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Specificity of human cytotoxic T lymphocytes induced by a human papillomavirus type 16 E7-derived peptide

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In order to establish tumour-specific cytotoxic T lymphocyte (CTL) cell lines, T cells from a human papillomavirus (HPV) type 16-positive patient with a cervical carcinoma *in situ* and from a healthy volunteer were stimulated *in vitro* with autologous dendritic cells loaded with peptides derived from the viral transforming proteins E6 and E7 and corresponding to potential HLA-A*0201-restricted T cell epitopes. From each donor a small number of low-affinity CTL lines against the peptide E7/86–93 was obtained, which specifically lysed HLA-A*0201-expressing B-lymphocytes (cell line 721) loaded with this peptide. Cytotoxicity was also observed against two HLA-A*0201-E7-positive epithelial cell lines, the cervical carcinoma cell line CaSki and the HPV-16-immortalized foreskin-keratinocyte cell line HPK IA. However, since none of the CTL recognized both cell lines, and E7-expressing 721 transfectants were never lysed, it was concluded that the reactivity against CaSki and HPK IA cells was due to cross-reactivity on allogeneic HLA molecules rather than to E7 recognition, which emphasizes that the specificity of tumour cell lysis by peptide-induced CTL has to be interpreted with caution.

Introduction

The role of the 'high risk' human papillomavirus (HPV) type 16 in the development of cervical cancer and its precursor lesions (high-grade cervical intraepithelial neoplasia, CIN) became evident from molecular and clinical investigations and was recently confirmed by epidemiological studies (for review see zur Hausen, 1996).

The transforming potential of this virus depends essentially on the expression of the two early viral open reading frames (ORFs) E6 and E7 (Mansur & Androphy, 1993). In pre-malignant and malignant cervical lesions, both proteins are constitutively expressed in the infected cells and therefore are considered to be the major tumour antigens. HPV-16 E6 and E7 proteins have already been shown to stimulate specific cytotoxic T lymphocytes (CTL) in mice which mediate rejection of tumours expressing either antigen (Chen *et al.*, 1991, 1992). In CIN and cervical cancer patients, however, the cellular immune response against HPV is obviously not strong enough to inhibit tumour growth, although E6 and E7 induce antibody reactivity and T cell proliferation, and hence are also immunogenic in humans (Jochmus-Kudielka *et al.*, 1989; Müller *et al.*, 1992; Kadish *et al.*, 1994; de Gruijl *et al.*, 1996).

A synthetic peptide from the HPV-11 E7 protein induced specific human CTL *in vitro* which recognized target cells infected with HPV-11 E7 recombinant vaccinia viruses (Tarpey *et al.*, 1994). In mice, protection against HPV-16-induced tumours could be achieved by immunization with a synthetic peptide that represents a naturally processed CTL epitope of the HPV-16 E7 protein (Sadovnikova *et al.*, 1994; Feltkamp *et al.*, 1995). To address the question of whether HPV-16 E6- and E7-derived peptides can activate protein-specific human CTL, we stimulated *in vitro* T cells from two HLA-A*0201-positive blood donors: a patient with an HPV-16-positive high-grade CIN (carcinoma *in situ*) and a volunteer without HPV-

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associated lesions. Five peptides were chosen for stimulation. These peptides contain characteristic amino acids in anchor positions predicted for HLA-A*0201-restricted epitopes according to Rammensee *et al.* (1995) (E7/7–15 and E7/82–90), and/or strongly bind to HLA-A*0201 (Kast *et al.*, 1994) (E7/11–20, E7/82–90 and E7/86–93). The peptide E6/18–26 has been identified by elution from MHC class I molecules of JY cells after infection with HPV-16 E6 recombinant vaccinia viruses (Bartholomew *et al.*, 1994). An HLA-A*0201-restricted CTL epitope of the influenza virus matrix protein (MP/58–66) served as a positive control (Bednarek *et al.*, 1991). The CTL obtained were tested against different HLA-A*0201-positive and HPV-16 protein-expressing target cells.

Methods

■ **Donors.** Informed consent for blood donation was obtained. A patient diagnosed with cervical carcinoma *in situ* donated blood before, and twice after, she underwent surgical treatment (large loop excision of the transformation zone). The HPV typing was done by PCR with DNA obtained from a cervical scrape and HPV-16 type-specific primers (van den Brule *et al.*, 1989). The healthy volunteer was a female co-worker in the laboratory without HPV-associated lesions, as determined by colposcopy and seronegativity against HPV-16 E7 protein in Western blot analysis (Jochmus-Kudielka *et al.*, 1989). Peripheral blood lymphocytes (PBL) prepared by Ficoll–Hypaque (Pharmacia) density gradient centrifugation were stored in 10% DMSO in liquid nitrogen. HLA typing was kindly done by Rudolf Wank, Institute of Immunology, University of Munich, Germany.

■ **Synthetic peptides.** The peptides were (i) obtained from the Deutsches Krebsforschungszentrum, Division of Peptide Synthesis, or (ii) produced by Stefan Stevanovic, Tübingen, Germany and were as follows: HPV-16 E6, aa 18–26 (KLPQLCTEL); HPV-16 E7, aa 7–15 (TLHEYMLDL), aa 11–20 (YMLDLQPETT), aa 82–90 (LLMGTLGIV) and aa 86–93 (TLGIVCPI); and influenza virus matrix protein (MP) aa 58–66 (GILGFVFTL). All peptides were synthesized by Fmoc Technology and were routinely analysed for purity by high performance liquid chromatography. Peptide solutions (1 mM) containing up to 50% DMSO were stored at −20 °C.

■ **Cell lines.** The cervical carcinoma cell lines CaSki (Patillo *et al.*, 1977), SiHa (Friedl *et al.*, 1970) and C33A (Auersperg, 1964), and the HPV-16-immortalized foreskin-keratinocyte cell lines HPK IA and HPK II (Dürst *et al.*, 1987), were cultivated as monolayers in DMEM containing 10% foetal calf serum (FCS). The lymphoblastoid B cell line 721 (Kavathas *et al.*, 1980) and the peptide-transporter deficient cells 174 × CEM.T2 (T2 cells) (Salter & Cresswell, 1986) were grown in RPMI 1640 containing 10% FCS. For the cultivation of 721 cells transfected with the HPV-16 E7 protein gene, G418 (0·8 mg/ml) was added to the medium.

■ **Establishment of cell line 721 clones transfected with HPV-16 E7.** An HPV-16 *Taq*I (#505)–*Pst*I (#875) fragment containing the E7 ORF was cloned into the eukaryotic expression vector pHβAPr1-neo (Gunning *et al.*, 1987), in which the polylinker had been replaced by the multiple cloning site of pSP72 (Promega). The resulting plasmid was kindly provided by Frank Momburg, Heidelberg, Germany.

721 cells transfected with pFM115.2 by electroporation (220 V) were selected in RPMI 1640 containing 10% FCS with 1 mg/ml G418, and subsequently cloned by seeding one cell per well into 96-well plates. Clones were cultured and tested for E7 protein expression.

■ **Western blot analysis.** Cell lysates derived from 1×10^5 cells were separated on 15% SDS–polyacrylamide gels and transferred onto a Nylon membrane (Millipore) by semi-dry blotting (45 min, 200 mA, 4 °C). The membrane was incubated for 30 min in 10% non-fat dry milk in PBS at room temperature, followed by overnight incubation at 4 °C with a monoclonal antibody (MAb) raised against HPV-16 E7 (50 µg/ml; Corning/Zymed) diluted 1:1000 in PBS, 5% BSA. After washing, a second peroxidase-labelled goat anti-mouse IgG antibody (0·8 mg/ml; Dianova), diluted 1:10000 in 10% non-fat dry milk in PBS, was added and incubated for 3 h at room temperature. Antibody reactivity was detected by enhanced chemiluminescence.

■ **Cultivation of dendritic cells.** Dendritic cells (DC) were obtained according to the protocol of Romani *et al.* (1994). Briefly, either 1×10^7 'low density cells' enriched from PBL by Metrizamide density gradient centrifugation (Tarpey *et al.*, 1994), or 1×10^7 monocyte-depleted PBL were cultivated per well of a 6-well plate in 3 ml of RPMI 1640 containing 10% FCS with 800 U/ml GM-CSF and 1000 U/ml IL-4 (both Promocell). Half of the medium was exchanged for fresh medium containing 1600 U/ml GM-CSF and 1000 U/ml IL-4 every other day. At day 7 the DC were harvested and DC derived from monocyte-depleted PBL were purified by Metrizamide density gradient centrifugation (Tarpey *et al.*, 1994).

■ **Cultivation of T cells.** T lymphocytes were separated from PBL by either Metrizamide density gradient centrifugation (Tarpey *et al.*, 1994) or by rosetting with sheep red blood cells. DC were incubated with peptides at a final concentration of 50 µM in RPMI 1640, 2% FCS for 2 h at 37 °C and washed once with RPMI 1640, 2% FCS. Peptide-pulsed DC ($3–10 \times 10^3$) were cultured together with 1×10^5 purified T cells per well in a 96-well plate in Iscove's modified Dulbecco's medium (IMDM) containing 10% human serum (HS) and incubated for 1 week with a medium change at day 4. Afterwards, the T cells were restimulated weekly in a 96-well plate with 7×10^4 autologous PBL per microwell as stimulator cells for the first and second restimulation, and a mixture of 4×10^4 T2 cells and 2×10^4 autologous or HLA-A*0201-matched PBL per microwell for all further restimulations. The stimulator cells were irradiated with 60 Gy, loaded with peptides at a concentration of 50 µM for 3 h at 37 °C and then washed three times with RPMI 1640 containing 2% FCS prior to adding them to the T cells. The restimulation was done in IMDM containing 10% HS. At days 1 and 4, half of the medium was exchanged for medium containing IL-2 (final concentration 4 U per well). By the second or third restimulation the T cells were split into sublines seeding $5–10 \times 10^3$ T cells per microwell. The sublines were then separately tested for cytotoxicity. The CTL were $CD3^+$ and $CD8^+$ as determined by FACScan analysis using undiluted hybridoma supernatants containing the respective antibodies (kindly provided by Stefan Meuer, Heidelberg, Germany) and an FITC-labelled goat anti-mouse IgG antibody (1·4 mg/ml; Diavova) diluted 1:1000 for staining.

■ **^{51}Cr-release assay.** The cytotoxicity assays were done from the third restimulation in 96-well plates. Peptides were added during the ^{51}Cr-labelling procedure at a concentration of 50 µM. For peptide titration, the ^{51}Cr-labelled cells were incubated with decreasing amounts of peptide for 45 min at 37 °C. Influenza virus infection of ^{51}Cr-labelled cells was done with 1 ml influenza virus strain PR8 supernatant containing 500 haemagglutinin (HA) U of the virus (kindly provided by Hansjörg Schild, Tübingen, Germany) for 90 min at 37 °C. Keratinocytes were treated with 50 U/ml γ-interferon for 24 h before labelling. T cells were added to $2·5–5 \times 10^3$ target cells in various effector:target (E:T) ratios (at least 4:1). The final volume per microwell was 200 µl. After 4 h, radioactivity was counted in a Microbeta (Wallac). Specific lysis (SL) was calculated from the experimental counts obtained, the spontaneous ^{51}Cr-release (target cells with medium) and total lysis (target cells plus 5%

Triton X-100), according to the formula: % SL = (experimental counts − spontaneous release) × 100/(total lysis − spontaneous release).

The spontaneous ^{51}Cr-release was always $\leqslant 20\%$. Only SL of $\geqslant 15\%$ was scored as positive. In the case of duplicates and triplicates the standard errors of the means were $< 15\%$ of the value of the mean. One lytic unit is defined as the number of T cells that lyse 30% of 10^3 target cells in a 4 h assay. For the calculation of mean values the lytic units were extrapolated to 10^6 effector cells.

Results

Establishment of peptide-specific CTL lines

Five different peptides, corresponding to putative HLA-A*0201-restricted CTL epitopes of the HPV-16 E6 and E7 proteins, were used to stimulate *in vitro* HPV-16-specific CTL lines from PBL of two HLA-A*0201-positive donors, a patient with a HPV-16-positive high-grade CIN lesion, and a healthy volunteer. The CTL epitope MP/58–66 derived from the influenza virus MP was used as a positive control. We failed to establish HPV-specific CTL lines by incubating PBL with peptides according to the method described by Plebanski *et al.* (1994), although this protocol was very successful (100%) when MP/58–66 was used as antigen (data not shown). Therefore, autologous dendritic cells were employed as potent antigen-presenting cells. By this method we obtained CTL lines against the peptides E7/86–93 and MP/58–66. None of the other peptides used as antigens (E6/18–26, E7/7–15, E7/11–20 and E7/82–90) were able to specifically induce CTL.

In each experiment more than 70% of the T cell sublines stimulated with MP/58–66 lysed target cells loaded with this peptide, whereas only 195 out of 1251 patient-derived sublines (15·6%, designated PA1–PA195) and 26 out of 196 sublines established from the healthy volunteer (13·3%, designated VO1–VO26) exhibited peptide-specific lytic activity after stimulation with E7/86–93. VO1–VO17 and PA1–PA158 were analysed further. VO1–VO17 could be cultured and continued to lyse E7/86–93-loaded target cells for 11–15 weeks. In contrast, CTL sublines established from the patient showed decreased lytic activity and stopped growing after 5–7 weeks of cultivation. Only six of them (PA1–PA6) could be tested separately. The remaining patient-derived sublines had to be pooled after they gave equal SL values in the first cytotoxicity assay in order to have enough effector cells in the subsequent assays.

Independent of the donor, SL of MP/58–66-pulsed target cells was always above 40%, even after short-term cultivation. In contrast, the cytotoxicity of E7/86–93-specific CTL lines was sometimes very low depending on the subline tested. Some of the sublines are shown in Fig. 1. The patient-derived sublines PA1–PA6 lysed E7/86–93-loaded target cells less efficiently than sublines from the healthy volunteer (Fig. 1, see also Fig. 4*a*) however, the average lytic units calculated from all SL values of 15% or more, after the third and all subsequent restimulations at the highest respective E:T ratio, did not differ

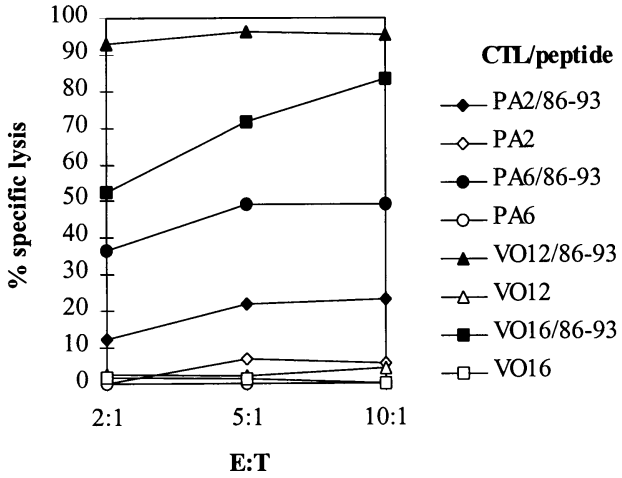

Fig. 1. Peptide-specific cytotoxicity of CTL lines obtained after *in vitro* stimulation with the HPV-16 E7 peptide E7/86–93: PA2 (◆, ◇), PA6 (●, ○), VO12 (▲, △), VO16 (■, □). 721 cells with (filled symbols) or without (open symbols) peptide were used as target cells.

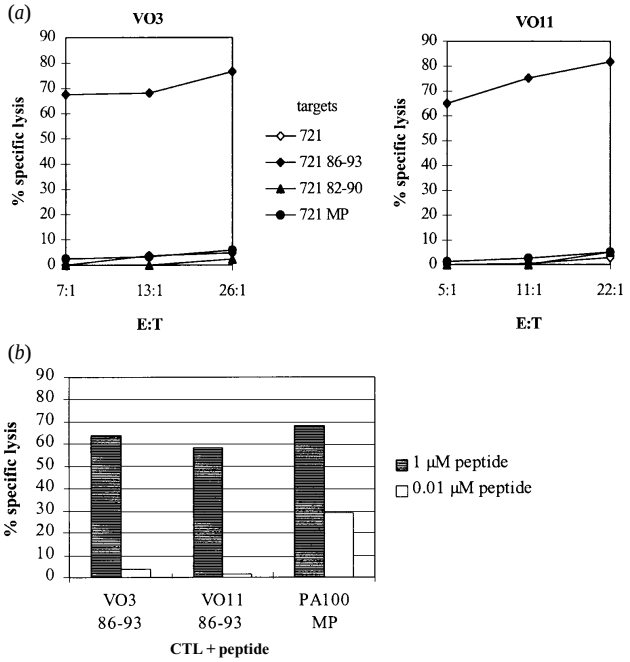

Fig. 2. (*a*) Cytotoxicity of the E7/86–93-specific CTL lines VO3 and VO11 against 721 cells (i) without peptide (◇), (ii) loaded with E7/83–93 (◆) or (iii) loaded with the peptides E7/82–90 (▲) and MP/58–66 (●), respectively. (*b*) Lysis of 721 cells pulsed with E7/86–93 or MP/58–66 at concentrations of 1 and 0·01 μM by two E7/86–93-specific CTL lines, VO3 and VO11 (E:T = 16:1 and 21:1), or the MP/58–66-specific patient-derived CTL line PA100 (E:T = 15:1), respectively.

significantly between the patient (141.9) and the volunteer (135.3). E7/86–93-specific CTL lines did not recognize 721 cells pulsed with other HLA-A*0201-restricted peptides (E7/82–90, MP/58–66; Fig. 2*a*). In peptide titration assays the E7/86–93-specific cytotoxicity had already disappeared at a peptide concentration of 10 nM (Fig. 2*b*), demonstrating a

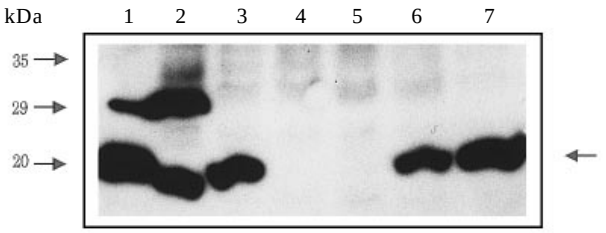

Fig. 3. Western blot analysis of HPV-16 E7 protein expression in the cervical carcinoma cell lines CaSki (lane 1) and SiHa (lane 7), the HPV-16-immortalized foreskin-keratinocyte cell lines HPK IA (lane 2) and HPK II (lane 3), and 721E7 transfectants (lane 6). Cell lysates of the HPV-16-negative cervical carcinoma cell line C33A (lane 4) and untransfected 721 cells (lane 5) were used as negative controls. The E7 protein is marked with an arrow on the right-hand side. Molecular masses (kDa) are given on the left-hand side.

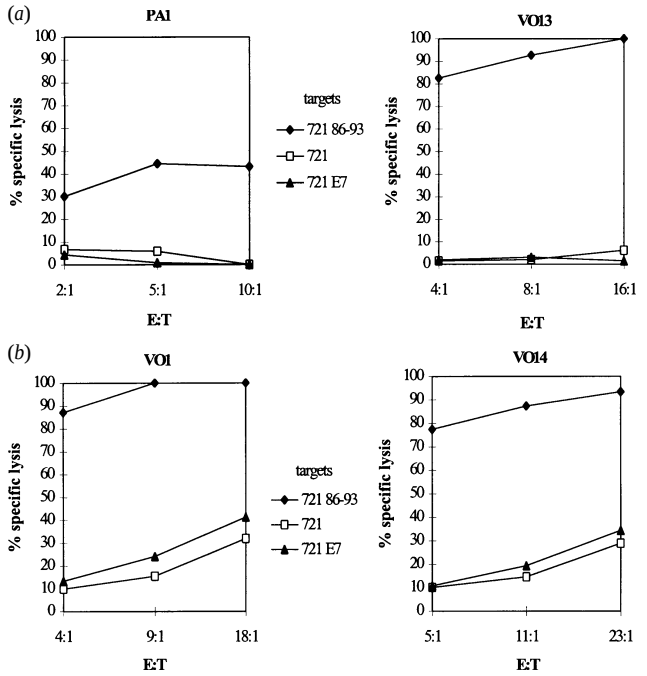

Fig. 4. (*a*) Lysis of 721E7 transfectants (▲) by the E7/86–93-specific CTL lines PA1 and VO13. 721 target cells loaded with E7/86–93 (◆) or without peptide (□) were included as positive and negative controls. (*b*) Nonspecific lysis of 721E7 transfectants (▲) and 721 cells without peptide (□) by the E7/86–93-specific CTL lines VO1 and VO14. The nonspecific reactivity was compared with the specific cytotoxicity against 721 cells loaded with E7/86–93 (◆).

low-affinity for the CTL towards their target cells. In contrast, MP/58–66 was still recognized by CTL at this concentration (Fig. 2*b*).

Cytotoxicity against antigen-expressing target cells

MP/58–66-specific T cell sublines lysed influenza virus (PR8)-infected 721 target cells, although less efficiently than peptide-loaded cells (for example, 45·8% SL of infected cells vs 75·0% SL of cells pulsed at a peptide concentration of 1 μM,

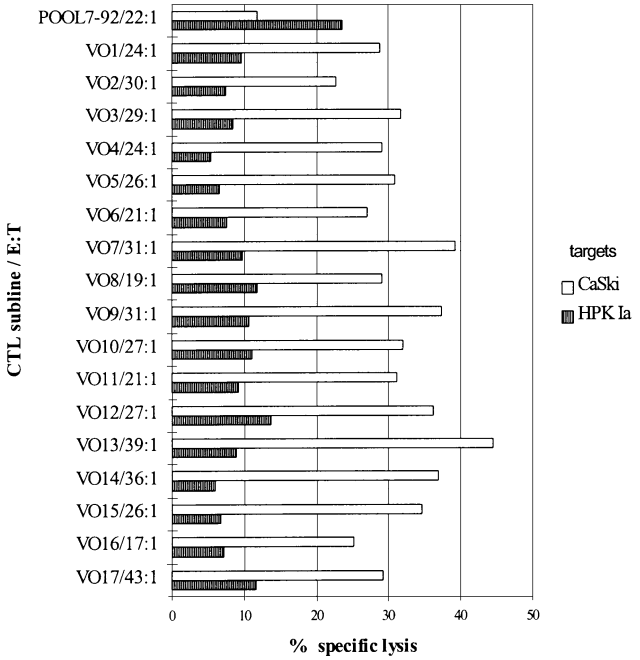

Fig. 5. Cytotoxicity of the volunteer-derived E7/86–93-specific CTL lines VO1–VO17 and POOL7-92 (PA7–PA92) against the HPV-16- HLA-A*0201-positive epithelial cell lines CaSki and HPK IA.

E:T ratio 5:1; data not shown). In contrast, CTL stimulated with E7/86–93 never specifically recognized 721 transfectants expressing the E7 protein (721E7) (Figs 3 and 4*a*). In two sublines (VO1 and VO14) with increased lysis of 721E7 transfectants, recognition of 721 cells without E7 protein or peptide was also elevated (Fig. 4*b*).

When E7-expressing keratinocytes (Fig. 3) were tested as target cells, all of the volunteer-derived sublines lysed the HPV-16- HLA-A*0201-positive cervical carcinoma cell line CaSki (20–45% lysis), but none of them recognized the HPV-16-immortalized HLA-A*0201-positive foreskin-keratinocyte cell line HPK IA (Fig. 5). One CTL subline pool established from the patient (POOL7-92 = PA7–PA92), which showed very weak cytotoxicity against E7/86–93-loaded 721 cells (19·3% lysis, E:T ratio 22:1), lysed HPK IA cells with a similar low efficiency (23·6% lysis; Fig. 5). These T cells did not recognize CaSki cells.

Discussion

CTL lines against the HPV-16 E7-derived peptide aa 86–93 and the peptide aa 58–66 of the influenza virus MP have been established from an HPV-16-positive high-grade CIN patient and a healthy donor by *in vitro* stimulation of PBL.

In comparison to the high number of T cell sublines that were cytotoxic after stimulation with MP/58–66, the percentage of E7/86–93-specific CTL sublines obtained from both donors was very small, which indicates the absence or low frequency of memory T cells recognizing E7/86–93, even in the HPV-16-positive patient. We hypothesize that in our

experiments the CTL against E7/86–93 were primarily stimulated *in vitro*, whereas MP/58–66-specific CTL probably represent restimulated memory T cells that were induced by previous influenza virus infections. This suggestion is further supported by the fact that CTL lines showing E7/86–93 SL of more than 20% were usually only obtained after at least four restimulations, and when dendritic cells were used initially for peptide presentation.

A CTL response was never observed against the other peptides used as antigens (see Methods). Even the peptides E7/82–90 and E7/11–20, which had previously been shown to activate CTL in HLA-A*0201 transgenic mice or human CTL *in vitro* (Ressing *et al*., 1995, 1996), or the peptide E6/18–26, which represents an endogenous protein-processing product (Bartholomew *et al*., 1994), were non-immunogenic in our experiments. This suggests that these peptides failed to induce a CTL memory and that the number of CTL precursors against the peptides was very small in the individuals tested.

The presence of memory CTL against the influenza virus peptide demonstrates that T cells of both donors are, in principle, responsive to antigens *in vivo*. One possible explanation for the minimal CTL response against HPV-16 E6 and E7 proteins in the patient could be T cell anergy induced by inadequate presentation of both antigens through epithelial cells, which are the exclusive targets for HPV infection. These cells do not express on their surface molecules like CD54 or CD80, which interact with receptor molecules on T cells, thereby inducing costimulatory signals that are critical for the outcome of T cell antigen recognition (Jenkins *et al*., 1991). Furthermore, MHC class I molecules are often downregulated on cells of CIN lesions and cervical cancer (Cromme *et al*., 1993). A larger number of patients with HPV-16-associated cervical lesions has to be investigated in order to finally clarify the question of whether T cell anergy is actually induced; our results, however, are in accordance with the recently published data by Ressing *et al*. (1996), who described a limited memory-CTL response against the peptide E7/11–20 in CIN and cervical cancer patients (four out of 22) after short-term cultivation.

In mouse experiments, Sadovnikova *et al*. (1994) identified a peptide within the HPV-16 E7 ORF that contains an amino acid motif characteristic of H2-K^b-restricted CTL epitopes (Rammensee *et al*., 1995), binds to the H2-K^b molecule and specifically stimulates CTL, but is obviously not produced in detectable amounts by natural processing of the E7 protein. Therefore, another possible explanation for CTL deficiency in the patient analysed in this study is that the peptides used for T cell stimulation in our experiments are not, or are only inefficiently, produced by endogenous protein processing. This could be true for all E7-derived peptides except E7/11–20, which had been demonstrated to induce a memory CTL response in CIN and cervical cancer patients, whereas E7/86–93 was negative in the same study (Ressing *et al*., 1996). In the case of E6/18–26, there may be no precursor T cells present with a T cell receptor able to bind corresponding HLA-A*0201 peptide complexes.

In a recent publication, CTL raised against E7/86–93 were demonstrated to lyse CaSki cells (Ressing *et al*., 1995). When we tested HLA-A*0201-positive, E7-expressing cell lines (CaSki, HPK IA and 721E7) as targets for E7/86–93-specific CTL, in order to determine whether this peptide is a relevant CTL epitope *in vivo*, the CTL lines were cytotoxic against either CaSki or HPK IA cells (Fig. 5). However, none of them recognized both epithelial cell lines, although CaSki, as well as HPK IA cells, were able to present the peptide E7/86–93 after external loading (data not shown). Furthermore, SL of 721E7 transfectants was never observed. E7 protein expression was slightly lower in 721E7 and HPK IA cells when compared to CaSki cells (Fig. 3), but this difference is unlikely to account for the total failure of 721E7 transfectants to serve as targets for E7-specific cytotoxicity, since HPK IA cells were lysed by CTL that did not recognize CaSki cells. It is impossible to decide whether lysis of CaSki and HPK IA cells is based on specific recognition of the E7 antigen, since E7-negative CaSki and HPK IA cells are not available as a control. However, the lack of detectable recognition of 721E7 transfectants and the low frequency of cytolysis against HPK IA cells by E7/86–93-specific CTL, and the fact that memory CTL against E7/86–93 have not been found in patients with HPV-16-associated diseases (Ressing *et al*., 1996), strongly suggest that this epitope is either absent or under-represented on E7-expressing cells and is very likely not a relevant CTL epitope *in vivo*. We therefore tend to interpret the cytotoxicity against CaSki cells observed by us and others (Ressing *et al*., 1995, 1996), as well as lysis of HPK IA cells, as cross- or alloreactivity. In order to investigate further the lytic activity of the E7/86–93-specific CTL, the two HPV-16-positive, but HLA-A*0201-negative, epithelial cell lines SiHa and HPK II were tested as target cells for all the CTL lines that lysed CaSki cells (data not shown). Seven of the CTL lines were cytotoxic against either HPK II or SiHa cells, which underlines their capacity to cross-react on allogeneic HLA molecules. It is very unlikely that the cross-reactivity observed is due to our restimulation protocol, since heterologous HLA-A*0201-matched stimulator cells were not employed before the third restimulation, and only for the cultivation of the patient-derived CTL. Peptide-induced low-affinity CTL have previously been demonstrated to cross-react with allogeneic target cells (Staerz *et al*., 1989). This kind of cross-reactivity therefore seems to represent a generic problem when peptides are used as antigens for T cell stimulation.

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