal controls after short-term *in vitro* peptide restimulation (17, 18).

Table 1 *Frequencies of HLA-A*0201/HPV 16 $E7_{11-20}$ tetramer-positive cells in PBMCs and $E7_{11-20}$ peptide-restimulated PBMCs*

Thawed HLA-A*0201$^+$ PBMCs were cultured overnight before two-color flow cytometric analysis using anti-CD8-FITC and HLA-A*0201/HPV 16 $E7_{11-20}$-PE tetramer. Percentage of $CD8^+$ tetramer$^+$ cells in small lymphocyte gate determined as for Fig. 3. Typically, percentage of gated cells constitutes half of all cells analyzed. No statistically significant difference in numbers of $CD8^+$ tetramer$^+$ cells found between patient and control groups either before or after peptide stimulation (*t* test, $P > 0.5\%$).

				PBMCs		Postpeptide stimulation[a]		
Subject	Histological grade/stage	Sex	Age (years)	$CD8^+$ tetramer$^+$ (%)	Reciprocal frequency	$CD8^+$ tetramer$^+$ (%)	Reciprocal frequency	Increase[b]
P1	CIN2/3	F	42	0.079	1,260	0.308	324	**3.89**
P2	CIN3	F	31	0.041	2,418	0.018	5554	0.44
P3	CIN3	F	30	0.039	2,546	0.162	617	**4.13**
P4	CIN3	F	24	0.030	3,328	0.105	953	**3.49**
P5	CaCx (IIb)	F	29	0.026	3,825	ND[c]	ND	ND
P6	CIN3	F	35	0.011	8,895	0.007	15200	0.59
P7	CIN3	F	25	0.009	10,928	0.014	7311	**1.49**
P8	CIN2	F	24	0.007	15,267	0.024	4223	**3.62**
P9	CIN3	F	56	0.006	17,954	0.005	19674	0.91
P10	CIN3	F	26	0.005	19,073	0.014	7295	**2.61**
C1	Control	F	NK	0.054	1,855	ND	ND	ND
C2	Control	M	52	0.03	3,095	0.04	2285	**1.35**
C3	Control	F	53	0.032	3,156	0.006	15648	0.20
C4	Control	F	NK	0.031	3,213	ND	ND	ND
C5	Control	F	42	0.009	11,078	0.004	27338	0.41
C6	Control	M	39	0.007	13,582	0.008	12715	**1.07**
C7	Control	F	28	0.006	17,765	0.003	29394	0.60
C8	Control	M	39	0.005	21,037	0.006	16036	**1.31**
C9	Control	M	34	0.002	42,004	0.003	32784	**1.28**

[a] Half of the thawed PBMCs were stimulated with HPV 16 $E7_{11-20}$ peptide for 1 week before analysis.
[b] Change calculated from [tetramer$^+$ cells (%) after peptide stimulation/tetramer$^+$ cells (%) before peptide stimulation]. Increases >1, bold.
[c] ND, not determined due to insufficient sample; NK, age not known.

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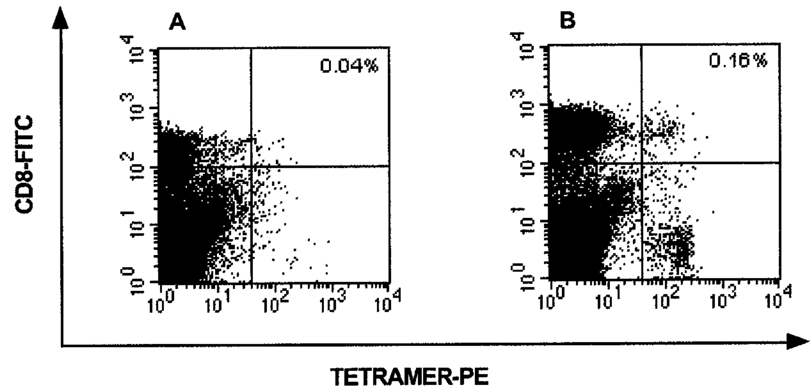

Fig. 2. Effect of peptide restimulation *in vitro* on tetramer staining of PBMCs. Tetramer staining of PBMCs from CIN3 patient 3. *A*, directly stained; *B*, after 7 days of *in vitro* restimulation with HPV 16 $E7_{11-20}$ peptide. The percentages of $CD8^+$ $tetramer^+$ cells using the small lymphocyte gate (24) are indicated in the *upper right corners*.

HPV 16 $E7_{11-20}$-specific CTL Isolated by Tetramer Lyse Targets Expressing Endogenous HPV Antigens. A potential pitfall for the use of synthetic peptides to restimulate CTLs *in vitro* is the selection of low-affinity CTLs that are peptide specific but unable to recognize endogenously processed antigens (42, 43). Previously, we demonstrated that HPV 16 $E7_{11-20}$-specific CTL lines were able to recognize targets expressing endogenous HPV antigens albeit at lower levels of killing than that seen for peptide-pulsed targets (Fig. 1, *A–E*; Ref. 18). To further investigate the fine specificity of HPV 16 $E7_{11-20}$ CTLs, we used the HPV 16 $E7_{11-20}$ tetramer to isolate purified populations of CD8+ CTLs (Fig. 3*A*) from the JJ CTL line (Fig. 1, *D* and *I*). The resulting polyclonal CTL line D4, and clone C6 stained with HPV 16 $E7_{11-20}$ tetramer at high levels (Fig. 3, *B* and *C*) and were phenotypically stable (92 and 99% TCR Vβ6, respectively) after prolonged tissue culture (data not shown).

Both D4 and C6 were tested for HLA-A**0201*-restricted cytotoxic recognition of peptide-pulsed targets and targets expressing endogenous HPV antigens; either after infection with recombinant vaccinia virus containing full-length HPV 16 E6 and E7 antigens (TA-HPV) or as a consequence of HPV 16 transformation (CaSki cells). Both D4 and C6 were able to recognize peptide-pulsed target cells more efficiently than the original JJ CTL line (Figs. 1*D*, 3*D*, and 3*E*). Furthermore, both D4 and C6 were able to recognize HLA-$A{*}0201^+$ target cells infected with TA-HPV vaccinia but not cells infected with control vaccinia (Fig. 3, *D* and *E*). However, lysis of TA-HPV-infected targets was lower than HPV 16 $E7_{11-20}$-pulsed target cells (Fig. 3, *D* and *E*). Both D4 and C6 populations were also able to lyse HLA-$A{*}0201^+$, HPV 16^+ CaSki cervical epithelial cell targets but not HLA-$A{*}0201^+$, HPV 16^- breast epithelial target cells (MDA-231). Pretreatment of CaSki and MDA-231 targets with IFN-γ prior to use in CTL assays (44) did not increase the level of CTL lysis despite increases in HLA class I expression (data not shown). Furthermore, there was no CTL recognition of HPV 16-negative target cells that were HLA matched with CaSki for one or more HLA class I alleles (data not shown). These results confirm that both clonal and polyclonal populations of HPV 16 $E7_{11-20}$-specific CTLs are able to recognize endogenously processed HPV 16 E7 antigens.

DISCUSSION

This study demonstrates for the first time the use of soluble MHC-peptide complexes or tetramers to study HPV-specific CTLs in patients with cervical neoplasia. A tetramer was constructed consisting of HLA-A**0201* and HPV 16 $E7_{11-20.}$ This tetramer combined the most frequently occurring HLA allele among Caucasians (33) together with the best-studied CTL peptide epitope (15, 17, 18, 45, 46) from the HPV type most frequently associated with CaCx (3). Furthermore, the HPV 16 $E7_{11-20}$ peptide epitope has been incorporated into peptide vaccines and has been the subject of several clinical trials (47). The HPV tetramer clearly identified 2–12% $CD8^+tetramer^+$ T cells in short-term CTL lines with known HPV 16 $E7_{11-20}$ specificity. These $CD8^+tetramer^+$ T cells could be purified and were shown to specifically recognize both HPV 16 $E7_{11-20}$ peptide-pulsed, HLA-A*0201^+ target cells and HLA-A*0201^+ target cells expressing endogenous HPV 16 E7 antigens. Previous studies using the HPV 16 $E7_{11-20}$ peptide have suggested that this epitope is processed endogenously, based on the recognition of either HPV-16-transformed CaSki target cells (17, 46) or vaccinia-HPV-infected target cells (18). However, recognition of CaSki cells may be due to alloreactive recognition of non-HLA-A**0201* molecules (15), and vaccinia-infected targets may express HPV antigens at higher levels than seen in cervical tumor cells. In this study we have used both types of target cell to show stringently that HPV 16 $E7_{11-20}$ peptide-specific CTLs (D4 and C6) can recognize endogenous HPV antigens in an HLA-A**0201*-restricted fashion. In peptide dose-response experiments, half-maximal lysis was achieved at ~10 pM for both CTL.[7] This is similar to the values obtained with melanoma-specific CTLs capable of recognizing endogenously processed tumor antigens (48).

Tetramer-directed cell sorting offers a precise method to generate both monospecific polyclonal lines (D4) and more importantly, clones such as C6. Both have been propagated for up to 10 months,[8] maintaining phenotype (CD8, TCR Vβ6) and specificity, thus suggesting that these CTLs could easily be propagated for adoptive immunotherapy. By contrast, the use of conventional limiting dilution methods to isolate CTLs from peptide-specific CTL lines is highly inefficient, with only 1% of CTL clones being able to recognize endogenous HPV antigens (49). Another drawback to the limiting-dilution cloning approach is the possibility that so-called "CTL clones" may actually be derived from mixed populations of $CD8^+$ and $CD4^+$ T cells, which may not be stable over time. Thus, the use of the HPV 16 $E7_{11-20}$ tetramer has efficiently isolated purified populations of stable, high-affinity HPV-specific CTLs not obtainable by conventional methods.

Both D4 and C6 CTLs were obtained from $CD8^{hi}tetramer^{hi}$ populations from the JJ CTL line (Fig. 3*A*). This CTL line had a heterogeneous pattern of tetramer staining (Fig. 1*I*) compared with other HPV 16 $E7_{11-20}$-specific CTL lines (Fig. 1, *F*, *H*, and *J*). This might reflect the presence of both $tetramer^{lo}$ or $tetramer^{hi}$ CTL subpopulations within the polyclonal CTL line as has been described recently for melanoma-specific CTLs (50). However, further *in vitro* culturing of the parental JJ CTL line in the absence of antigen resulted in a more homogeneous population of predominantly $CD8^{lo}tetramer^{hi}$ T cells.[9] This might reflect the presence of nonsynchronously activated CTL

[7] S. Youde, unpublished observations.
[8] S. Youde and P. R. Dunbar, unpublished observations.
[9] P. R. Dunbar, unpublished observations.

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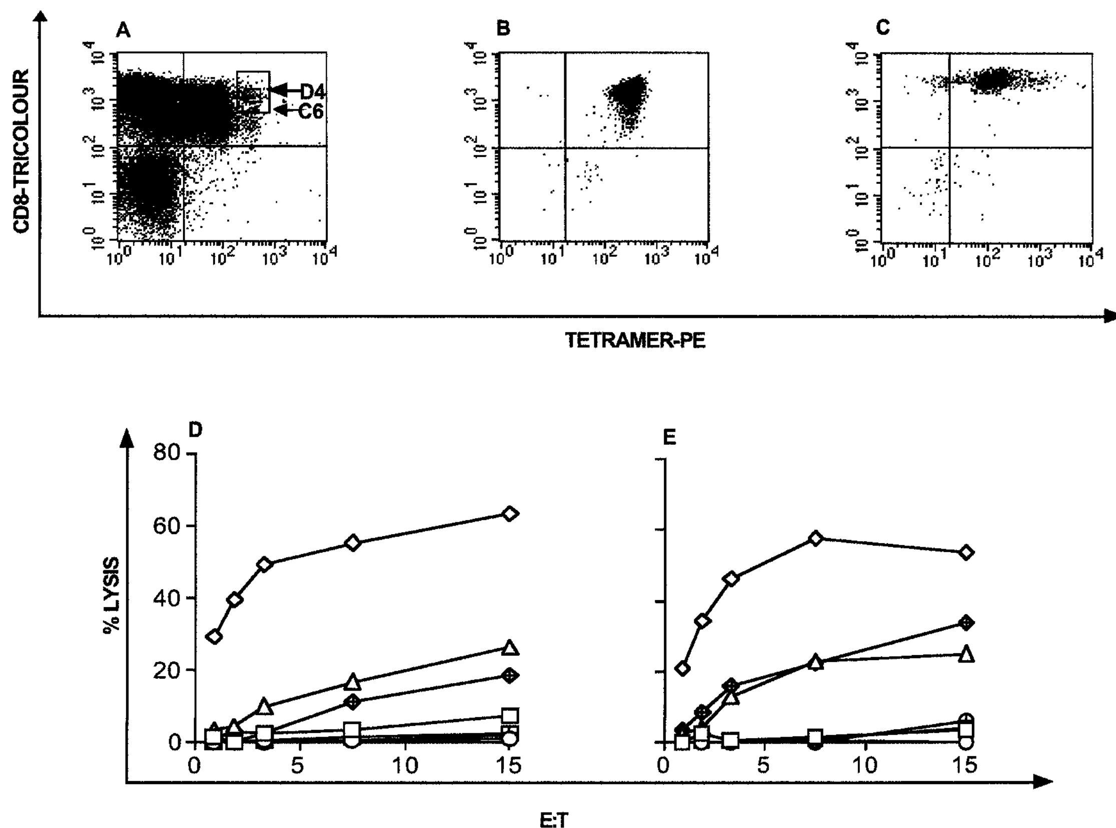

Fig. 3. Isolation of polyclonal and clonal populations of HPV 16 $E7_{11–20}$-specific CTLs using tetramer. The HPV 16 $E7_{11–20}$ peptide-specific line generated from patient JJ, with stage IIB CaCx, was double-stained with the HPV tetramer and CD8-tricolour; the FACS profile is shown in *panel A*. Using the gates shown in *A*, this population was FACS sorted to create the clone C6 and the polyclonal, monospecific line D4. The tetramer staining profiles for these populations are shown in *panels B* (C6) and *C* (D4). The *dot plots* show lymphocytes gated by forward and side scatter, with tetramer-PE staining on the *X axis* and CD8-tricolour on the *Y axis*. The lytic activity of C6 (*D*) and D4 (*E*) was assessed in a standard 4-h chromium-release assay at various E:T ratios. Target cells were C1R-A2 transfectants (□) pulsed with the HPV 16 $E7_{11–20}$ peptide (◇) and as the negative control, the HLA-A2-binding peptide TP from TAP_2 (○). Recognition of endogenously processed viral antigens was assayed on C1R-A2 cells infected with recombinant vaccinia virus expressing HPV 16 and 18 E6 and E7 (TA-HPV; △) or the HPV-expressing CaCx cell line CaSki (⊕). Controls were C1R-A2 cells infected with wild-type vaccinia virus (⊠) or the breast carcinoma cell line MDA-231 (⊗). Percentage of lysis was calculated as follows: 100 × [(specific release − spontaneous release)/(maximal release − spontaneous release)].

subpopulations (with differing levels of TCR expression) rather than distinct $tetramer^{lo}$ or $tetramer^{hi}$ populations. Alternatively, $tetramer^{hi}$ T cells may have a longer life span *in vitro* than $tetramer^{lo}$ T cells.

The results discussed above established the specificity of the tetramer for detecting effector CTLs from *in vitro* restimulated PBMCs. A goal of this study was to develop tetramer reagents that allow direct quantitation of HPV 16-specific CTLs from *ex vivo* blood or tissue biopsy samples, as has been demonstrated for melanoma (28, 29, 51) and viral antigens (29, 38, 52). This would be particularly beneficial for rapid monitoring of HPV-specific $CD8^+$ T-cell frequencies either in epidemiological studies in developing countries (53) or for vaccine trials (21). Analysis of *ex vivo* PBMC samples revealed that staining of $CD8^+$ T cells with the HPV 16 $E7_{11–20}$ tetramer was rare (1 of 1,260 to 1 of 42,000) in both CIN3 patients and controls. On the basis of epidemiological data, HPV would be detected in 65% (54) and 5% (55) of CIN3 patients and controls respectively. Although only small numbers of patients and controls were studied, there was no correlation between the numbers of $tetramer^+$ cells detected and HPV-associated disease. It was not possible to examine the HPV 16 $E7_{11–20}$ cells for phenotypic markers associated with T-cell memory because of the low frequency of cells detected and the limited PBMC samples available. However, the enhanced detection of $tetramer^+$ cells after peptide stimulation of patient samples suggests that these were more readily able to proliferate *in vitro*. This observation is consistent with previous findings that HPV 16 $E7_{11–20}$-specific CTLs can be generated in patients, but not controls, by standard restimulation protocols (17, 18).

The staining patterns of *ex vivo* PBMCs were not as clear as for established HPV 16 $E7_{11–20}$-specific CTL lines (Fig. 1). This is particularly problematic where the numbers of $CD8^+tetramer^+$ cells are close to the limit of detection by FACS (Table 1). However, it is not surprising that such low frequencies were seen in PBMCs in the absence of *in vitro* restimulation (Table 1) because similar results have been observed in melanoma (28). It may be that for HPV-specific CTLs, as for melanoma, PBMCs will not be the best compartment for study. Higher frequencies of tumor-specific CTLs may be detected among tumor-infiltrating lymphocytes (18) or in lymph nodes that have been infiltrated with tumor (28).

The frequencies detected for HPV 16 $E7_{11–20}$ $CD8^+$ cells (1 of 1,260 to 1 of 42,000) are an order of magnitude lower than found for other viral antigens and tumor antigens. For example, memory CTLs recognizing influenza A $M1_{58-66}$ can be found in 1 of 500 small lymphocytes (29), whereas for EBV $EBNA3C_{325–333}$, up to 1 of 25 T cells can be detected using tetramer (41). However, it should be noted that both of these viruses generate strong systemic CTL responses, whereas HPV may have evolved mechanisms to avoid immune recognition (56). Furthermore, the immunodominant peptide epitopes of influenza A and EBV were used to construct tetramers. It is not clear whether HPV 16 $E7_{11–20}$, which was defined using the reverse immunogenetic approach (33), is immunodominant. Even for immunodominant viral epitopes, at least 10-fold variation has been observed in the frequency of CD8 T cells detected by tetramer (29, 41). The variation in the frequency of $CD8^+$ HPV 16 $E7_{11–20}$ $tetramer^+$ T cells among patient PBMCs in this study might explain the contrasting

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results obtained for HPV 16 $E7_{11-20}$-specific CTL detection (15, 17, 18). However, the current study is limited by the use of tetramers incorporating a single peptide epitope from HPV, and additional HPV CTL epitopes need to be defined. In the future, multiple tetramers comprising different CTL epitopes may allow more precise assessment of the role of CTL in cervical disease, including the possibility that HPVs can induce immunological tolerance (56). Nevertheless, the HPV tetramer we have characterized may be beneficial for monitoring patients who have been immunized with the HPV 16 $E7_{11-20}$ peptide as part of ongoing clinical trials (47).

Recently, tetramer-driven sorting has been used to derive melanoma-specific CTLs suitable for use in adoptive immunotherapy (36, 40, 50). Regardless of the natural role of HPV-specific CTLs, our current studies demonstrate that it is possible to use a HPV tetramer to select high-affinity HPV-specific CTLs, which have potential application in the immunotherapy of CaCx. However, the frequent down-regulation of HLA class I molecules in cervical tumors (57) and the low levels of HPV E6 and E7 expression (58) may confound attempts at CTL-based immunotherapy. The use of the HPV 16 $E7_{11-20}$ tetramer to derive large numbers of stable HPV-specific CTL clones will now allow detailed investigation of the factors influencing CTL recognition of HPV-transformed tumor cells.

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