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Definition of Immunogenic Determinants of the Human Papillomavirus Type 16 Nucleoprotein E7

Annette Altmann, Ingrid Jochmus-Kudielka, Rainer Frank, Heinrich Gausepohl, Ulrich Moebius, Lutz Gissmann and Stefan C. Meuer

Specific T lymphocyte lines and T cell clones were established from peripheral blood mononuclear cells of asymptomatic seropositive individuals employing synthetic peptides which correspond to the sequence of the human papillomavirus (HPV) type 16 transforming protein E7. Specificity analysis of T cells as determined by means of [^{3}H] thymidine incorporation after stimulation with individual peptides revealed three immunogenic determinants of E7 that are recognised in association with at least two different HLA haplotypes. One N-terminal region (aminoacids 5–18) was recognised by one T cell line. T cell clones and the corresponding T cell line established from another donor responded to a different N-terminal (17–38) and to a C-terminal region (69–86). The N-terminal sequence 5–18 and the C-terminal determinant contain a periodicity of hydrophilic and hydrophobic residues that have been found in many T cell epitopes. Phenotypic characterisation of T cell clones by indirect immunofluorescence revealed that the T cell clones expressed the CD4 surface glycoprotein suggesting that the specific E7 determinants were recognised in association with major histocompatibility complex (MHC) class II molecules. With regard to functional properties, at least three T cell clones exhibited specific cytotoxic activity towards autologous B lymphocytes transformed by Epstein–Barr virus in the presence of the relevant HPV16 E7 peptides. The implications of these results regarding the development of vaccination strategies and host–virus interaction are discussed.
Eur J Cancer, Vol. 28, No. 2/3, pp. 326–333, 1992.

INTRODUCTION

THE GENOME of human papillomavirus (HPV) has been detected in the majority of cervical carcinomas. Of more than 60 characterised HPV types HPV16 is most commonly associated with severe dysplasias and malignant tumours of the uterine cervix [1].

The nuclear protein E7 of HPV16 has been demonstrated to play a key role in both the transformation and maintenance of the malignant phenotype in cell culture [2–4]. Most recently, it has been demonstrated that the E7 oncoprotein can form complexes with p105-RB (the gene product of the RB1 retinoblastoma gene) similar to the adenovirus E1A protein and the simian virus 40 (SV40) large T antigen [5, 6]. These findings suggest that these three DNA viruses may employ similar mechanisms in transforming host cells and implicate RB-binding as a possible step in human papilloma virus-associated carcinogenesis.

Immunisation of mice with a syngenic non-tumorigenic fibroblast-cell line that contains the transfected HPV16 E7 gene confers protection against transplanted cells from an HPV16 E7-positive syngeneic tumour suggesting that the human papilloma virus type 16 nuclear protein E7 represents a 'tumour specific' antigen which can serve as a target for cellular immune responses [7]. These findings could provide a basis for the development of highly specific means of immunotherapy against HPV16 associated tumours and, in addition, for their prevention by means of vaccination.

In this study we have employed a series of HPV16 E7 analogous synthetic peptides to identify the regions that are recognised by human T lymphocytes. To this end, human T cell lines and T cell clones were established from HPV16 E7 seropositive individuals and their specificity was determined *in vitro*. One T cell line generated with synthetic peptides also recognised the recombinant E7 protein expressed in two different vector systems.

The T cell clones were of the $CD4^+$ phenotype and proliferated specifically following incubation with the respective peptides presented by autologous antigen-presenting cells. Moreover, some of the $CD4^+$ T cell clones generated were capable of lysing autologous MHC class II-positive B cell lines transformed by Epstein–Barr virus in the context of the same antigenic peptide determinants.

MATERIALS AND METHODS

Blood donors

Sera and venous blood samples were taken from donors with no obvious papillomavirus associated disease. Therefore 10 ml blood modified with heparin (Thrombophob) was diluted in 25 ml culture medium supplemented with 2% fetal calf serum (FCS). Peripheral blood mononuclear cells (PBMC) were separated by density centrifugation on Ficoll-Hypaque. Purified PBMC were used for proliferation assays or for generation of T cell lines or were cryopreserved in liquid nitrogen in 90% FCS and 10% dimethylsulphoxide (DMSO).

Identification of seropositive donors

HPV16 E4 and E7 seropositive individuals were identified by Western blot analysis [8]. Briefly, HPV16 E4 and HPV16 E7 expressed as MS2- or cII-fusion proteins were separated by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (15%) and blotted onto nitrocellular filters [9]. Subsequently, sera obtained from various individuals were screened for antibodies specific for recombinant proteins [8].

The determination of the human leucocyte antigen (HLA) phenotype of donors which were selected for the generation of T cell lines and clones was kindly performed by W. Opelz, Institute of Immunology and Serology, University of Heidelberg: donor 1(D1) = A24, B35, Bw41, Cw4, DR4, DRw13 and donor 2 (D2) = A3, B27, B44, Cw2, Cw5, DR1, DRw11.

Generation of T cell lines and clones

Optimal peptide concentrations for the generation of T cell lines and clones as well as for the lymphoproliferation assay was determined by titration experiments (data not shown).

T lymphocyte lines were established by stimulation of 10^5 freshly prepared peripheral blood mononuclear cells per well (96-well U-bottom-plates) with 10 to 30 μmol/l synthetic peptide pool in 0.2 ml RPMI1640 culture medium supplemented with 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% human serum. Proliferating T cells were restimulated every 7–10 days by incubating 3×10^4 T cells with antigen and 7×10^4 irradiated autologous PBMC. Interleukin 2 (IL-2 supernatant: 2 to 6%; purified IL-2 (Biotest): 10 μg/ml) was added on day 5 and thereafter fresh IL-2-containing culture medium was supplied every 3–4 days.

T cell clones were isolated from T cell lines by limiting dilution. Therefore different concentrations of T lymphocytes (0.3–10 cells per well) were stimulated with antigen in presence of 9×10^4 irradiated autologous PBMC in 0.2 ml interleukin-2 (IL-2) containing culture medium and propagated by restimulation as described above.

Lymphoproliferation assay

T lymphocyte lines or clones were assayed for specific proliferative response against the total peptide pool (33 μmol/l), a panel of peptide pools consisting of five or six different peptides (33 μmol/l), single peptides (6.6 μmol/l) or fusion proteins. Proliferation of peripheral blood mononuclear cells was assayed against fusion proteins. Therefore, 5×10^4 PBMC or 3×10^4 T cells per well (96-well U-bottom-plate) and 7×10^4 irradiated autologous PBMC as antigen-presenting cells were incubated with antigen in culture medium supplemented as above. Control wells contained either T cells and irradiated PBMC with or without IL-2 or PBMC with or without Phytohemagglutinine (0.1 μg/ml). The plates were pulsed for 24 h with [methyl-^{3}H]-thymidine (37 kBq/well) after 3 days (T cells) or 5 days (PBMC) and harvested onto glass fibre filters. [^{3}H]thymidine incorporation, expressed as counts per minute (cpm), was measured in a scintillation spectrometer.

Transformation with Epstein–Barr virus (EBV)

Transformation of resting B cells with EBV was performed after preparation of PBMC and removal of T lymphocytes by rosetting with sheep red blood cells [10]. The EBV containing supernatant from the marmoset cell line B95/8 was used to transform 10^7 cells.

Cytotoxicity assay

T cell clones were tested for cytolytic activity in a cromium release assay. Following preincubation with peptide 10^3 autologous ^{51}Cr-labeled (7.4 MBq) B lymphocytes transformed by EBV per well were cultured with different concentrations of T cells in 0.2 ml culture medium supplemented with 3% FCS (96-well V-bottom plates). Spontaneous lysis and maximal lysis of target cells was determined by incubating labelled B cells in culture medium with or without 50% NP40. Control wells were also prepared containing T cells and target cells cultured with or without concanavalin A (10 μg/ml) but without peptide. After an incubation period of 3.5 h radioactivity in 0.1 ml supernatant per culture was measured in a gamma counter.

Synthetic peptides

Synthetic peptides as shown in Figure 1 and Table 1 were prepared as recently described [11]. HPV16 E7 was synthesised as 22 synthetic peptides corresponding to the aminoacid sequence expressed as MS2- or cII-fusion protein lacking the first eight N-terminal aminoacids. Individual peptides consisting of 14 residues overlapped by 10 aminoacids. The *Eco*RI- and polylinker present between the MS2- or cII-fusion part and the E7 insert was prepared as one peptide consisting of 9 aminoacids that was used as control peptide in proliferation assays.

Peptides were dissolved in H_2O, 50% DMSO and 0.1% β-mercaptoethanol.

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Revised 28 Oct. 1991; accepted 5 Nov. 1991.

Table 1. *Amino acid sequences of HPV16 E7 peptides*

Peptide	Sequence
E7:1–14	MHGDTPTLHEYMLD
E7:5–18	TPTLHEYMLDLQPE
E7:9–22	HEYMLDLQPETTDL
E7:13–26	LDLQPETTDLYCYE
E7:17–30	PETTDLYCYEQLND
E7:21–34	DLYCYEQLNDSSEE
E7:25–38	YEQLNDSSEEEDEI
E7:29–42	NDSSEEEDEIDGPA
E7:33–46	EEEDEIDGPAGQAE
E7:37–50	EIDGPAGQAEPDRA
E7:41–54	PAGQAEPDRAHYNI
E7:45–58	AEPDRAHYNIVTFC
E7:49–62	RAHYNIVTFCCKCD
E7:53–66	NIVTFCCKCDSTLR
E7:57–70	FCCKCDSTLRLCVQ
E7:61–74	CDSTLRLCVQSTHV
E7:65–78	LRLCVQSTHVDIRT
E7:69–82	VQSTHVDIRTLEDL
E7:73–86	HVDIRTLEDLLMGT
E7:77–90	RTLEDLLMGTLGIV
E7:81–94	DLLMGTLGIVCPIC
E7:85–98	GTLGIVCPICSQKP

The nomenclature of the peptides indicates their respective aminoacid residues.

Expression and preparation of fusion proteins

The expression vectors pEX12mer-HPV16 E4, -HPV16 L1/N, -HPV16 L1/C, and pEX8mer-HPV16 E6, -HPV16 E7 have been constructed by Seedorf *et al.* [12]. They contain the HPV16 open reading frame fused to the first 100 aminoacids of the bacteriophage MS2 polymerase. Growth of transfected *Escherichia coli* C600/537 cells and induction of protein synthesis were performed as described by Remault *et al.* [13]. For preparation of fusion proteins the bacteria from 400 ml cultures were sedimented by low-speed centrifugation and resuspended in 2 mmol/l EDTA, 10 mmol/l Tris–HCl (pH 8.0), treated with a final concentration of 0.5 mg/ml lysozyme for 15 min followed by DNase I ingestion in presence of 20 mmol/l $MgCl_2$ for 30 min. Cells were lysed by the addition of 0.5% Triton X-100, 150 mmol/l NaCl, 1 mmol/l dithiothreitol (DTT), and 10 mmol/l Tris–HCl (pH 8.0) for 15 min at 0°C, 30 min at 37°C, and 15 min at 0°C, and cleared twice by centrifugation at 9871 *g* in 1 mmol/l DTT, and mol/l Tris–HCl (pH 8.0). The lysate was resuspended in 100 mmol/l SDS, 2.7 mmol/l glycerine, 125 mmol/l Tris–HCl (pH 6.8), 3% β-mercaptoethanol, and bromophenol blue and separated on a preparative SDS–PAGE (12.5%; [14]). Subsequently, the fusion proteins were purified from the gel by electroelution in 2 mM glycine and 0.25 mmol/l Tris followed by dialysis in phosphate-buffered saline.

The HPV16 fusion proteins cll-E4 and cll-E7 as well as the control protein cllras consisting of the v-Ha-ras gene product fused to the truncated cll-protein were prepared as described [15].

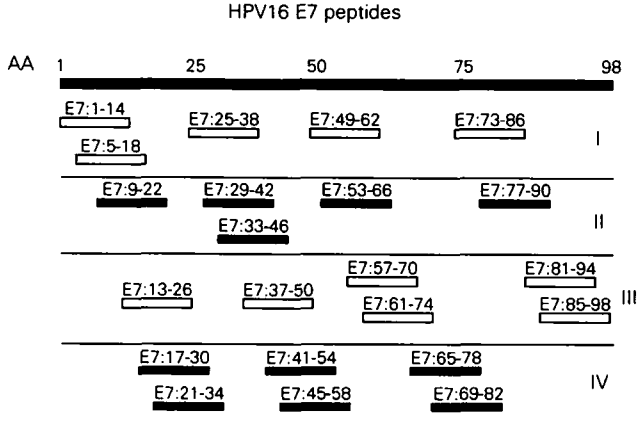

Fig. 1. Arrangement of E7 peptides. The complete peptide pool was subdivided into four smaller pools consisting of 5 (Pp I, Pp II) or 6 peptides (Pp III, Pp IV) that were used for stimulation in proliferation assays.

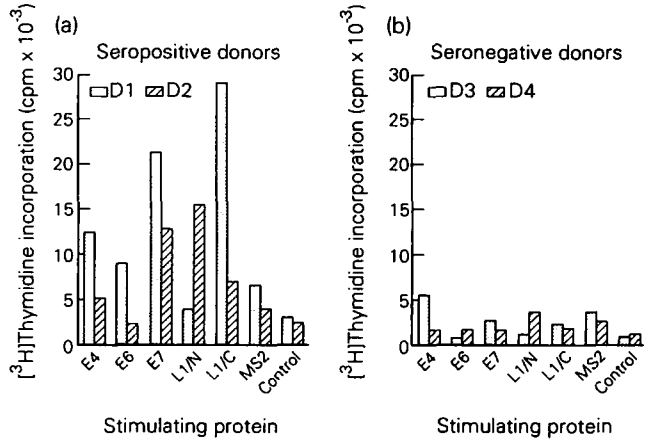

Fig. 2. Proliferative response of PBMC from seropositive and seronegative donors to HPV16 MS2-fusion proteins or the MS2-fusion part. Bars represent proliferation of PBMC from 2 donors of each group to 2 μg/ml protein. Proliferation obtained in the absence of antigen is also shown (Control). Each bar represents the mean of two values (S.D. < 15%).

RESULTS

Proliferative responses of PBMC to HPV16 fusion proteins

HPV16 E7 seropositive donors were identified by western blot analysis. To this end, sera of various individuals were obtained and tested with nitrocellulose filters onto which the MS2- or cll-fusion protein of HPV16 E4 or E7 had been blotted after SDS–PAGE according to the approach of Jochmus-Kudielka *et al.* [8]. Three out of 33 tested serum samples reacted with both, the E4 and E7 fusion proteins (data not shown). Peripheral blood mononuclear cells of individuals producing high titres of HPV16 E4- and E7-specific antibodies as well as PBMC from seronegative donors were prepared and stimulated with predetermined optimal concentrations of various HPV16 MS2-fusion proteins (E4, E6, E7, L1/N, L1/C, respectively). As shown in Fig. 2a for two of three seropositive donors, a significant proliferative response to MS2-E7 was obtained at a concentration of 2 μg/ml. In addition, peripheral blood lymphocytes incorporated [^{3}H]thymidine also in response to E4, L1/N and L1/C fusion proteins, although the pattern of responses to these antigens varied among donors. Proliferation to the N-terminal fusion part MS2 without the HPV16 insert was rather low as compared with the HPV16 fusion proteins. As shown in Fig. 2b, HPV16-induced proliferation was much lower in

Table 2. Proliferative responses

(a) T cell line	Stimulating peptide (cpm) Il-2	Control	E7-Pep	E7-Pep + Il-2	Cont. Pep	Cont. Pep + Il-2
A52	7.886	181	4.862	16.960	1.812	6.165
A57	7.694	172	17.983	48.493	4.347	n.d.
T cell clone						
10C5	6.063	367	19.800	41.090	298	
10C19	3.703	260	3.255	18.141	271	
10C23	8.675	180	41.420	91.950	165	
10C24	1.481	83	23.515	51.530	117	
100C2	6.354	205	1.462	26.805	108	
100C3	1.920	97	16.907	46.210	56	

(b) T cell line	Stimulating antigen (cpm) Il-2	Control	E7-Pep	MS2-E7	cII-E7
A50	9.885	498	16.990	10.970	10927
	Pp I	Pp II	Pp III	Pp IV	
	2.951	14.116	6.800	16.305	

[^{3}H]Thymidine incorporation (cpm).
(a) Proliferative response of T cell line A57 from donor D2 or T cell line A52 and corresponding clones from donor D1 to 33 μmol/l E7 peptide or 6.6 μmol/l control peptide (Cont. Pep). Cultures containing T cells and antigen-presenting cells with or without Il-2 were also prepared (Control, Il-2). Values represent the mean of triplicates (S.D. < 10%). n.d. = not done. (b) Proliferation of T cell line A50 obtained after stimulation with E7 fusion protein (MS2-, cll-; 2 μg/ml), with E7 peptide pool (33 μmol/l) or with different E7 peptide pools (Pp I – Pp IV; 33 μmol/l). Cultures containing T cells and antigen-presenting cells with or without Il-2 are indicated as 'Il-2' and 'control'. Values represent the mean of triplicates.

seronegative as compared with seropositive donors (compared with Fig. 2a).

Establishment of HPV16 E7-specific T cell lines and clones

Investigations of antigen-specific T cell responses are limited by low frequencies of T cells specific for a given antigenic determinant. To overcome this problem, HPV16 E7-specific T cell lines and clones from peripheral blood of seropositive donors were established. E7 was selected as antigen, because recent work of Jochmus-Kudielka *et al.* [8], Chen *et al.* [7], and data presented in Fig. 2a point strongly towards the relevance of this early HPV16 gene product for HPV-directed immune responses. To obtain E7-specific T cell lines and clones PBMC from three HPV16 E7-seropositive donors were stimulated with HPV16 E7 analogous synthetic peptides. To this end, 22 overlapping 14mers (see Table 1 and Fig. 1) were synthesised by standard methods [11] and as a pool incubated with freshly isolated PBMC. T cells were repetitively restimulated with peptides in the presence of IL-2. To isolate T cell clones activated T cells were cloned by limiting dilution. As shown in Table 2a, T cell lines A57 and A52 as well as a series of six T cell clones derived from line A52 responded to E7 peptides, E7 peptides plus IL-2

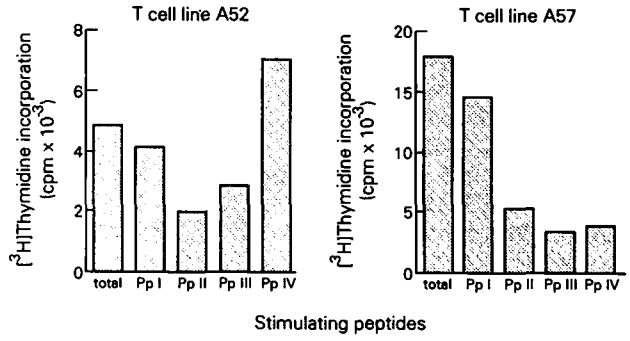

Fig. 3. Proliferative response of T cell lines A52 (donor D1) and A57 (donor D2) against different E7 peptide pools (Pp I–PpIV; see Fig. 2) or against the total peptide pool (total) at a concentration of 33 μmol/l. Bars represent the mean of three wells (S.D. < 10%).

and to a lesser extent to IL-2 alone, but not to a control peptide representing the *Eco*RI- and polylinker of the E7 fusion protein. T cell line A50 proliferated to E7 peptides and to the recombinant E7 gene product expressed as cll- or MS2-fusion protein (see Table 2b). These data indicate the specificity of these T cells for E7.

Identification of epitopes recognised by E7-specific T cell lines and clones

Antigenic sites recognised by T lymphocytes specific for a particular protein generally consist of regions restricted to 8–15 aminoacids [16]. In order to identify E7 sequences which can be recognised by human T lymphocytes the whole peptide pool was subdivided into four individual smaller pools each consisting of five or six different peptides (see Fig. 1). Following proliferation analysis with these small pools (Pp I to IV) as antigens single peptides of those pools that had induced a strong proliferation of individual lines or clones were assayed for their stimulatory capacity. T cell line A50 showed a strong response to peptide pools II and IV as compared with proliferation obtained after stimulation with peptide pools I and III (see Table 2b). Figures 3 and 4 summarise the results of the specificity analysis for two different T cell lines which underwent further characterisation. Line A57 proliferated most to peptide pool I. The peptide E7:5–18 (aminoacids 5–18 at the N-terminal region) was recognised by these T cells. Cell line A52 established from another donor (D1) responded to a different N-terminal and to a C-terminal epitope that were contained in the peptide pools I and IV in association with different HLA molecules. The epitopes recognised by this line were contained within the aminoacid sequences 17 to 34 and 69 to 86 represented by the overlapping peptides E7:17–30 and E7:21–34 or E7:69–82 and E7:73–86. As expected, T cell clones derived from line A52 showed the same specificity pattern as found for the original cell line (A52) responding either to both, the N-terminal and C-terminal determinants, or to one of these sequences (see Table 3). Comparing both regions no aminoacid sequence homology can be observed. Other clones, however, such as 10C5 and 100C2 recognised only one of these epitopes, the N-terminal (100C2) or the C-terminal sequence (10C5). Interestingly, T cell clones specific for the N-terminal region differed in reactivity to peptides spanning this region. Clones 10C24 and 100C3 proliferated to peptide E7:21–34. In contrast, clones 10C19 and 10C23 were stimulated by two overlapping peptides E7:17–30 and E7:21–34 or E7:21–34 and E7:25–38. In view of the fact that T

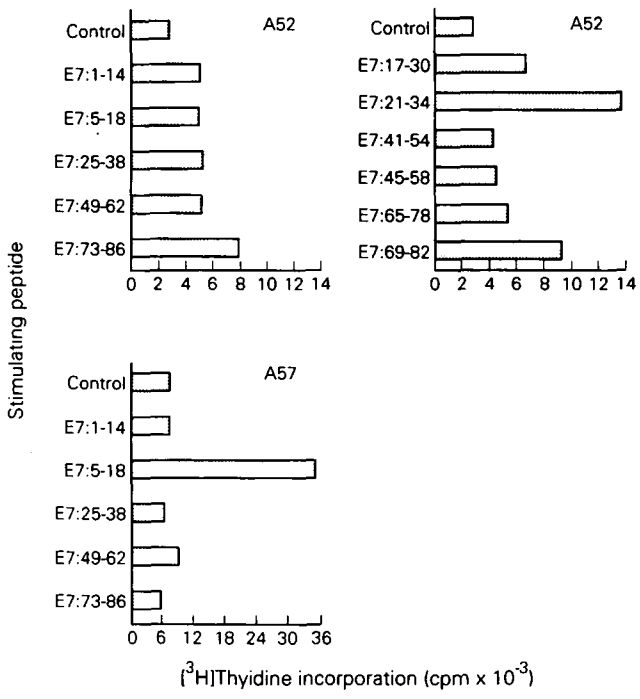

Fig. 4. Proliferation of T cell lines A52 and A57 obtained after stimulation with E7 peptides of peptide pool I (A57) or peptide pool I and IV (A52) at a concentration of 6 μmol/l. Proliferation obtained in wells without peptide is indicated as 'Control'. Values represent the mean of triplicates (S.D. < 12%).

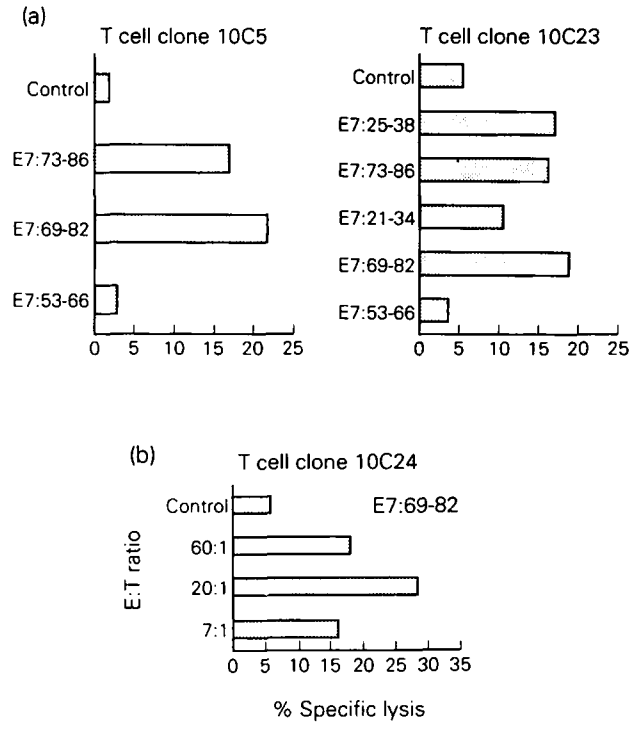

Fig. 5. Cytolytic activity of T cell clones 10C5 and 10C23 (A) or 10C24 (B) against B cell targets plus peptide. Autologous B lymphocytes transformed by EBV were pulsed with 10 μmol/l individual specific or nonspecific E7 peptide for 1.5 h, radiolabeled and used as targets in standard ^{51}Cr release assays with T cell clones. The T cell to target cell ratio (E:T) was 20:1 (a) or 60:1, 20:1 and 7:1 (b). Control cultures contained T cells and target cells without peptide [E:T 20:1 (a); E:T 60:1 (b)].

cell epitopes can consist of a minimum of five residues [17] these clones might have responded to entirely different epitopes within the N-terminal aminoacids 17–38.

The phenotypic characterisation of the E7-specific T cell clones by indirect immunofluorescence revealed that they expressed the CD3 and CD4 but not the CD8 surface molecules (data not shown).

In conclusion, we identified three immunogenic determinants of HPV16 E7 that were recognised by human T cells. The fact that T cell clones expressed CD4 surface molecules strongly suggests that they responded to the specific peptide in association with MHC class II molecules. This point will require further investigation.

Table 3.

	T cell clone (cpm)					
Peptide	10C5	10C19	10C23	10C24	100C2	100C3
Pp I						
E7:1–14	690	459	256	172	n.d.	108
E7:5–18	186	516	207	190	n.d.	128
E7:25–38	216	668	3.515	472	n.d.	823
E7:49–62	315	828	351	218	n.d.	121
E7:73–86	6.580	1.604	11.922	17.363	n.d.	3.983
Pp IV						
E7:17–30	423	1.670	206	157	228	135
E7:21–34	205	1.250	32.930	4.014	4.821	17.508
E7:41–54	234	598	179	417	165	190
E7:45–58	154	577	211	163	213	120
E7:65–78	181	756	191	348	205	173
E7:69–82	24.230	2.253	12.003	30.570	139	6.468

[^{3}H]-Thymidine incorporation (cpm).

Cytolytic activity of E7-specific T cell clones

T cell clones specific for HPV16 E7 were also characterised with respect to cytolytic activity. To this end, autologous B lymphocytes transformed by EBV were individually prepulsed with single E7 peptides and employed as targets for individual T cell clones in ^{51}Cr release assays. As shown in Fig. 5a, clones 10C5 and 10C23 possessed significant cytolytic activity towards B cells that had been prepulsed with specific E7 peptides E7:69–82 or E7:73–86 (10C5), and E7:21–34, E7:25–38, E7:69–82 or E7:73–86 (10C23). In contrast, no lysis toward B cells that had been prepulsed with the E7 peptide E7:53–66 occurred indicating an antigen-specific cytolytic activity of these T cell clones. Figure 5 b demonstrates the dose dependent cytolytic activity of clone 10C24 towards B cells that had been prepulsed with the specific E7 peptide E7:69–82. The exact reasons why at higher E:T ratios cytotoxicity is reduced are not known at present. One possibility might be that (MHCII positive) activitated T cells present peptide to each other which could lead to effector cell damage or, alternatively, could mediate cold target inhibition. In contrast with clones 10C5, 10C23 and 10C24, clone 10C19 showed little if any cytolytic activity (data not shown) consistent with the notion of functional heterogeneity among $CD4^+CD8^-$ T cells.

The results shown before indicate that the presentation of a relevant E7 peptide by autologous antigen-presenting cells can

not only lead to proliferation of $CD4^+$ T cells but also at least in the case of certain E7-specific T cell clones to a direct lysis of autologous targets expressing HPV16 gene products. It is worth mentioning that those peptides which mediated the cytotoxic effector function corresponded to the sequences that were previously found to induce clonal proliferation.

DISCUSSION

Cellular immune reactions play an important role in the protection against latent viral infections including human papillomaviruses. There is a higher prevalence of HPV-induced lesions in patients with cell-mediated immune deficiencies caused by immune-suppressive drugs [18] or diseases that are associated with disorders of the cell-mediated immune response [19]. HPV infections are one of the most frequent viral complications affecting immunosuppressed individuals [20]. However, virtually nothing is known about cellular immune reactions to HPV in man. The strong association of HPV16 with cervical cancer and its wide spread in the population requires the development of vaccines to prevent HPV16 infection or to modulate pre-existing infections. Therefore, cellular immune reactions need to be clarified with respect to immunogenic viral gene products and the mechanisms involved in immune responses.

HPV16 DNA has been demonstrated in the majority of malignant cervical tumours and also in a high percentage of asymptomatic individuals [21, 22]. Moreover, antibodies specific for HPV16 E4, E7 [8], E2 [23] and L2 [24] have been identified in sera from asymptomatic individuals and in carcinoma patients, in particular anti-E7 and anti-E2 antibodies being more prevalent in patients.

We, therefore, investigated HPV16-specific T cell responses in healthy asymptomatic donors. Peripheral blood lymphocytes from seropositive individuals were shown to respond to the recombinant HPV16 early protein E7 expressed as MS2-fusion protein. Other recombinant HPV16 gene products such as MS2-E4 and the N- or C-terminal part of MS2-L1 were also capable of stimulating PBMC although the pattern of responses to these antigens varied among donors. E7 seems to play a central role in the genesis of HPV16 infection for several reasons: specific mRNA has constantly been identified in biopsies of cervical carcinomas as well as in carcinoma-derived cell lines [25, 26]. It is also known to have immortalising and transforming capacity [2, 3, 27]. Furthermore, recent work concerning cellular immune responses to HPV16 [7] points strongly to the immunological significance of E7.

In order to identify immunogenic determinants of HPV16 E7 a series of E7 analogous synthetic peptides were employed to establish specific T cell lines and clones from peripheral blood of seropositive donors. The use of peptides as antigens, provides a reasonable approach to screen for T cell epitopes or to establish specific lines and clones, particularly in cases such as HPV16 where the lack of a suitable culture system makes it impossible to obtain sufficient viral protein. At least one of the T cell lines generated with E7 peptides recognised the recombinant protein expressed in two different vector systems.

With regard to specificity, we have identified within E7 a 14 aminoacid epitope spanning the N-terminal residues 5–18 that was recognised by the T cell line from one donor. We have also shown that a different N-terminal epitope within the aminoacids 17–38 and a C-terminal epitope within the residues 69–86 was recognised by the T cell line and derived clones from another individual. This result extends the previous findings that T cells restricted to different HLA haplotypes can respond to entirely different regions of a given protein antigen [28–30]. The fact that the last-mentioned cell line as well as some of the corresponding clones responded to non-homologous regions of E7 suggests that they were composed of at least two different E7-specific clonal cell populations.

Some of the T cell clones responding to the N-terminal determinant were shown to proliferate to different but overlapping peptides of this region. Given the fact that T cell epitopes may consist of a minimum of five aminoacids [17], we consider them being specific for distinct regions within this definite sequence.

The precise delineation of the immunogenic E7-determinants enabled us to compare their structure with that of epitopes defined for several protein antigens in the murine system and for a small number of viral and bacterial proteins in the human system. Berzofsky *et al.* [31] noted a periodicity of hydrophilic and hydrophobic residues to form an amphipathic α-helix in many T cell epitopes. It is of interest that one of the N-terminal epitopes, the aminoacids 5–18, and the C-terminal epitope identified within the E7 protein contain the residue motif predicted by Berzofsky.

The finding that certain E7-specific T cell clones exhibited antigen-specific cytolytic activity is interesting for several reasons. HPV16 infection may induce epithelial lesions that sporadically progress to malignant tumours despite the presence of an antiviral antibody response. In contrast, cell-mediated immune responses may be an essential antiviral mechanism for controlling infection, because an HPV16-specific T cell response has been demonstrated in healthy asymptomatic individuals. Studies concerning the investigation of HPV16-specific T cell responses in patients with severe cervical dysplasia and cancer are underway.

It is of further interest that E7-specific cytotoxic T cell clones isolated here belonged to the $CD4^+CD8^-$ T cell subpopulation responding to the specific antigen in association with MHC class II molecules [32]. Although $CD4^+CD8^-$ T cells are often referred to as to belong to the helper/inducer subset this population is heterogenous in terms of their effector functions such as lymphokine productoin and secretion or cytolysis.

In humans $CD4^+$ cytotoxic T cells have been described as an effector cell population in a variety of virus infections including EBV [33], hepatitis B [34], herpes simplex [35], measles [36], influenza [37], and probably HIV [38]. The above data indicate that $CD4^+$ cytotoxic T lymphocytes might be important in the control of HPV infection. The finding that some of these clones possessed cytotoxic activity while others did not point to a functional heterogeneity of the cells.

The $CD4^+CD8^-$ phenotype of E7-specific clones described here suggests that lytic activity of these clones is directed against class II expressing target cells, although this point remains to be proved formally. In humans, besides B cells, macrophages, activated T cells, and a variety of endothelial and epithelial cells also keratinocytes, the natural host cells of papillomaviruses, express MHC II molecules under certain conditions [39]. Given that an appropriate peptide epitope in association with MHC molecules might be expressed on the surface of infected keratinocytes, lysis of these cells by $CD4^+$ cytotoxic T lymphocytes might be an important mechanism for the protection against HPV infection *in vivo*.

In summary, we have shown that a HPV16 E7-specific T cell response exists in peripheral blood of seropositive donors. Employing E7 analogous synthetic peptides three immunogenic

determinants that are recognised by specific T cell lines and clones in association with different HLA haplotypes could be identified. We have also shown that the HPV16 E7-specific T cell response probably included class II-restricted cytotoxic T lymphocytes. More donors with different HLA haplotypes will have to be investigated in order to confirm, whether the epitopes identified here are immunodominant with regard to eliciting T cell responses. Furthermore, experiments are under way to characterise immunoregulatory phenomena in the environment of HPV-induced lesions such as the expression of adhesion molecules or the production of lymphokines. Recently, keratinocytes have been shown to present antigenic peptides in association with MHC molecules which, however, induces tolerance rather than clonal expansion of specific T lymphocytes [40]. Obviously, additional activation signals are required for clonal propagation of specific T cells *in vivo*. In cases of HPV16 infections that favour malignant conversion of the lesions the definition of additional factors necessary for an optimal T cell response is required.

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Mutations in the *ras* Protooncogenes are Rare Events in Renal Cell Cancer

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Mutations in codon 12, 13 or 61 of one of the three *ras* genes, Ha-*ras*, Ki-*ras*, and N-*ras*, convert these genes into active oncogenes. To determine the role mutated *ras* genes play in the carcinogenesis of renal cell carcinoma, we analysed tumour DNA and unaffected renal tissue derived from 55 patients. The polymerase chain reaction technique was used to amplify DNA fragments containing Ki-, Ha-, and N-*ras* codons 12, 13, and 61. The amplified fragments were then probed on slot-blots with labeled mutation-specific oligomers. A single Ki-*ras* mutation (codon 12, gly-> val) was detected in a patient with a pT2N2M1 tumour. We concluded that *ras* oncogene mutations do not play an important role in the initiation of renal cell carcinoma.
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INTRODUCTION

ONCOGENE ACTIVATION has been recognised as part of a multistep process in the carcinogenesis of many human tumours. Members of the *myc*- and the *ras*-gene family are the oncogenes most frequently encountered in human neoplasms, with amplification, overexpression, and single base mutations being the most important mechanisms of their activation [1, 2]. The overall incidence of *ras* oncogene activation by mutations of single bases is 15–30% in human malignancies [1, 3]. The functional and structural resemblance of the ras proteins with the G-proteins has led to the proposal that normal ras proteins are involved in the transduction of growth stimulatory signals through the cell membrane. Mutated ras proteins have lost their ability to become inactivated and thus stimulate growth or differentiation autonomously, leading to the malignant transformation of the cell [1].

These mutations commonly occur in the codons 12, 13, and 61 of the Ki-, Ha-, and N-*ras* oncogenes and are found in both epithelial and haematopoietic tumours. For example, approximately 90% of pancreatic carcinomas [4], 50% of colorectal tumours [5, 6] 35% of rhabdomyosarcomas [7] and 25% of acute myelogenic leukaemias [8, 9] contain mutated *ras* genes. However, in some tumours such as carcinoma of the breast [10] or ovary [11], *ras* gene activation appears to be a rare event.

The biological relevance of these differences remains unclear. In myelodysplastic syndromes, N-*ras* mutations seem to be associated with progression to frank acute leukaemia [12] and in chronic myelogenic leukaemia *ras* mutations occur in late stages only [13]. In colorectal neoplasms and some other solid tumours, however, *ras* oncogene mutation seems to be an early step in the carcinogenic process [14].

Several molecular alterations of oncogenes and tumour suppressor genes have been described in renal cell cancer (RCC) [15–20]. Among these, point mutations in codons 12 and 61 of the Ha-*ras* oncogene in 2 of 16 kidney tumours have been reported in a previous study that used the relatively unsensitive DNA transfection assay to determine oncogenic changes [21].

To evaluate the role ras oncogene mutations play in the initiation of renal cell carcinoma, we analysed codons 12, 13, and 61 of the three *ras*-oncogenes by polymerase chain reaction (PCR) and mutation-specific oligonucleotide hybridisation in 55 patients with this disease.

MATERIALS AND METHODS

Tumours

55 consecutive patients operated in the Department of Urology of the University of Düsseldorf, West Germany, were included in the study. No patient had received chemotherapy or radiotherapy prior to operation. The histopathology and the clinical

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