Evaluation of immunological cross-reactivity between clade A9 hrHPV-types on basis of E6-specific CD4+ memory T-cell responses

Muriel van den Hende, Anke Redeker, Kitty M.C. Kwappenberg, Kees L.M.C. Franken, Jan W. Drijfhout, Jaap Oostendorp, A. Rob P.M. Valentijn, Lorraine M. Fathers, Marij J.P. Welters, Cornelis J.M. Melief, Gemma G. Kenter, Sjoerd H. van der Burg, Rienk Offringa

Adapted from J Infect Dis 2010; 202:1200-1211

Abstract

CD4+ T-cell responses against the E6 oncoprotein of HPV16 and 5 closely related members of clade A9 (HPV31, 33, 35, 52, 58) were charted in PBMC cultures from healthy subjects and patients that underwent HPV16E6/E7 specific vaccination. Initial analyses with overlapping peptide arrays showed that approximately half of the responding subjects displayed reactivity against corresponding E6 peptides from two or more HPV types. This suggested immunological cross-reactivity and complicated retrospective evaluation of the infection history of the healthy subjects concerned. Importantly, further dissection of the response by means of enriched and clonal T-cell cultures, using protein antigen instead of peptides, revealed that CD4+ T cells capable of efficiently reacting against E6 antigen from multiple HPV types are rare, and only occur when epitope sequences are highly conserved. Our data indicate that natural and vaccine-induced HPV16E6-specific CD4+ T-cell responses are unlikely to mediate efficient cross-protection against other clade A9 members.

Introduction

Comparison of HPV-specific T-cell immunity in patients and healthy controls revealed that HPV16-positive cervical neoplasia is associated with failure of the CD4+ T-cell response against HPV16 early antigens E6, E7 and E2. In contrast, healthy subjects commonly display strong CD4 Th1/Th2 memory responses against HPV16, bearing witness to encounter and successful immune control of this virus [de Jong 2002a; de Jong 2004; van Poelgeest 2006; Welters 2003]. The frequency by which the latter responses are detected (approximately 50%) is in line with the estimated lifetime incidence of HPV16 infections [Jenkins 1996]. Our recent finding of a strong correlation between the strength of vaccine-induced HPV16E6/E7-specific CD4+ T-cell responses and full regression of HPV16-positive lesions in patients [Kenter 2009] lends further support to the functional importance of CD4 T-cell responses against HPV early antigens.

The existence of multiple closely related high-risk (hr) HPV types [Munoz 2003], the antigens of which display up to 70% homology, suggests that a considerable degree of immunological cross-reactivity towards these viruses may exist. Increased insight in this matter is important for two reasons. First, this will facilitate correct interpretation of analyses of the HPV-specific T-cell response in relation to health and disease, such as for assessing the role of T-cell immunity in clearance of HPV infections and regression of premalignant lesions. Second, detailed information on the specificity of hrHPV-specific T-cell responses can inform us on whether the impact of HPV-specific vaccines would be limited to the HPV types comprised, or could target a broader range of hrHPV types. We therefore charted the incidence of CD4+ memory responses against HPV16 and highly similar hrHPV types [Bernard 2005; Chan 1995; de Villiers 2004] in healthy donors as well as in patients that received HPV16 E6/E7 specific vaccination. We focused on clade A9, encompassing HPV types 16, 31, 33, 35, 52, 58, because HPV16 is the most prevalent type in cervical carcinoma and anogenital neoplasia [De Vuyst 2009; Smith 2007]. Furthermore, the degree of sequence conservation within this clade is higher than that between clades A9 and A7, the latter of which comprises HPV types 18 and 45. We choose to study responses against the E6 antigen, because its expression is associated with all stages of neoplasia, and T-cell responses against E6 are more commonly found than against E7 in both healthy subjects and patients [de Jong 2004; Welters 2003].

Materials and Methods

Subjects

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy blood donors (n = 44), as well as from a group (n = 10) of HPV16 positive patients with high-grade vulvar intraepithelial neoplasia (VIN) who participated in our immunotherapeutic vaccination trial [Kenter 2009], after informed consent.

Antigens

To examine HPV clade A9 specific T-cell responses, sets of overlapping 32-mer peptides (14-amino acid overlap) spanning the entire E6 proteins of HPV16, 31, 33, 35, 52 and 58 were used (Figure 1). The peptides, recombinant E6 proteins and memory response mix (MRM), consisting of a mixture of tetanus toxoid, *Mycobacterium tuberculosis* sonicate and *Candida albicans* were produced and used as described previously [de Jong 2002a; de Jong 2004; Franken 2000; van der Burg 1999; van der Burg 2001; Welters 2003].

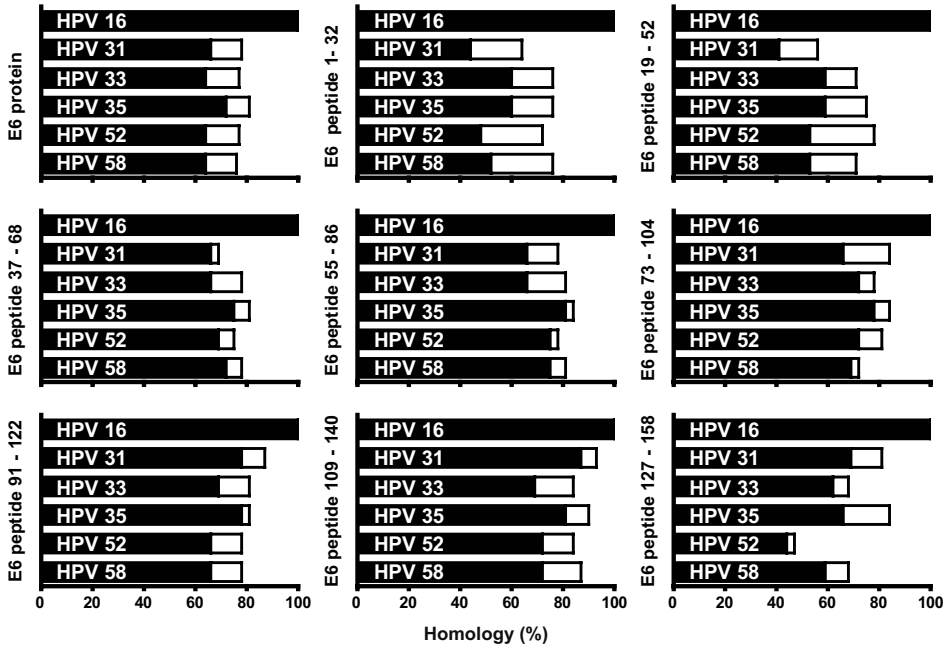

Figure 1. Sequence homology between E6 antigens of human papillomavirus (HPV) clade A9. Analysis of sequence homology of E6 protein and overlapping clade A9 peptides performed in SwissProt protein database using Basic Local Alignment Search Tool (BLAST) [Altschul 1997] showed >70% amino acid homology with HPV16E6. Both percentages of identical amino acids (black bars) and percentages of amino acids with similar properties (open bars) are depicted.

T-cell cultures and clones

Enriched HPV16E6 specific T-cell cultures were isolated by magnetic cell sorting (Miltenyi Biotech) based on IFNγ secretion after an extended period of in vitro stimulation as described by de Jong *et al.* [de Jong 2005]. T-cell clones were isolated from the enriched HPV16E6 specific T-cell cultures using limiting dilution [van der Burg 1999].

Analysis of T-cell responses

All analytical protocols used are part of standard operating procedures established in our laboratory, for which detailed descriptions have been published elsewhere. The presence of HPV clade A9 specific memory T cells in healthy donors and HPV16 positive VIN patients was analyzed by IFNγ enzyme-linked immunosorbent spot (ELISPOT) as described previously [van der Burg 2001; van Poelgeest 2006; Welters 2003; Welters 2006]. Antigen-specific T-cell responses were considered positive when T-cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x background.

T-cell cultures and clones were tested for their specificity and function by IFNγELISPOT, intracellular cytokine staining (ICS) and in a 3-day proliferation assay. When analyzed by ICS [de Jong 2002a; de Jong 2005], positive response was defined as at least three times the percentage of IFNγ producing CD4+ T cells found with the medium-only control. When analyzed in 3-day proliferation assays [de Jong 2005; van der Burg 2001], responses were defined positive if the stimulation index (SI) ≥ 3. Antigen-specific IFNγ secretion into the supernatant was measured by enzyme-linked immunosorbent assay (ELISA) or cytometric bead array (Becton Dickinson) as reported previously [de Jong 2004; van der Burg 1999].

Results

HPV clade A9 E6-specific T-cell memory in healthy subjects

Memory T-cell responses to the E6 antigens of HPV clade A9 members (types 16, 31, 33, 35, 52 and 58) were evaluated in primary PBMC cultures from 30 healthy donors. T cells responding to pools of overlapping peptides (Figure 1) were enumerated by means of IFNγELISPOT assays. We have previously shown that these assays primarily detect CD4+ memory T-cell responses [van der Burg 2001; van Poelgeest 2006; Welters 2003; Welters 2006]. In line with the notion that a vast majority of the population encounters a hrHPV type during life time (80-85% [Jenkins 1996]), immune responses to E6 of one or more clade A9 members were detected in 19 (63%) of 30 subjects (Table 1). Responses to HPV16 were observed in 40% of the healthy subjects (12 of 30), in accordance with our previous studies [de Jong 2002a; de Jong 2004; Welters 2003]. Peptides recognized differed between subjects, in line with natural human leukocyte antigen (HLA) variation in the human population. Responses against E6 of the other clade A9 members were found in a smaller fraction of donors (4-9 of 30 donors). In this latter group, responses to HPV58E6 were the most prevalent (found in 9 (30%) of 30), and their frequency approximated that of responses against HPV16. As indicated in bold in Table 1, 11 (58%) of the 19 responding subjects displayed T-cell responses against corresponding peptide pools from two or more clade A9 HPV types, which suggests immunological cross-reactivity. For instance, donor 15 shows reactivity against E6 peptide pool 3 of HPV16 and 33, while donor 26 reacts against pool 2 of HPV16 and 58. In all but one case (donor 28), reactivity against corresponding peptide pools was limited to two clade A9 members.

Table 1. HPV clade A9 specific CD4+ Th1 responses in healthy subjects

Donor[a]	HPV16 E6				HPV31 E6				HPV33 E6				HPV35 E6			
	1-50[c]	37-86	73-122	109-158	1-50	37-86	73-122	109-156	1-50	37-86	73-122	109-156	1-50	37-86	73-122	109-156
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	**11**[d]	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	**87**	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	**11**	-	-	-	-	-	**10**	-	**15**
6	-	-	-	-	-	-	-	*21*	-	-	-	-	-	-	-	-
7	-	-	-	*86*	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	*12*	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	*13*	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	**17**	-	-	-	-	-	-	-	**13**	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	**28**	-	-	-	-	-	-	-	**19**	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	**12**	-	-	-	-	-	-	-	**12**	-	-
19	-	-	*23*	-	-	-	-	-	-	-	-	-	-	*21*	-	-
20	-	-	*13*	-	-	-	-	-	-	-	-	-	-	-	-	*10*
21	-	-	**14**	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	25	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	**17**	-	-	-	-	-	-	-	**17**	-	-	-	-	-	-
26	-	**21**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	*13*	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	**20**	*73*	-	-	-	-	-	-	**18**	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Frequency[c] of responders	**40%** (12/30)				**17%** (5/30)				**17%** (5/30)				**13%** (4/30)			

[a] PBMC from 30 healthy blood donors were tested against E6 peptides of HPV clade A9 (HPV16, 31, 33, 35, 52 and 58) by IFNγ ELISPOT. [b] Encounter of HPV types. Defenite: showing ELISPOT response towards one or different peptide pools of one or more HPV types. Response can – with reasonable probality – be ascribed to infection with this particular HPV type. Ambigous: ELISPOT response towards similar peptide pool of 2 or more HPV types. Responses can reflect encounter of either one or both of the viruses. [c] Indicated are the first and last amino acid in the peptide pool used. [d] Specific spots were calculated by subtracting the mean number of spots + 2xSD of the medium control from the mean number of spots in

	HPV52 E6				HPV58 E6					Encounter of HPV type(s)[b]	
	1-50	37-86	73-122	109-155	1-50	37-86	73-122	109-156	MRM	definite	ambigous
	-	-	-	-	-	-	-	-	**408**	-	-
	-	-	-	-	-	-	**12**	-	**150**	-	31/58
	-	-	-	-	-	-	-	-	**35**	-	-
	-	-	**102**	-	-	-	-	-	**215**	-	31/52
	-	-	-	-	-	**41**	-	-	**19**	-	31/35/52
	-	-	-	-	-	-	-	-	**139**	31	-
	-	-	-	-	-	*21*	*20*	-	**177**	16; 58	-
	-	-	-	-	-	-	-	-	**107**	16	-
	-	-	-	-	-	-	-	-	**251**	-	-
	-	-	-	-	-	-	-	-	**81**	-	-
	-	-	-	-	-	-	-	-	**21**	33	-
	-	-	-	-	-	-	-	-	**50**	-	-
	-	**11**	-	-	-	**19**	-	-	**114**	-	16/33; 52/58
	-	-	-	-	-	-	-	-	**34**	-	-
	-	-	-	-	-	-	-	-	**83**	-	16/33
	-	-	-	-	-	-	-	-	**54**	-	-
	-	-	-	-	-	-	*12*	-	**75**	58	-
	-	-	-	-	-	-	-	-	**95**	-	31/35
	-	-	-	-	-	-	-	-	**132**	16; 35	-
	-	-	-	-	-	-	-	-	**230**	16; 35	-
	-	-	**21**	-	-	*15*	-	-	**57**	58	16/52
	-	-	-	-	-	-	-	-	**125**	-	-
	-	-	-	-	-	-	-	-	**135**	-	-
	-	**47**	-	-	-	**35**	-	-	**107**	16	52/58
	-	-	-	-	-	-	-	-	**198**	-	16/33
	-	-	-	-	-	**81**	-	-	**27**	-	16/58
	-	*17*	-	-	-	-	-	-	**209**	16; 52	-
	100	-	-	-	-	-	**34**	-	**147**	52	16/33/58
	-	-	-	-	-	-	-	-	**134**	-	-
	-	-	-	-	-	-	-	-	**24**	-	-
		20% (6/30)				**30%** (9/30)					

experimental wells. The number of spots per 100.000 PBMC are given. Antigen-specific T cell responses were considered to be positive when T cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x background [van der Burg SH 2001]. These values are indicated: coinciding responses against corresponding E6 peptide pools are depicted in **bold** (possible crossreactivity) and responses not matched by coinciding reactivity against corresponding peptide pools are depicted in *italics*. A dash indicates no measurable specific T-cell reactivity and memory recall mix (MRM) was used as a positive control. [e] The total number of donors responding per HPV type are depicted.

The patterns of HPVE6-specific responses as collected in Table 1 were used to reconstruct (with reasonable probability) the HPV clade A9 infection history for each of the donors. This was possible for 8 of 19 subjects (Table 1): 4 donors (6, 8, 11 and 17) whose responses are limited to E6 of a single HPV type, and 4 donors (7, 19, 20 and 27) who responded against non-matching E6 peptide pools from 2 different HPV types. For instance, donor 7 responds to peptide pool 4 of HPV16 and peptide pools 2 and 3 of HPV58, arguing that this donor has encountered both HPV types and that the resulting E6-specific T-cell responses are not cross-reactive. In the remaining 11 cases, coinciding responses against corresponding peptide pools from different HPV types caused ambiguity with respect to the HPV types encountered. For instance, the reactivity by donor 4 against peptide pool 3 of HPV 31 and 52 could reflect encounter of either one or both of these viruses. Our data demonstrate that ELISPOT data shown in Table 1 cannot be readily used for retrospective evaluation of the infection history of subjects.

HPV clade A9 cross-reactivity in cultures enriched for HPV16E6-specific T cells

We conducted a more detailed analysis with cultures enriched for HPV16E6-specific T cells by means of magnetic cell sorting (MACS). The resulting 14 cultures (from donors 31-44) contained 2.3% (range 0.4-6.9%) HPV16E6 specific T cells before and 45.6% (range 16.5-65.3%) HPV16E6 specific T cells after MACS, representing a 9-60 fold enrichment. The first 10 enriched cultures were analyzed for immune responses against the different E6 antigens by IFNγELISPOT. The use of single peptides, rather than peptide pools, increased the resolution of the analysis. As expected on basis of enrichment, all cultures displayed a HPV16 specific immune response against one of more peptides. Importantly, 8 of 10 cultures also responded against one or more non-HPV16 peptides (Table 2), which supports the notion that coincidence of T-cell responses against related HPV types is a frequent event. In total, 34 HPV16E6 peptide-specific responses were detected. Because we have used

Table 2. Screening of HPV clade A9 cross reactive immune responses in HPV16E6 enriched T cell cultures by Elispot

	HPV16 Elispot			Potential cross-reactivity				
Donor[a]	E6 peptide		response	HPV31	HPV33	HPV35	HPV52	HPV58
31	37-68[b]	}	**33**[c]	-	-	-	-	-
	55-86	}	**32**	-	-	**62**	-	-
	73-104	} [d]	**359**	-	**33**	-	**142**	**46**
	109-140	}	**38**	-	-	-	-	-
	127-158	} •	**63**	-	-	-	-	-
32	55-86	}	**158**	-	-	-	-	**168**
	73-104	}	**209**	-	-	-	**264**	-

	HPV16 Elispot			Potential cross-reactivity				
Donor[a]	E6 peptide		response	HPV31	HPV33	HPV35	HPV52	HPV58
33	73-104	}	**151**	-	-	-	-	**91**
	109-140	}	**117**	-	-	-	-	-
	127-158	} ●	**291**	-	-	-	-	-
34	37-68	}	**235**	-	-	-	-	-
	55-86	}	**239**	-	-	**175**	-	-
	73-104	}	**225**	-	-	-	-	-
	109-140	}	**140**	-	-	-	-	-
	127-158	} ●	**329**	-	-	-	-	-
35	73-104	}	**160**	-	-	-	-	**84**
	91-122	}	**130**	-	-	-	-	-
	109-140	} ●	**66**	-	-	-	-	-
36	37-68	●	**120**	-	-	-	-	-
	109-140	}	**122**	-	-	-	-	-
	127-158	} ●	**290**	-	-	-	-	-
37	37-68	}	**17**	-	-	-	-	-
	55-86	}	**42**	-	-	**19**	-	-
	73-104	}	**17**	-	-	-	-	-
	109-140	}	**21**	-	-	-	-	-
	127-158	}	**69**	**32**	-	**41**	-	-
38	55-86	}	**169**	-	-	**10**	-	-
	73-104	}	**65**	-	-	-	-	-
	109-140	}	**511**	-	-	-	-	-
	127-158	} ●	**354**	-	-	-	-	-
39	127-158		**215**	-	-	**226**	-	-
40	55-86	} ●	**111**	-	-	-	-	-
	73-104	}	**160**	-	-	-	-	-
	91-122	} ●	**197**	-	-	-	-	-

[a] Enriched HPV16E6 specific T cell cultures of 10 healthy donors evaluated by IFNγ ELISPOT for HPV clade A9 cross reactive immune responses. [b] All peptides (32 mer) of the E6 protein of HPV16, 31, 33, 35, 52 and 58 were tested (single peptide, no pool). Only peptides showing a response are depicted, indicated by the first and last amino acid. [c] Specific spots were calculated by subtracting the mean number of spots + 2xSD of the background from the mean number of spots in experimental wells. The number of spots per 100.000 PBMC are given. Antigen-specific T cell responses were considered to be positive when T cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x background [van der Burg SH 2001]. These values are indicated in **bold**. All donors show a HPV16E6 specific response. In 8 out of 10 donors, this response is matched by a response to one or more of the other clade members. [d] Brackets are placed over potentially overlapping epitopes and HPV16E6 specific responses not matched by reactivity against matching E6 peptides of the other clade members are indicated with a dot (●).

overlapping peptides, it is conceivable that many of these responses are directed against the same (overlapping) epitope (Table 2). In view of the latter, we can discern at least 21 distinct responses. Of these, 43% (9 of 21) are not paralleled by a response against a corresponding peptide of one of the other clade A9 members tested, strengthening the notion that these responses were primed by encounter of HPV16 (Table 2). In the other 57% cases (12 of 21), reactivity against a corresponding peptide from another HPV type was detected. In all but 2 cases (donor 31, peptide 73-104; donor 37, peptide 127-158), reactivity was limited to the corresponding peptides of HPV16 and one other clade A9 member. This indicates that, if these coinciding responses are due to cross-reactivity between HPV types, this cross-reactivity is not broad (see also Table 1).

To assess whether the coincidence of T-cell responses against E6 of different HPV types reflects the reactivity of highly potent T cells, we measured the response of the 4 remaining enriched T-cell cultures in the presence of peptide arrays as well as full-length E6 proteins. Our prior studies have shown that the repertoire of antigen-specific T cells responding to peptide-pulsed antigen-presenting cells (APCs) is generally larger than that responding to protein-pulsed APCs. We use this comparison to distinguish between highly potent T cells, capable of responding against limiting quantities of naturally processed antigen, and T cells that require greater quantities of cognate antigen [de Jong 2002a; van der Burg 2001; van der Burg 2007]. Although T cells that are capable of responding against peptide-pulsed (but not protein-pulsed) APCs may reflect physiologically relevant responses, these are less likely to play a prominent role in the immune defense in case antigen presentation is suboptimal, due to either limitations in quantity or differences in antigen structure. Analysis of the responses by ICS allowed more accurate comparison of the T-cell fractions responding to protein and peptide antigens and, in addition, discrimination between CD4+ and CD8+ T-cell responders.

In accordance with our prior studies [de Jong 2002a; Welters 2003; Welters 2008], the latter discrimination showed that all IFNγ producing T cells were detected in the CD4+ T-cell gate, confirming that our methodology using long peptide or protein pulsed APCs is geared towards detection of CD4+ T-cell responses. All 4 T-cell cultures responded not only to HPV16E6 peptide but also to HPV16E6 protein (Figure 2), arguing that at least a major fraction of the HPV16E6 peptide-reactive T cells reflect a potent immune response to naturally processed antigen. As expected on basis of the data in Tables 1 and 2, the 4 cultures also responded to E6 peptides of related HPV types. However, only one of the enriched T-cell cultures responded to the matching E6 proteins (Figure 2B), which suggests that this culture represents a T-cell response capable of reacting against HPV types 16, 31 and 35 with similar efficiency. On basis of this criterion, the HPV16-enriched cultures of the other three donors, which display strong reactivity towards HPV16E6 protein only, represent T-cell responses that will readily react when encountering HPV16, but much less

efficient against the closely related HPV types. Our findings indicate that cross-reactivity of HPV16E6-specific T cells to one or two other HPV types, even though commonly detected in peptide pulsed cultures, occurs less frequent in the presence of limiting quantities of naturally processed peptide-epitopes such as achieved when the HPV proteins are used to stimulate the T cells.

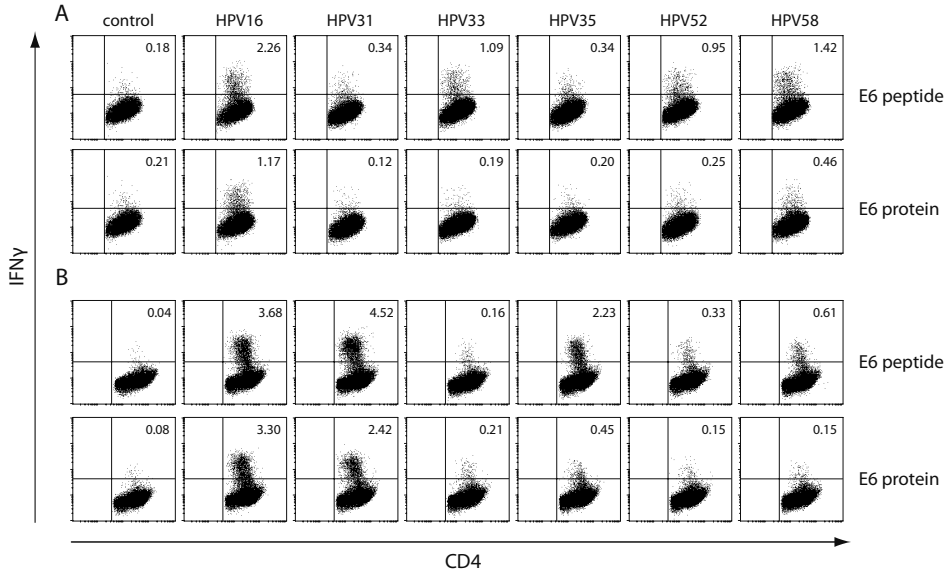

Figure 2. Human papillomavirus (HPV) clade A9 cross-reactivity in HPV16E6 enriched T-cell cultures. Peptide- and protein-specific recognition was analysed in 4 HPV16E6 enriched T-cell cultures (donors 41-44) by ICS. The percentage of IFNγ producing cells in the CD4+ fraction is depicted in the upper right corner. A positive response was defined as at least three times the percentage of IFNγ producing CD4+ T cells as in the medium control. Data shown are for donors 44 (A) and 42 (B).

T-cell cross-reactivity at the clonal level

The polyclonal T-cell cultures studied so far leave the possibility that reactivity against E6 antigens of more than one HPV type is due to distinct subpopulations of T cells within these cultures. We therefore examined the aspect of cross-reactivity by means of HPV specific T-cell clones isolated from the aforementioned enriched T-cell culture that was reactive to E6 of HPV types 16, 31 and 35 (donor 42; Figure 2B). Furthermore, we used shorter overlapping 10-mer peptides to minimize the possibility that reactivity would be due to distinct, partially overlapping epitopes encompassed by a single peptide.

In total, 73 different HPV16 peptide-reactive clones were isolated by limiting dilution, 14 of which were responsive to both HPV16 peptide and protein. In view of the likelihood to find cross-reactivity against proteins of other HPV types, we focused our further analyses on the latter 14 clones, which were able to recognize antigen when present at limiting quantity. Clonality was confirmed by V-beta staining (data not shown). Clone 105 (Figure 3A) is an example of 1 of 7 clones that exclusively responded against peptide and protein of HPV16. Clone 20 (Figure 3A) is 1 of 6 clones that responded to HPV16 (peptide and protein) as well as to other clade A9 peptides, but not to the other clade A9 proteins. Only one of the clones isolated (Figure 3A, Clone 8) responded to E6 of HPV types 16, 31 and 35 at both the peptide and protein level which suggests that efficient cross-reactivity is not only a rare event at the population level but also at the clonal level within the T-cell response of a single individual.

Additional analysis of clone 8 with major histocompatibility complex class II-blocking antibodies revealed that this response was restricted by HLA-DP (data not shown). Fine mapping of the HPV16 specific immune response by means of HPV16E6 10-mer peptides identified peptide LKFYSKISEY as the minimal epitope recognized by the T-cell clone (Figure 3B). This epitope sequence is largely conserved between the three E6 proteins recognized, whereas the non-recognized E6 proteins share a prominent tyrosine to leucine substitution at position 77 (Figure 3C).

HPV clade A9 cross-reactivity in HPV16 E6/E7-vaccinated patients

A clear disadvantage of charting hrHPV-specific T-cell responses in healthy donors is that the HPV infection history of these subjects is unknown. As illustrated in Table 1, this makes it difficult to causally link detection of memory T-cell responses to encounter of specific HPV types. Our laboratory recently performed a phase II therapeutic vaccination study in which patients who received a diagnosis of HPV16+ vulvar intraepithelial neoplasia grade 3 (VIN-III) received 3-4 consecutive doses of a synthetic peptide vaccine encompassing the complete amino acid sequences of HPV16E6 and E7 [Kenter 2009]. This study offered the unique opportunity to monitor the CD4+ T-cell reactivity against the E6 antigen of different clade A9 members in subjects known to have encountered both HPV16 and multiple doses of the HPV16E6 antigen. Before vaccination, none of the patients displayed IFNγ responses against E6/E7 peptides of HPV16, in accordance with failure of this immune response as documented in our prior studies [de Jong 2004; Welters 2003]. In contrast, the majority of the patients displayed HPV16-specific T-cell responses, predominantly reacting against E6, after the last vaccination [Kenter 2009].

For 10 of these vaccinated patients, who displayed strong HPV16-specific reactivity after vaccination, we charted T-cell reactivity against E6 peptide pools from the different clade A9 members (Table 3). In 5 patients, the vaccine-induced response against HPV16E6 was accompanied by reactivity to E6 peptides of one or more other clade A9 members (e.g. patient 6; Figure 4). For the other 5 patients, the post-vaccination E6-specific response was restricted to HPV16 (e.g. patient 6; Figure 4). Interestingly,

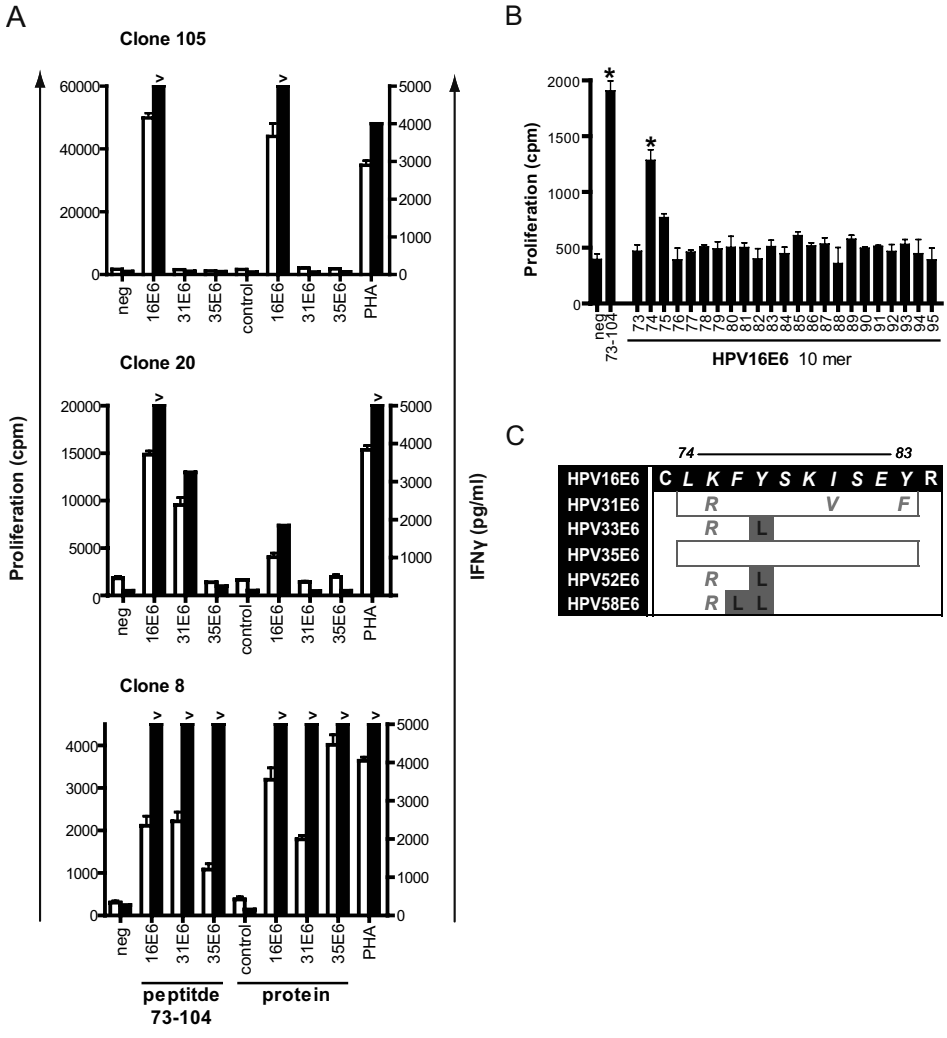

Figure 3. Clade A9 cross-reactivity in human papillomavirus (HPV) 16E6 specific T-cell clones. A. T-cell clones (n = 73) isolated by limiting dilution from the enriched HPV16, 31 and 35-reactive T-cell culture of donor 42 (Figure 2) were tested for their capacity to proliferate upon stimulation with monocytes pulsed with peptide (5 µg/ml) or protein (10 µg/ml). Phytohemagglutinine (PHA) served as a positive control. Proliferation was measured by [^{3}H] thymidine incorporation and was defined specific if stimulation index (SI) ≥ 3 (open bars). Supernatants were analysed for IFNγ by ELISA (black bars). Clones representative of three reactivity patterns found are shown. B. Fine mapping of the HPV16E6-specific immune response of clone 8 by means of HPV16E6 10-mer peptides in a 3-day proliferation assay. Specific responses (SI ≥ 3) are marked with an asterisk. C. The identified minimal epitope (74-83, LKFYSKISEY) is largely conserved between the three clade A9 types recognized by this T-cell clone (HPV16, 31 and 35). Depicted are the minimal epitope ( ), amino acids with similar properties ( ) and amino acid mismatches ( ).

Table 3. HPV clade A9 specific CD4+ Th1 responses in HPV16+ VIN patients before and after HPV16 peptide vaccination

Patient[a]		HPV16E6		HPV31E6		HPV33E6		HPV35E6		HPV52E6		HPV58E6		
	Vaccination	1-86[b]	73-158	1-86	73-156	1-86	73-156	1-86	73-156	1-86	73-155	1-86	73-156	MRM
01	pre	-	-	-	**11**	-	**11**	**10**	**10**	-	-	**15**	**13**	**73**
	post	**90**[c]	**167**	-	**120**	**39**	**116**	**87**	**121**	**69**	**65**	**66**	**89**	**78**
02	pre	-	-	-	-	-	-	-	-	-	-	-	-	-
	post	*30*	-	-	-	-	-	-	-	-	-	-	*10*	-
03	pre	-	-	-	-	-	-	-	-	-	-	-	-	**26**
	post	*53*	**50**	-	-	-	-	-	-	-	-	-	**10**	-
04	pre	-	-	-	-	-	-	-	-	-	-	-	-	-
	post	**84**	**42**	**13**	-	-	**19**	-	**24**	-	-	-	-	-
06	pre	-	-	-	-	-	-	-	-	-	-	-	-	-
	post	*62*	*128*	-	-	-	-	-	-	-	-	-	-	-
07	pre	-	-	*11*	-	-	-	-	-	-	-	-	-	**15**
	post	*82*	**74**	-	-	-	**13**	-	-	-	**14**	-	**26**	**60**
08	pre	-	-	-	-	-	-	-	-	-	-	-	-	-
	post	**73**	*51*	-	-	-	-	-	-	-	-	**10**	-	**17**
13	pre	-	-	-	**10**	-	-	-	-	-	**15**	-	**11**	**13**
	post	*37*	*23*	-	-	-	-	-	-	-	-	-	-	-
16	pre	-	-	-	-	-	-	-	-	-	-	-	-	-
	post	*79*	-	-	-	-	-	-	-	-	-	-	-	-
30	pre	-	-	-	-	-	-	-	-	-	-	*20*	-	**24**
	post	*79*	*37*	-	-	-	-	-	-	-	-	-	-	-

[a] Clade A9 cross reactive T cell responses evaluated by IFNγ ELISPOT in PBMC from 10 HPV16+ VIN patients before and after HPV16 E6/E7 peptide vaccination. [b] Indicated are the first and last amino acid in the peptide pool used. [c] Specific spots were calculated by subtracting the mean number of spots + 2xSD of the medium control from the mean number of spots in experimental wells. The number of spots per 100.000 PBMC are given. Antigen-specific T cell responses were considered to be positive when T cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x background [van der Burg SH 2001]. These values are indicated: coinciding responses against corresponding E6 peptide pools are depicted in **bold** (possible crossreactivity) and responses not matched by coinciding reactivity against corresponding peptide pools are depicted in *italics*. A dash indicates no measurable specific T-cell reactivity and memory recall mix (MRM) was used as a positive control.

the incidence by which primary PBMC cultures display reactivity against E6 peptides of both HPV16 and one or more other clade A9 members is very similar for the HPV16-vaccinated VIN patients (5 [50%] of 10) and the healthy donors with unknown HPV infection history (5 [42%] of 12; Table 1). In 4 patients (patients 1, 7, 13, 30), modest pre-vaccination responses were detected against E6 of HPV 31, 33, 35, 52 and/or 58, but not against HPV16. Notably, the detection of these pre-vaccination responses was not correlated with post-vaccination responses (Table 3). Taken together, these data show that HPV16 vaccination of patients can induce IFNγ-responses against E6 of HPV16 and related clade A9 members independent of pre-existing immunity, and that the detection of post-vaccination IFNγ responses against E6 of the related clade A9 members varies greatly between patients.

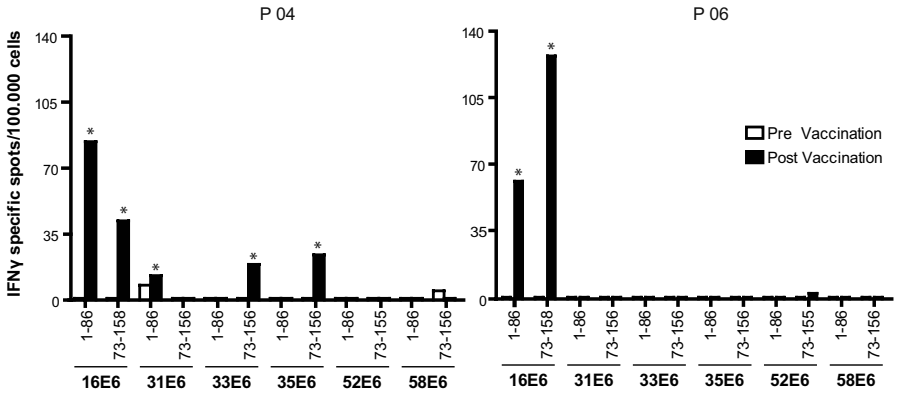

Figure 4. Human papillomavirus (HPV) clade A9 immune responses in HPV16E6/E7 vaccinated patients with vulvar intraepithelial neoplasia (VIN). Peripheral blood mononuclear cell (PBMC) samples of HPV16-positive patients with VIN who participated in an HPV16E6/E7 vaccination study were isolated before the first and after the fourth vaccination. IFNγELISPOT analysis was performed against peptide pools specified. Responses were considered positive when T-cell frequencies were ≥ $1/10^4$ PBMC and at least ≥ 2x medium control value (marked with an asterisk). All patients tested (n = 10) displayed strong HPV16E6-specific reactivity after vaccination (Table 3). In half of the patients, this vaccine-induced response was also accompanied by reactivity to E6 peptides of one or more other clade A9 members (patient 4 is shown as a representative example). For the other five patients, the post-vaccination response was restricted to HPV16E6 only (patient 6 is shown as a representative example).

Discussion

Charting of the CD4+ memory T-cell responses in healthy subjects against the E6 antigen of HPV16 and 5 closely related hrHPV types by means of overlapping peptide arrays revealed that responders frequently display reactivity against corresponding E6 peptides from two HPV types. This initially suggested immunological cross-reactivity between the E6 antigens. However, further dissection of these responses by means of HPV16-enriched and clonal T-cell cultures, as well as by using whole protein antigens,

indicated that CD4+ T cells capable of reacting efficiently with limiting quantities of naturally processed E6 antigens from two or more HPV types are only rarely observed. This finding implies that natural CD4+ T-cell immunity elicited through encounter of a given hrHPV type is unlikely to provide efficient cross-protection against closely related hrHPV types. In accordance with our data, analysis of sequential acquisition of HPV types indicated that risk of acquiring a new HPV type was not decreased among those with prior infection by a related type [Thomas 2000; Woo 2009

Our findings furthermore demonstrate that detection of memory CD4+ T-cell responses against a given HPV type cannot be readily used as an indicator that the subject concerned has encountered this particular HPV type. For 11 of 19 HPV-specific responders tested, the detection of responses against corresponding E6 peptides from two or more HPV types resulted in ambiguity with respect to number and identity of HPV types encountered (Table 1). In 6 cases (donors 13, 15, 21, 25, 26, 28), HPV16 was among the HPV types recognized. In view of the highest prevalence of HPV16, these 'overlapping' responses are most likely primed through encounter of HPV16. This notion is supported by our data showing that about half of the T-cell clones reacting to peptides of HPV16 and other clade A9 members respond to HPV16-derived E6 protein only. Our data also imply that detection of CD4+ memory T-cell responses by means of peptide arrays cannot readily be used for determining the prevalence of HPV types. This is expected to result in an overestimation of the prevalence of HPV types closely related to HPV16. For instance, we found HPV58 E6-specific T-cell reactivity in 9 (30%) of 30 subjects (Table 1), a frequency approximating that found for HPV16 (12 (40%) of 30 subjects). However, HPV58 prevalence on basis of DNA testing was found to be 5-6 fold lower than that of HPV16 [Clifford 2005].

With respect to our methodology of analysis, the following can be noted. First, HPV types included in our screening share more sequence homology with each other than with any of the other types. Therefore, we deem it unlikely that cross-reactivity against HPV types excluded from our study would play a significant role. Accordingly, we did not find indications for immunological cross-reactivity between E6 of HPV16 and 18 [Welters 2006]. Second, it is conceivable that charting of HPV-specific immunity with single and/or shorter peptides, instead of pools comprising two 32-mer peptides, would result in fewer coinciding responses, and will thereby allow for greater resolution with respect to the infection history of subjects concerned. Notably, the larger numbers of PBMC required for this fine mapping can generally not be obtained in the context of cohort studies.

The availability of PBMCs from HPV16E6/E7 vaccinated patients allowed analysis of the E6-specific reactivity in subjects known to have encountered the HPV16E6 antigen. In addition to the vaccine-induced HPV16E6-specific T-cell responses, these patients showed post-vaccination reactivity to E6 peptides from the other clade A9 members. Importantly, the overall frequency and breadth of these additional responses were not greater than those detected alongside naturally induced HPV16E6-specific responses

in healthy subjects. Our findings in vaccinated patients strengthen the notion that the majority of coinciding responses against corresponding E6 peptides of HPV16 and one or more other clade A9 members as found in healthy subjects have been induced by HPV16 encounter, and do not bear witness to encounter of the less prevalent HPV types. Consequently, screening for CD4+ memory T-cell responses with peptide arrays against, for example, HPV31 or HPV58 in the absence of parallel screening with a HPV16 peptide array could readily lead to misinterpretation of the resulting data. Furthermore, the resemblance between the clade A9 response pattern in healthy controls and HPV16 vaccinated patients suggests that the vaccine-induced CD4+ T-cell responses are unlikely to have therapeutic impact against neoplastic lesions positive for HPV types other than HPV16. More definitive information on the cross-reactivity of the vaccine-induced responses would require measurements of reactivity against full-length antigens. Unfortunately, insufficient PBMCs were available from the vaccinated patients to perform analysis on enriched T-cell cultures, while primary PBMC cultures are not suitable for measuring specific T-cell responses against recombinant proteins due to the high background levels associated with the particular experimental setting [Britten 2008]. We were therefore not able to assess whether responses against the non-HPV16E6 peptides as recorded in the HPV16E6/E7 vaccinated patients reflect functional, protective immunity against clade A9 members other than HPV16. However, based on our current data on T-cell responses from healthy subjects we deem this unlikely.

In conclusion, in spite of considerable sequence homology between the E6 antigens of clade A9 hrHPV types, E6-specific CD4+ T cells induced by HPV16 generally do not efficiently cross-react against the corresponding sequences of closely related hrHPV types. In view of our findings, and the similar, somewhat lower degree of sequence conservation between E7 proteins, the same is to be expected for E7-specific CD4+ T-cell responses. Therefore, the therapeutic efficacy of vaccines comprising E6 and E7 antigens of a given hrHPV type is likely to be limited to anogenital lesions positive for the specific hrHPV type concerned.