Phase I Immunotherapeutic Trial with Long Peptides Spanning the E6 and E7 Sequences of High-Risk Human Papillomavirus 16 in End-Stage Cervical Cancer Patients Shows Low Toxicity and Robust Immunogenicity

Gemma G. Kenter,[1] Marij J.P. Welters,[2] A. Rob P.M. Valentijn,[3] Margriet J.G. Löwik,[1] Dorien M.A. Berends-van der Meer,[1] Annelies P.G. Vloon,[2] Jan W. Drijfhout,[2] Amon R. Wafelman,[3] Jaap Oostendorp,[3] Gert Jan Fleuren,[4] Rienk Offringa,[2] Sjoerd H. van der Burg,[5] and Cornelis J.M. Melief[2]

Abstract

Purpose: To determine the toxicity, safety, and immunogenicity of a human papillomavirus 16 (HPV16) E6 and E7 long peptide vaccine administered to end-stage cervical cancer patients.

Experimental Design: Three groups of end-stage cervical cancer patients (in total $n = 35$) were s.c. vaccinated with HPV16 E6 combined with or separated from HPV16 E7 overlapping long peptides in Montanide ISA-51 adjuvant, four times at 3-week intervals. Group 1 received 300 μg/peptide at a single site and group 2 received 100 μg/peptide of the E6 peptides in one limb and 300 μg/peptide of the E7 peptides in a second limb. Group 3 received separate injections of E6 and E7 peptides, each at a dose of 50 μg/peptide. The primary end point was to determine safety and toxicity of the HPV16 long peptides vaccine. In addition, the vaccine-induced T-cell response was assessed by IFNγ enzyme-linked immunospot.

Results: No toxicity beyond grade 2 was observed during and after four vaccinations. In a few patients, transient flu-like symptoms were observed. Enzyme-linked immunospot analysis of the vaccine-induced immune response revealed that coinjection of the E6 and E7 peptides resulted in a strong and broad T-cell response dominated by immunity against E6. Injection of the E6 and E7 peptides at two different sites increased the E7 response but did not affect the magnitude of the E6-induced immune response.

Conclusions: The HPV16 E6 and E7 long peptide-based vaccine is well tolerated and capable of inducing a broad IFNγ-associated T-cell response even in end-stage cervical cancer patients.

Close to 100% cervical cancers are caused by persistent infection with high-risk human papillomaviruses (HPV; ref. 1). Of the different high-risk HPV types that can cause cervical cancer (2), HPV16 alone is responsible worldwide for more than half of all cases of cervical cancer (1, 2). Genital infection with high-risk HPV is very common and normally cleared within 1 year. However, in a minority (~1%) of the infected individuals, the HPV persists, ultimately resulting in genital neoplastic lesions.

Currently, a preventive HPV vaccine is on the market, which effectively prevents this persistent infection and associated disease by the induction of neutralizing antibodies against the envelope proteins of HPV16 and HPV18, the HPV type second on the list of most frequent HPV types associated with cancer. Although these vaccines are very efficient in the prevention of persistent infection by HPV16 and HPV18 (3–8) and have potential for prevention of cervical intraepithelial neoplasia (9–11), there is no evidence for efficacy against established HPV16 and HPV18 genital lesions (3–11).

It is generally accepted that virus-infected cells can only be effectively dealt with by cell-mediated adaptive T-cell immunity. Consequently, immunotherapy capable of inducing robust HPV16-specific T-cell immunity is highly desirable for eradication of established HPV16 infection and diseases caused thereby, such as cervical intraepithelial neoplasia, vulvar intraepithelial neoplasia, cervical cancer, other anogenital lesions, and a high percentage of head and neck cancers. In animal models, we have shown that immunotherapy with long peptide vaccines consisting of HPV16 E7 peptides was capable of eradicating established HPV16$^+$ tumors in mice (12), whereas a cottontail rabbit papillomavirus (CRPV)

Authors' Affiliations: Departments of [1]Gynaecology, [2]Immunohematology and Blood Transfusion, [3]Clinical Pharmacy and Toxicology, [4]Pathology, and [5]Clinical Oncology, Leiden University Medical Center, Leiden, the Netherlands

Received 7/31/07; revised 9/18/07; accepted 10/5/07.

Grant support: Dutch Cancer Society grant RUL 2000-2200 (G.G. Kenter, R. Offringa, and C.J.M. Melief) and ISA Pharmaceuticals B.V., Bilthoven, the Netherlands.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: G.G. Kenter and M.J.P. Welters contributed equally to this work.

Requests for reprints: C.J.M. Melief, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, P. O. Box 9600, 2300 RC Leiden, the Netherlands. Phone: 31-71-5263133; Fax: 31-71-5265267; E-mail: C.Melief@lumc.nl.

doi:10.1158/1078-0432.CCR-07-1881

Table 1. Enrollment of patients in phase I study with long peptide HPV16 E6 and E7

Study	ID	Age	FIGO stage	Primary treatment	Recur	Secondary treatment	Vaccination (*n*)	Clinical status	Follow-up (mo)
1	1	53	IB	RH	LR	CHRT	4	Died	9
	2	47	IB1	RH/RT	D	RT	2	Died	3
	3	33	IIB	RT/HT	D	CH/HT	4	Died	9
	4	42	IB2	RH	D		0	Died	1
	5	59	IV	CH	—		0	—	—
	6	53	III	CHRT	D		4	Died	11
	7	44	IB1	RH	D		1	Died	1
	8	32	—	—	—		2	Lost to follow-up	1
	9	41	IB2	RH	LR	CH/HT/SUR	4	Died	7
	10	72	IIA	RT/HT	LR	SUR	4	Died	4
	11	48	—	—	—		3	Died	3
	12	58	III	RT	D	CH	4	Died	6
	13	56	IB1	RH/RT	LR	SUR	3	Died	4
2	14	58	IIB	CHRT	LR	CH	5	Alive, CR	36
	15	35	—	—	D		3	Died	2
	16	36	IB1	RH/RT	LR	CHRT	4	Died	4
	17	48	IB2	CH/RH	LR	RT/HT/SUR	4	Died	9
	18	44	—	CHRT	—		4	Died	4
	19		—	—	—		0	—	—
	20	39	—	—	—	CH/RT/HT	4	Died	2
	21	48	IIA	CHRT	LR	CH	2	Died	2
	22	34	IB1	RH	LR	CH	4	Died	3
	23	35	IIIB	CH/RT/HT	D	CHRT	2	Died	3
	24	44	IV	CH	—		3	Died	2
	25	44	IA2	RH	D	CH	0	Died	0
	26	44	IA	RH	LR	RT	4	Died	2
	27	60	IB1	RH	D	CH	4	Alive, stable	26
	28	39	—	RH	D	CHRT	3	Lost to follow-up	2
	29	56	IV	CH	LR	RT	4	Died	2
	30	48	IB1	RH/RT	D	RT	4	Alive, stable	24
	31	51	IB	RH	LR	CHRT	4	Died	3
	32	57	IB	RH/RT	D	RT	4	Alive, stable	24
3	33	33	IB2	RH/RT	LR	RT	1	Died	1
	34	58	IIA	RH/RT	LR	RT/HT	4	Died	3
	35	30	IIA	CH	LR	CH	1	Withdrawn	0
	36	48	IB1	RH/RT	LR		2	Died	8
	37	54	IIB	CH/RH	LR	CHRT	4	Died	3
	38	42	IIIB	CHRT	LR	CH	4	Died	7
	39	41	IB1	RH	LR	RT/HT	4	Died	17
	40	34	IIB	CH/RH	LR	RT/HT	4	Died	4
	41	32	IB1	RH	LR	SUR	4	Alive, stable	18
	42	42	IB2	CH/RH	LR	RT/SUR	4	Alive, stable	18
	43	29	IB1	RH/RT	LR	CH	4	Died	5
	44	43	IB2	RH/RT	LR	CH	4	Died	7
	45		—	—	—	CH	0	—	—
	46	49	—	CHRT	—	CH	4	Died	2
	47	36	IIA	RH/RT	LR	CH/HT/SUR	4	Died	7
	48	34	IIIB	RH/RT	D	RT	4	Died	8

NOTE: Numbers 1 to 13 were vaccinated with 300 μg E6 and E7 each in a combined vaccination (patient group 1), numbers 14 to 32 were vaccinated with 100 μg E6 and 300 μg E7 in a separate vaccine (group 2), and numbers 33 to 48 were vaccinated with 50 μg E6 and E7 each in a separate vaccine (group 3). The stage of the disease is given as the International Federation of Gynecologists and Obstetricians stage. The primary treatment, the localization of the recurrence, the secondary treatment, and the number of vaccinations in the current immunotherapeutic trial are shown. The follow-up time is depicted in months after the first vaccination; however, some of the patients were lost to follow-up after receiving one or more vaccinations. All death was because of progressive disease. From the six patients still alive at least one and half year after the vaccinations (i.e., July 2007), patient 14 is a complete responder. Patients 27, 30, 32, and 41 have received chemotherapy after the vaccinations and have stable disease. Patient 42 received radiotherapy after the immunotherapy and also has stable disease.

Abbreviations: LR, locoregional; D, distant metastasis; RH, radical hysterectomy; RT, radiotherapy; CH, chemotherapy; CHRT, chemoradiation; HT, hyperthermia; SUR, surgery; FIGO, International Federation of Gynecologists and Obstetricians; CR, complete responder.

E6/E7 long peptide vaccine suppressed CRPV-induced skin papillomas in rabbits (13), which was associated with robust type 1 T-cell immunity. Moreover, in the course of our studies looking at the natural history of immunity to HPV16 in healthy blood bank donors and in patients with HPV16-induced diseases, we have noted that a robust virus-specific T-cell response, characterized by IFNγ production, against the early proteins E2 and E6 of HPV16, is associated with protection against persistent infection and disease (14–18). This led to the hypothesis that immunotherapy with long

overlapping peptides of the E6 and E7 oncoproteins of HPV16 might induce a type 1–polarized antiviral T-cell response associated with therapeutic effects.

In a first step toward such immunotherapeutic vaccination, we treated patients with end-stage cervical cancer in a phase I toxicity study with a therapeutic vaccine consisting of the complete set of long overlapping peptides (25-35 amino acids long) of the oncogenic proteins E6 and E7 of HPV16.

Materials and Methods

Patients. Patients with histologically proven advanced or recurrent carcinoma of the lower female genital tract ($n = 43$) without options for further treatment were included in the study between May 2003 and September 2006 after oral and written informed consent. Eligibility also required the following criteria: (*a*) performance status of WHO 1 to 2 and/or Karnofsky score ≥60; (*b*) pretreatment laboratory findings of leukocytes >3 × 10^9/L, lymphocytes >1 × 10^9/L, thrombocytes >100 × 10^9/L, and hematocrit >30%; (*c*) no radiotherapy, chemotherapy, or other potentially immunosuppressive therapy administered within 4 weeks before the immunotherapy; and (*d*) life expectancy of more than 3 months. The patient characteristics are summarized in Table 1. The study was approved by the medical ethical committee of the Leiden University Medical Center.

Vaccine and vaccination scheme. The vaccine consisted of 13 peptides together representing the entire sequence of the E6 and E7 proteins of HPV16 (Human Papillomavirus Compendium 1997, Los Alamos National Laboratory)[6] formulated in Montanide ISA-51 adjuvant and as such contained all potential T-cell epitopes, irrespective of the HLA type of the patient. The clinical-grade peptides (nine E6 and four E7 peptides of 25-35 amino acids long with an overlap of 10-14 amino acids) were prepared at the Leiden University Medical Center Interdepartmental Good Manufacturing Practice facility by the Department of Clinical Pharmacy and Toxicology. Synthesis was done using a CS Bio CS536 solid-phase peptide synthesizer (CS Bio) according to the Fmoc protocol. After purification by reversed-phase high-performance liquid chromatography, the peptides underwent quality control, including identity by Voyager DE-PRO matrix-assisted laser desorption/ionization time-of-flight (PE Biosystems) and quadrupole time-of-flight mass spectrometry (Micromass UK Ltd.) and Edman degradation. The peptides were also sequenced on a HP gas-phase sequencer (Hewlett-Packard) and stored as freeze-dried powder at -20°C until use. At the day of vaccination, the 13 peptides (0.3 mg/peptide) are dissolved in DMSO (final concentration is 20%) and admixed with 20 mmol/L phosphate buffer (pH 7.5) and Montanide ISA-51. The E6 vaccine consisted of nine overlapping HPV16 E6 peptides DMSO/20 mmol/L PBS/Montanide ISA-51 adjuvant (20:30:50, v/v/v) in a total volume of 0.9 mL and the E7 vaccine consisted of four overlapping HPV16 E7 peptides DMSO/20 mmol/L PBS/Montanide ISA-51 adjuvant (20:30:50, v/v/v) in a total volume of 1.5 mL. All vaccinations were administered s.c. four times at 3-week intervals (Fig. 1A).

The first group of patients (group 1, $n = 11$) has been vaccinated with a mix of E6 and E7 peptides at a dose of 300 μg/peptide in Montanide ISA-51. The second group of patients (group 2, $n = 17$) received E6 peptides at a dose of 100 μg/peptide in Montanide ISA-51 in one limb and E7 peptides at a dose of 300 μg/peptide in Montanide ISA-51 in the other limb. In the third group (group 3, $n = 15$), the vaccine was given at separate injection sites, similar to second group, but at a dose of 50 μg/peptide in Montanide ISA-51. Patients were accrued in each group until safety, tolerability, and the immune response were evaluated in at least five patients per group.

[6] http://hpv-web.lanl.gov

Safety and tolerability monitoring. Prompted and spontaneous adverse events, injection site reactions, clinical assessments, and clinical laboratory variables were monitored (Fig. 1A). Injection site reactions were defined as induration, erythema, and tenderness. Beside the medical history, the patients were thoroughly examined physically and hematologically before and after each vaccination. An electrocardiogram was made before and 4 h after vaccination. Further vital sign examination included weight, temperature, pulse, blood pressure, oxygen saturation, and respiratory frequency before and following 1 and 4 h after vaccine administration. Blood samples were drawn before and 1 to 3 weeks after the vaccination for the analysis of leukocyte, lymphocyte, erythrocyte, and thrombocyte counts; hematocrit; hemoglobin; prothrombin time; and partial thrombin time. Clinical chemistry analysis was done for alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, anorganic phosphate, total bilirubin, γ-glutamyl transpeptidase, sodium, potassium, creatinine, lactate dehydrogenase, and urea. During the vaccination, the women were admitted in day care and under observation in the hospital. Electrocardiogram, pulse and blood pressure, temperature, respiratory frequency, and oxygen saturation were monitored during the day. After finishing the vaccinations, the patients were seen at least every 3 months during the regular follow-up visits to the hospital. Only those patients who completed all four vaccinations were eligible for safety evaluation.

Lymphocytes. Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood samples using Ficoll density gradient according to the manufacturer. The PBMCs were directly thereafter cryopreserved using a controlled freezing machine and stored in the vapor phase of liquid nitrogen until use.

HPV-specific T-cell immunity monitoring. Analysis of T-cell responses using 4-day IFNγ enzyme-linked immunospot (ELISPOT) was conducted as described previously (19). The following peptide pools indicated by the amino acid sequence within the antigen were used at a 5 μg/mL concentration: E6.1: 1-22, 11-32, 21-42, and 31-52; E6.2: 41-62, 51-72, 61-82, and 71-92; E6.3: 81-102, 91-112, 101-122, and 111-132; E6.4: 111-132, 121-142, 131-152, and 137-158; E7.1: 1-22, 11-32, 21-42, and 31-52; and E7.2: 41-62, 51-72, 61-82, 71-92, and 77-98. As a positive control, PBMCs were cultured in the presence of a recall antigen mixture [memory response mix (MRM)] consisting of tetanus toxoid (0.75 LF/mL; National Institute of Public Health and Environment, Bilthoven, the Netherlands), *Mycobacterium tuberculosis* sonicate (5 μg/mL; generously donated by Dr. P. Klatser, Royal Tropical Institute, Amsterdam, the Netherlands), and *Candida albicans* (0.015%; HAL Allergenen Lab; ref. 20). The number of spots was analyzed with a fully automated computer-assisted video imaging analysis system (Biosys 4000). Specific spots were calculated by subtracting the mean number of spots of quadruplicate wells plus 2 × SD of the medium only control from the mean number of spots of experimental wells. Antigen-specific T-cell responses were considered to be positive when T-cell frequencies were ≥$1/10^4$ PBMCs (19). A vaccine-induced response was defined as a 3-fold increase in T-cell frequency after vaccination compared with the baseline sample (21, 22).

Identification of T-cell type producing IFNγ using intracellular cytokine staining. PBMCs were stimulated for 4 days with the peptide pools as described for the ELISPOT analysis (see above) before they were harvested and stained intracellularly for IFNγ, CD4, and CD8 as described previously (16). Briefly, autologous monocytes were loaded overnight with 5 μg/mL of the peptide pools of HPV16 E6 and E7 or 10 μg/mL of HPV16 E6 or E7 recombinant protein. After washing, the stimulated PBMCs were cocultured with these loaded monocytes for 1 h and another 5 h after the addition of 10 μg/mL brefeldin A (Sigma) to prevent cytokine secretion. Then, the cells were harvested and stained with IFNγ-FITC (clone 4S.B3; BD PharMingen), CD4-APC (clone RPA-T4; BD PharMingen), and CD8-PerCP (clone SK1; BD PharMingen). A positive response was defined as at least twice the percentage of IFNγ-producing $CD4^+$ or $CD8^+$ T cells as in the medium only control and the response should be visible as a clearly distinguishable population of IFNγ-producing cells separated from the nonproducing cells.

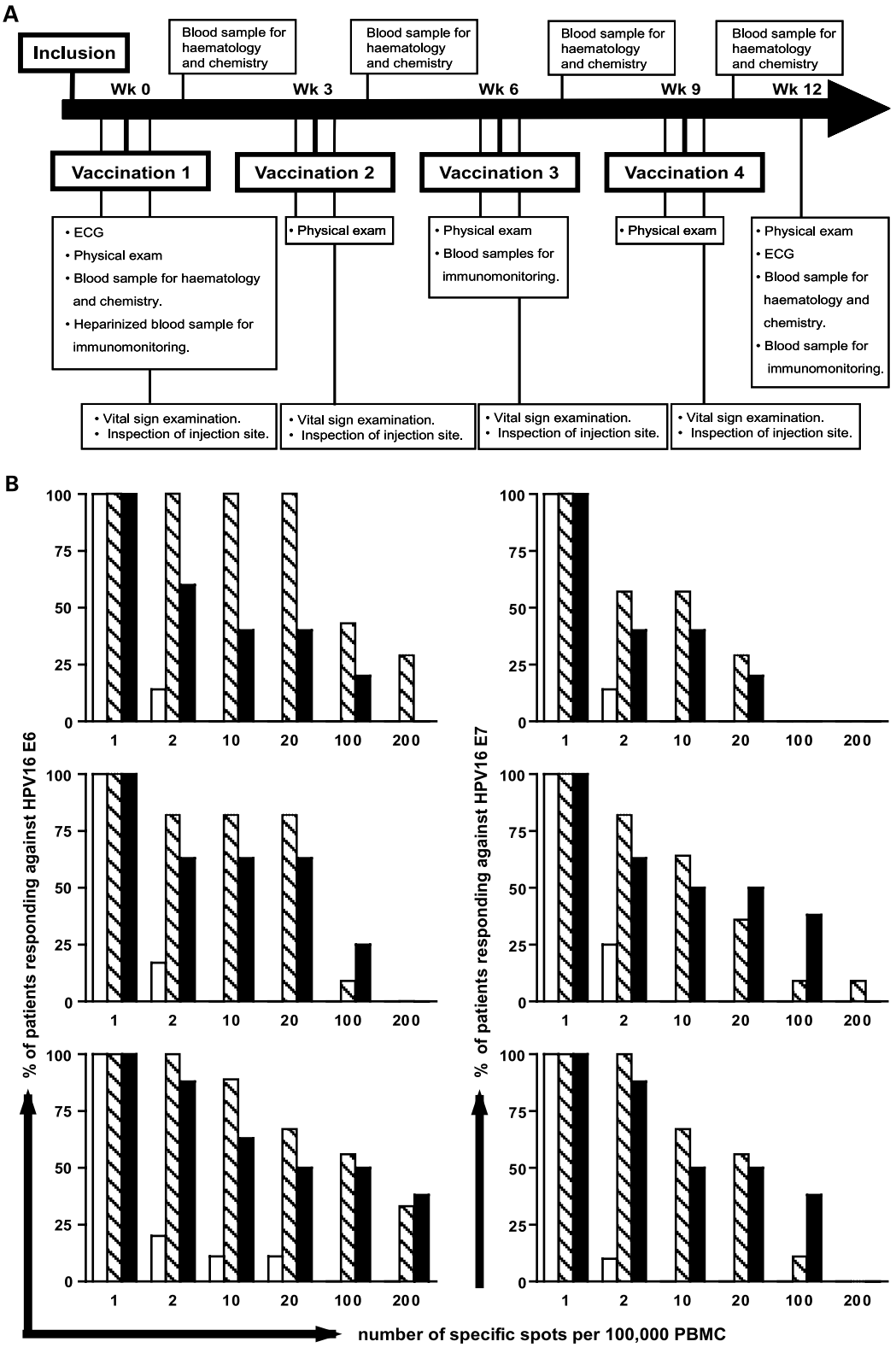

Fig. 1. Vaccination scheme and HPV16-specific T-cell responses. *A,* schematic overview of vaccination scheme. ECG, electrocardiogram. *B,* for each patient group (*top*, group 1; *middle*, group 2; *bottom*, group 3), the number of patients responding to the HPV16 antigens E6 and E7 as analyzed in PBMC before vaccination (*white columns*), after two vaccinations (*striped columns*), and after four vaccinations (*black columns*) is shown versus the number of specific spots scaled from 1 to 200 as determined by IFNγ ELISPOT. As described in Materials and Methods, a positive response is defined as at least 10 specific spots per 100,000 PBMCs. Thus, the observed T-cell response is stronger at the right side. Notably, the peak T-cell responses are used to determine the strength of the response.

Table 2. Adverse events after each vaccination in the three groups of patients receiving HPV16 peptide vaccination

Adverse events	1st vaccination				2nd vaccination				3rd vaccination				4th vaccination			
	p	r	sw	sys	p	r	sw	sys	p	r	sw	sys	p	r	sw	sys
Group 1: no. patients			11				10				8				6	
Grade 1	4	4	4	1	5	3	7	6	4	4	5	6	3	1	3	4
Grade 2		1	6	1			3				3				3	
Group 2: no. patients			15				15				13				10	
E6																
Grade 1	2	6	1	3	1	6	7	5	3	4	5		2	2	5	
Grade 2			6				2				2				1	
E7																
Grade 1	2	7	7		2	3	7		2	3	6		1	1	5	
Grade 2		1									1				1	
Group 3: no. patients			15				13				12				12	
E6																
Grade 1	6	7	7		5	5	7		2	5	6			5	5	
Grade 2			1				1									
E7																
Grade 1		4	3		2	3	5		3	3	1			2	4	
Grade 2		1														

NOTE: The numbers represent number of patients with adverse events.
Abbreviations: p, pain at vaccination site; r, redness at vaccination site; sw, swelling at vaccination site (scored as grade 2 when swelling >4 cm, although it was without necrosis); sys, systemic responses (fever, chills, odorous breath, itching, and malaise).

Statistical analysis. Differences in the overall magnitude of the vaccine-induced immunity (as determined by IFNγ ELISPOT) between the three patient groups were analyzed by comparing the mean number of specific spots for E6 or E7 immunity between the groups using nonparametric *t* test (unpaired, with Welch's correction). A *P* value of <0.05 was considered significant.

Results

Safety and tolerability. In total, 43 patients were included in the study (Table 1). Thirty of them completed all four vaccinations. An additional five patients received in total three vaccinations. Five patients received two vaccinations and three patients received only one vaccination. In the blood samples drawn before and after every vaccination, no significant differences were observed in the hematology and chemistry values due to the vaccination. Overall, the vaccine was well tolerated. All patients experienced the vaccination as mildly painful. The pain vanished within 10 to 15 min. The local pain was graded lower than grade 2 according to the common toxicity criteria. Vaccination was mostly accompanied by redness of the skin at the injection site, which was scored as grade 1 to 2 toxicity. Toxicity and side effects beyond grade 2 were not observed. All patient deaths occurred due to progressive disease. A summary of adverse events is shown in Table 2. Notably, the second, third, or fourth vaccination was associated with a painless swelling and sometimes redness of the previous injection sites in patient groups 1 and 2 but not in patients who received the lowest dose of the vaccine (patient group 3). Five patients experienced fever after one or more vaccinations. In these cases, body temperature did not exceed 40°C, was effectively treated with paracetamol, and was scored as grade 2 toxicity. Flu-like symptoms, such as fatigue, malaise, and chills (grade 1), were reported by seven patients and not necessarily after each vaccination.

Currently (July 2007), six patients are still alive 18 to 25 months after vaccination, one with a complete remission and five with stable disease (defined as persistence of the disease without progression; Table 1). Four of them (ID 27, 30, 32, and 41) were treated with chemotherapy after immunotherapy.

Immunogenicity of long peptides vaccine. PBMCs isolated from blood samples drawn before the first, before the third, and after the last vaccination were subjected to a single IFNγ ELISPOT analysis. In addition, patients who received only three vaccinations were included. In none of the patients in the three groups, circulating HPV16 E6- and E7-specific IFNγ-producing T cells were detectable before vaccination.

In the first study group (patient group 1), seven of the seven analyzed patients showed T-cell reactivity against HPV16 E6 and four against E7 after two vaccinations. After four vaccinations, three of six analyzed patients responded to HPV16 E6 and two of the six patients against E7. The decline in number of responding patients after four vaccinations was probably due to the fact that their last blood sample was taken too soon (i.e., 7 days) after vaccination, whereas the other blood samples were drawn 3 weeks after vaccination. Therefore, the blood samples of patients in groups 2 and 3 were drawn 3 weeks after the last vaccination. The frequencies ranged between one HPV-specific T-cell among 10,000 PBMCs up to 1 of 400 (see Table 3 for complete overview). In Fig. 1B, the percentage of patients with a vaccine-induced HPV16 E6-specific (left graphs) or E7-specific (right graphs) T-cell response was depicted for the three patient groups versus the strength of this antigen-specific immunity, as represented by the peak response to a peptide pool measured by the ELISPOT. In patient group 1, the response to E6 was more vigorous than against E7, both in number of patients responding and in the strength of response (Fig. 1B).

Table 3. IFNγ ELISPOT analysis of PBMC before and after two and four vaccinations of patients with long peptide HPV16 E6 and E7

Study	ID	Prevaccination							After two vaccinations							After three or four vaccinations						
		E6.1	E6.2	E6.3	E6.4	E7.1	E7.2	MRM	E6.1	E6.2	E6.3	E6.4	E7.1	E7.2	MRM	E6.1	E6.2	E6.3	E6.4	E7.1	E7.2	MRM
1	1	—	—	—	—	—	—	**28**	**169**	**183**	**62**	**55**	—	**13**	**35**	**57**	**48**	**24**	—	—	—	—
	3	4	8	—	—	4	—	—	**168**	**225**	**256**	**72**	**14**	5	3	**103**	**163**	**147**	**55**	3	**25**	2
	6	—	—	—	—	—	—	**10**	—	9	**41**	—	—	—	3	—	—	—	—	—	—	**12**
	9	—	—	—	—	—	—	4	—	**57**	**10**	—	—	—	4	NA						
	10	—	—	—	—	—	—	**11**	**10**	**29**	**124**	**104**	6	**24**	9	—	—	—	—	—	—	—
	12	—	—	—	—	—	5	9	—	**46**	**11**	—	—	—	4	—	—	—	—	—	—	—
	13	—	—	—	—	—	—	—	**155**	**210**	**121**	**72**	—	**37**	—	**22**	**41**	**26**	—	3	**12**	—
2	14	—	—	—	—	—	—	—	—	**37**	**93**	—	**21**	**73**	—	—	**16**	**48**	—	4	**46**	**75**
	17	7	7	6	6	—	3	**51**	**29**	**12**	**31**	3	—	—	**14**	**33**	**27**	4	3	—	9	**83**
	18	—	—	—	—	—	—	**242**	NE							—	—	—	—	—	—	**85**
	20	—	—	—	—	—	—	—	—	—	—	—	—	—	8	—	—	—	—	—	—	5
	22	—	—	—	—	—	—	3	2	**26**	4	6	4	—	5	6	**11**	4	1	2	**52**	3
	24	—	—	—	—	—	—	4	4	**176**	**43**	—	2	**55**	—	NA						
	26	—	—	—	—	—	—	**21**	7	**16**	**42**	—	—	**12**	—	**20**	**51**	**91**	—	—	**104**	**29**
	27	—	—	4	—	—	6	**152**	**74**	**82**	**70**	**21**	**61**	**261**	**86**	**48**	**172**	**53**	8	**75**	**318**	**103**
	28	—	—	—	—	—	2	**38**	**11**	**62**	3	—	—	9	6	NA						
	29	—	—	—	—	—	—	5	—	—	—	—	—	—	**14**	—	—	—	—	—	—	—
	30	—	—	—	—	—	—	**55**	6	**16**	**54**	—	6	**57**	**41**	**43**	**165**	**48**	**23**	**22**	**173**	**117**
	32	—	—	—	—	—	—	—	—	**31**	2	2	—	**11**	**11**	6	**123**	**19**	**19**	1	**120**	**70**
3	34	—	—	—	—	—	—	—	NE							—	—	—	—	—	—	—
	37	—	—	—	—	—	—	—	6	**20**	**13**	—	—	4	—	—	**18**	**14**	**14**	—	5	—
	38	—	5	6	—	3	3	**579**	**35**	**74**	**142**	4	—	**54**	**467**	NE						
	39	—	—	—	—	—	—	**37**	9	2	2	—	—	6	**46**	—	3	—	—	—	1	**138**
	40	NE							—	**138**	**13**	**10**	2	4	**20**	—	1	—	—	—	—	4
	41	—	—	—	—	—	—	—	**10**	**10**	8	—	2	**61**	**13**	2	2	**13**	—	2	**29**	5
	42	—	—	—	—	—	—	—	**365**	**315**	**25**	**73**	—	**16**	**19**	**255**	**364**	**11**	**38**	—	6	**14**
	43	—	**10**	—	**31**	—	—	**59**	**12**	**50**	**98**	**296**	—	**24**	**61**	**17**	**73**	**74**	**118**	4	**137**	**31**
	46	—	—	—	—	—	—	—	**15**	**97**	**15**	—	—	**44**	—	**70**	**357**	**145**	6	**20**	**172**	3
	48	—	—	—	2	—	—	**168**	**28**	**360**	**29**	—	**13**	**150**	**80**	**84**	**417**	**27**	2	**111**	**131**	**116**

NOTE: The PBMCs were tested against four peptide pools of HPV16 E6 (E6.1-E6.4) and two peptide pools of E7 (E7.1 and E7.2). MRM was taken along as a positive control. In bold, the positive responses (definition is described in Materials and Methods) are depicted as number of specific spots per 10^5 PBMCs.

Abbreviations: NE, not evaluable ELISPOT analysis; NA, no blood sample was available of that specific time point; —, the number of specific spots is lower than 1 per 100,000 PBMCs.

We reasoned that the lower response to HPV16 E7 could be due to antigen competition and this prompted us to reconsider both the dose of the antigens as well as the administration of the two antigens at the same injection site. Therefore, the dose of HPV16 E6 peptides was lowered from 300 to 100 μg/peptide and the E6 peptides were injected at a site distinct from the site of the E7 peptides. In this second study group (patient group 2), after two vaccinations, 9 of the 11 analyzed and evaluable patients displayed circulating HPV16 E6-specific T cells and 7 of the 11 patients against E7, and following four vaccinations, specific T-cell immunity directed against E6 was observed in 7 of the 10 patients analyzed and against E7 in 6 of 10 (see also Table 3). The response rate is not different between two and four vaccinations, suggesting that the timing of blood sampling is indeed important.

Because of the adverse events, associated with the injection of a large s.c. volume of Montanide ISA-51, observed in groups 1 and 2, a third group of patients was injected with a lower dose of antigen and, therefore, also a smaller amount of Montanide ISA-51. Patients in group 3 were vaccinated with HPV16 E6 separated from E7 at a dose of 50 μg/peptide of each antigen. After two vaccinations, eight of nine analyzed patients responded to E6 and six of nine patients to E7 and, after four vaccinations, six of nine to E6 and four of nine to E7, similar to what was observed in the previous group. In addition, the peak IFNγ-producing T-cell frequencies observed in patient group 3 were in the same order of magnitude as observed in patient group 2 (Table 3; Fig. 1B). In conclusion, lowering of the dose of the antigens did not significantly reduce the number of responding patients or lower the peak of vaccine-induced responses to E6 and E7.

Analysis of the overall magnitude of vaccine-induced immunity by comparison of the median ELISPOT reactivity to E6 and E7 between the three groups showed that separation of the E7 peptides from the E6 peptides resulted in a significant increase in magnitude of the HPV16 E7-specific T-cell response ($P = 0.02$), whereas the median number of specific spots to E6 did not change (Fig. 2).

We deviated from the protocol for patient 14 because this patient reached a complete response as shown by computed tomography scan 4 weeks after the last vaccination and remains disease-free until now (July 2007). To maintain her immune response, she received a fifth vaccination 3 months after the fourth vaccination. After this fifth vaccination and also at 5 months of follow-up, HPV16 E6- and E7-specific immunity was still detectable. To confirm that vaccination with long peptides resulted in an immune response to naturally processed antigen, the remaining cells after the 4-day incubation of the ELISPOT

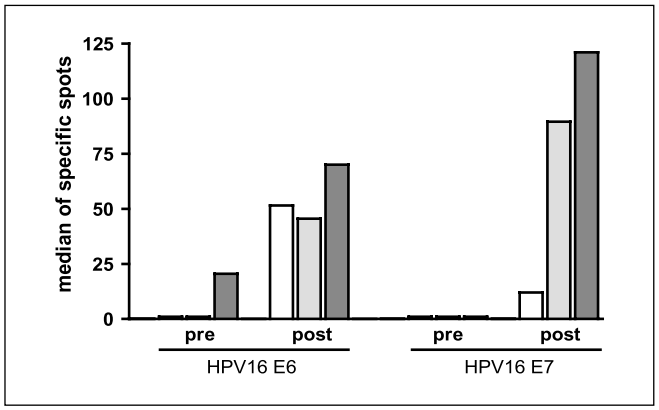

Fig. 2. Antigen competition between E6 and E7 when administered admixed. The median of positive T-cell responses as measured by IFNγ ELISPOT in blood sample before vaccination (*pre*) and after three or four vaccinations (*post*) is depicted for HPV16 E6 (*left*) and E7 (*right*) for the three patient groups (*white columns*, group 1; *gray columns*, group 2; *black columns*, group 3). This shows that after vaccination significant induction of type 1 T-cell immunity is found for E6 in all patient groups. The results obtained for each patient group did not differ. However, only after separation of the E7 from the E6, which was the case in patient groups 2 and 3, a significant enhancement of the E7 response was observed ($P = 0.02$).

assay (Fig. 3A) were subjected to an intracellular cytokine analysis that showed that the vaccine-induced HPV16 E6-specific T cells also recognized their cognate antigen when processed and presented by autologous monocytes loaded with HPV16 E6 recombinant protein (Fig. 3B). Moreover, also, HPV16 E7 peptide- and protein-specific $CD4^+$ T cells were observed. In conclusion, these results show that the long peptide-based vaccine against HPV16 E6 and E7 is indeed capable of inducing broad and vigorous antigen-specific T-cell immunity.

Discussion

In this phase I study, immunotherapy with synthetic long peptides representing the sequence of the oncogenic proteins E6 and E7 of high-risk HPV16 is proven to be safe and highly immunogenic in formulation with Montanide ISA-51 adjuvant. The maximal toxicity seen was grade 2 and consisted of discomfort and swelling at the vaccination sites and low-grade fever in the first 48 h after injection. These results fit with the clinical experience gathered thus far with peptide vaccination in Montanide adjuvant, showing only low-grade toxicity and strong immunogenicity (23–27).

In the quoted studies, mostly exact HLA class I–binding peptides were used for immunotherapy of metastatic melanoma, and the overall clinical results with such exact HLA-fitting peptides in patients with melanoma have been very disappointing worldwide (28). However, our preclinical studies in mice indicate that immunization with extended peptides is superior to that with exact MHC class I–fitting peptides (refs. 12, 29, reviewed in ref. 30). First, exact MHC class I–binding peptides are exogenously loaded on all cells that express MHC class I. This leads to antigen presentation by nonprofessional antigen-presenting cells, a tolerizing immunization mode because only professional antigen-presenting cells can provide the proper costimulatory context for productive immune responses. Second, exact MHC-binding peptides lack helper epitopes for induction of $CD4^+$ T cells, required for CD8 memory responses (31, 32). Indeed, by vaccination with a long peptide containing both a helper and a CTL epitope of HPV16 E7, established HPV16 E6/E7-transformed tumors in mice were eradicated efficiently by the long peptide with CpG adjuvant (12). In the absence of CpG, the *in vivo* CD8 CTL response induced by the long peptide in this study was abolished in MHC class II knockout or CD40 knockout mice (12), underscoring the important role of the CD4 helper response in the induction of a CD8 CTL response.

Long peptide vaccination has a strong antitumor activity not only in our preclinical mouse model (12) but also in a more realistic papillomavirus infection model in rabbits, in which skin warts with a tendency toward cancer progression can be induced by CRPV. Established skin lesions induced by CRPV were successfully eradicated by vaccination with long overlapping peptides representing the entire sequence of CRPV E6 and E7, associated with viral clearance as established by PCR in a high percentage of lesions (13). Vaccination with CRPV E6 and E7 peptides induced a robust T-helper type 1 response, which was associated with clearance of the CRPV-induced warts (13). In line with these preclinical results, the current phase I clinical study shows that vaccination with multiple overlapping peptides representing the entire E6/E7 sequence of HPV16 induces a robust IFNγ ELISPOT response against multiple epitopes of both the E6 and E7 proteins. Moreover, the current

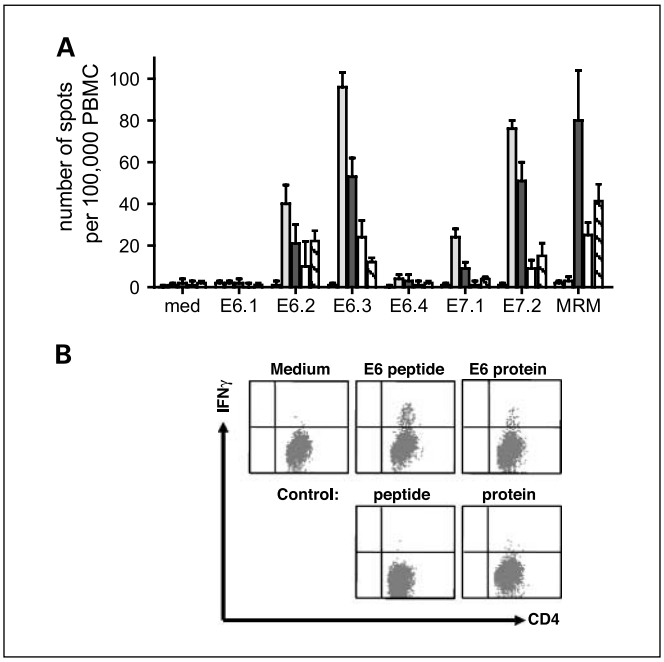

Fig. 3. Detailed analysis of HPV16-specific T-cell response of patient 14. *A,* IFNγ ELISPOT analysis of the HPV16-specific T-cell response after five vaccinations. The number of spots per 10^5 PBMCs is given before vaccination, after two vaccinations (*light gray columns*), after four vaccinations (*dark gray columns*), after five vaccinations (*white columns*), and 5 mo after this fifth vaccination (*striped columns*). Shown are the T-cell responses against the four peptide pools of HPV16 E6 and the two peptide pools of HPV16 E7. Medium only was taken along as negative control and the MRM as a positive control. *B,* vaccine-induced T cells recognize naturally processed HPV16 E6 protein. PBMCs left over after doing the ELISPOT analysis were subjected to an intracellular cytokine staining as described by de Jong et al. (16). The cells were stained for IFNγ and CD4 and analyzed by flow cytometry. Typical flow cytometric dot plots are shown of the responses obtained in follow-up blood sample of patient 14 after she received five vaccinations. Similarly, also, HPV16 E7-specific IFNγ-producing $CD4^+$ T cells were observed.

clinical HPV E6/E7 long peptides vaccine also induces both HPV E6/E7-specific CD4 and CD8 T-cell responses (33).

Interestingly, our studies on the immune response to HPV16 (15, 16) convincingly showed that patients with cervical cancer fail to spontaneously mount IFNγ-producing T-cell responses against the early HPV16 proteins E2, E6, and E7. Indeed, the cervical cancer patients studied in this phase I trial all lacked robust IFNγ T-cell responses to HPV16 E6 and E7 before vaccination but mounted robust T-cell responses to E6 and E7 following HPV16 E6/E7 long peptide immunotherapy. These immune responses are higher than the memory T-cell response detected in healthy women that have spontaneously cleared the virus (14–18).

Our results clearly show that the response to E6 dominates over that against E7 when the HPV16 peptides are injected in a single s.c. site in most patients, suggesting antigenic competition at the level of the local draining lymph nodes. When the s.c. injections of E6 and E7 peptides were separated, the response to E7 became more robust with significantly more patients responding to E7 and also a more vigorous IFNγ-producing T-cell immunity compared with the E7-specific response following coinjection of E6/E7 in a single site. These results indicate that the use of a synthetic long peptides vaccine is a robust way to prime (and boost) therapeutic cell-mediated immune responses and its design allows swift changes (e.g., separation of E6 and E7 peptide antigens and inclusion of other antigens and adjuvants) and, therefore, supports rapid translation of new immunologic concepts into phase I/II trials in humans. Furthermore, these vaccines are easily produced, are chemically stable, and are devoid of oncogenic potential as well as free of bacterial/viral contaminating substances, hereby avoiding the antigenic competition often seen against viral vector-based vaccines expressing tumor-associated antigens or HIV antigens (19, 34–36) or between simultaneously injected antigens such as is the case with fusion protein or gene products (20–22). Other approaches at therapeutic vaccination in cancer include administration of antigen-loaded dendritic cells; however, this requires specialized laboratories that prepare autologous dendritic cells for each individual patient (37, 38).

The first published attempt at immunotherapy of (pre-) malignant lesions induced by high-risk HPV concerns vaccination of patients with early-stage cervical cancer with recombinant vaccinia virus expressing detoxified HPV16/18 E6/E7 (39). In this study, a few patients showed a HPV-specific $CD8^+$ T-cell response and one patient experienced a complete remission. In our previous clinical vaccination study in end-stage cervical cancer patients with exact HLA class I–binding E7 peptides, we did not observe HPV-specific immunity (27). Our current data indicate that this does not mean failure of patients with end-stage cervical cancer to respond to peptide vaccination because they responded vigorously to both E6 and E7 long HPV peptides; the T-cell response rate was at least 80% and the same long peptide vaccine showed a 100% HPV16-specific $CD4^+$ and $CD8^+$ T-cell response in cervical cancer patients vaccinated postoperatively (33). Therefore, in these categories of patients, the use of a mix of E6 and E7 long peptides seems to be very successful at inducing both strong $CD4^+$ and $CD8^+$ T-cell responses.

It seems likely that long peptide immunotherapy by itself cannot exert an effective therapeutic action in most patients with established cervical cancer. Reasons for this may be improper polarization of tumor-specific immune responses, including induction of Foxp3-positive HPV-specific (regulatory) T cells (30, 33, 40, 41), immunoediting, including down-regulation of MHC class I molecules or processing molecules (42–44), and failure of effector T cells to properly home into cancer tissues (40, 45, 46). In the present study, one patient experienced a complete remission confirmed by computed tomography scan, whereas five others remained stable over a long period. The patient with a complete remission had been treated with chemoradiation before immunotherapy and four of the five others were treated with platin-containing chemotherapy after the vaccination. Although anecdotal and not an end point in the present study, this kind of long-lasting responses in cervix carcinoma are very rare. Recent publications in preclinical models indicate the benefit of combined chemotherapy or irradiation with immunotherapy (47, 48). A recent study reported that patients with lung cancer showed no survival benefit following vaccination with a recombinant adenovirus expressing wild-type p53. However, subsequent chemotherapy bestowed a significant survival advantage onto the vaccinated patients, whereas chemotherapy alone was of no benefit (49, 50).

Immunotherapy alone may be more successful in patients with premalignant HPV16-induced lesions, such as vulvar intraepithelial neoplasia. In a phase I trial with recombinant HPV16 E6/E7 vaccinia virus, this vaccine was well tolerated in patients with early-stage cervical cancer and induced HPV-specific CTL responses (51). Subsequently, 12 women with high-grade anogenital intraepithelial neoplasia were treated with this vaccine and 5 patients experienced at least 50% reduction in lesions with 1 patient achieving complete remission of her lesion (21). In a similar study with the same vaccine in 18 patients with HPV16-positive high-grade vulvar intraepithelial neoplasia, 8 of 18 patients showed at least 50% lesion reduction and 13 women showed an increased HPV-specific immune response by one or more immune assays (52). Likewise, of 29 women immunized with a recombinant HPV16 L2/E6/E7 fusion protein followed by boosting with the same recombinant HPV16 E6/E7 recombinant vaccinia virus, HPV16-specific immune responses were recorded but there was no simple relationship between clinical responses and HPV-specific immune responses (22).

In conclusion, our study shows that HPV16 E6/E7 long peptides vaccination is safe and highly immunogenic. It was found to induce robust IFNγ T-cell responses to HPV16 E6/E7 antigens in end-stage cervical cancer patients. Failure to generate such responses in HPV16-infected women is a risk factor for the development of cervical cancer and such responses are generally absent or weak in cervical cancer patients. For patients with cervical cancer and other HPV-related neoplasias, we advocate exploration of potential benefit of combined treatment modalities, including conventional treatments by surgery, chemotherapy, and/or irradiation, in conjunction with the currently described immunotherapy. In patients with premalignant lesions, immunotherapy with long peptides alone is attractive and a trial to explore therapeutic benefit in such patients has been initiated.

Acknowledgments

We thank all patients who participated in this study and Kees Franken and Willemien Benckhuijsen for protein and peptide synthesis.

References

1. Bosch XF, De Sanjosé S. Human papillomavirus and cervical cancer—burden and assessment of causality. J Natl Cancer Inst Monogr 2003;31:3–13.
2. Muñoz N, Bosch XF, De Sanjosé S, et al. Classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003;348:518–27.
3. Koutsky LA, Ault KA, Wheeler CM, et al. A controlled trial of a human papillomavirus type 16 vaccine. N Engl J Med 2002;21:1645–51.
4. Harper DM, Franco EL, Wheeler C, et al. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet 2004;364:1757–65.
5. Villa LL, Costa RLR, Petta CA, et al. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet 2005;6:271–8.
6. Harper DM, Franco EL, Wheeler CM, et al. Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. Lancet 2006;367:1247–55.
7. The Future II Study Group. Quadrivalent vaccine against human papillomavirus to prevent high-grade cervical lesions. N Engl J Med 2007;356:1915–27.
8. Garland SM, Hernandez-Avila M, Wheeler CM, et al. Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases. N Engl J Med 2007;356:1928–43.
9. Mao C, Koutsky A, Ault KA, et al. Efficacy of human papillomavirus-16 vaccine to prevent cervical intraepithelial neoplasia. A randomized control trial. Obstet Gynecol 2006;107:18–27.
10. Schiller JT, Lowy DR. Prospects for cervical cancer prevention by human papillomavirus vaccination. Cancer Res 2006;66:10229–32.
11. Stanley MA. Human papillomavirus vaccines. Rev Med Virol 2006;16:139–49.
12. Zwaveling S, Ferreira Mota SC, Nouta J, et al. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. J Immunol 2002;169:350–8.
13. Vambutas A, DeVoti J, Nouri M, et al. Therapeutic vaccination with papillomavirus E6 and E7 long peptides results in the control of both established virus-induced lesions and latently infected sites in a pre-clinical cottontail rabbit papillomavirus model. Vaccine 2005;23:5271–80.
14. de Jong A, van der Burg SH, Kwappenberg KM, et al. Frequent detection of human papillomavirus 16 E2-specific T-helper immunity in healthy subjects. Cancer Res 2002;62:472–9.
15. Welters MJP, de Jong A, van den Eeden SJF, et al. Frequent display of human papillomavirus type 16 E6-specific memory T-helper cells in the healthy population as witness of previous viral encounter. Cancer Res 2003;63:636–41.
16. de Jong A, van Poelgeest MIE, van der Hulst JM, et al. Human papillomavirus type 16-positive cervical cancer is associated with impaired $CD4^+$ T-cell immunity against early antigens E2 and E6. Cancer Res 2004;64:5449–55.
17. Welters MJP, van der Logt P, van den Eeden JF, et al. Detection of human papillomavirus type 18 E6 and E7-specific $CD4^+$ T-helper 1 immunity in relation to health versus disease. Int J Cancer 2006;118:950–6.
18. van Poelgeest MIE, Nijhuis ER, Kwappenberg KMC, et al. Distinct regulation and impact of type 1 T-cell immunity against HPV16 L1, E2 and E6 antigens during HPV16-induced cervical infection and neoplasia. Int J Cancer 2006;118:675–83.
19. van der Burg SH, Kwappenberg KMC, O'Neill T, et al. Pre-clinical safety and efficacy of TA-CIN, a recombinant HPV16 L2E6E7 fusion protein vaccine, in homologous and heterologous prime-boost regimens. Vaccine 2001;19:3652–60.
20. de Jong A, O'Neill T, Khan AY, et al. Enhancement of human papillomavirus (HPV) type 16 E6 and E7-specific T-cell immunity in healthy volunteers through vaccination with TA-CIN, an HPV16 L2E7E6 fusion protein vaccine. Vaccine 2002;20:3456–64.
21. Baldwin PJ, Van der Burg SH, Boswell CM, et al. Vaccinia-expressed human papillomavirus 16 and 18 E6 and E7 as a therapeutic vaccination for vulval and vaginal intraepithelial neoplasia. Clin Cancer Res 2003;9:5205–13.
22. Smyth LJC, van Poelgeest MIE, Davidson EJ, et al. Immunological responses in women with human papillomavirus type 16 (HPV-16) associated anogenital intraepithelial neoplasia induced by heterologous prime-boost HPV-16 oncogene vaccination. Clin Cancer Res 2004;10:2954–61.
23. Rosenberg SA, Sherry RM, Morton KE, et al. Altered $CD8^+$ T-cell responses when immunizing with multiepitope peptide vaccines. J Immunother 1997;29:224–31.
24. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998;4:269–70.
25. Speiser DE, Liénard D, Rufer N, et al. Rapid and strong human $CD8^+$ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest 2005;115:739–46.
26. van Driel WJ, Ressing ME, Kenter GG, et al. Vaccination with HPV16 peptides of patients with advanced cervical carcinoma: clinical evaluation of a phase I-II trial. Eur J Cancer 1999;35:946–52.
27. Ressing ME, Van Driel WJ, Brandt RM, et al. Detection of T helper responses, but not of human papillomavirus-specific cytotoxic T lymphocyte responses, after peptide vaccination of patients with cervical carcinoma. J Immunother 2000;23:255–66.
28. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy moving beyond current vaccines. Nat Med 2004;10:909–15.
29. Bijker MS, Van den Eeden SJ, Franken KL, et al. $CD8^+$ CTL priming by exact peptide-epitopes in IFA induces a vanishing CTL response, while long peptides induce sustained CTL reactivity. J Immunol 2007;179:5033–40.
30. Van der Burg SH, Bijker MS, Welters MJP, et al. Improved peptide vaccine strategies, creating synthetic artificial infections to maximize immune efficacy. Adv Drug Deliv Rev 2006;58:916–30.
31. Janssen EM, Lemmens EE, Wolfe T, et al. $CD4^+$ T cells are required for secondary expansion and memory in $CD8^+$ T lymphocytes. Nature 2003;421:852–6.
32. Janssen EM, Droin NM, Lemmens EE, et al. $CD4^+$ T cell help controls $CD8^+$ T cell memory via TRAIL mediated activation-induced cell death. Nature 2005;434:88–93.
33. Welters MJP, Kenter GG, Piersma SJ, et al. Induction of tumor-specific $CD4^+$ and $CD8^+$ T-cell immunity in cervical cancer patients by an HPV16 E6 and E7 long-peptide vaccine. Clin Cancer Res 2008;14.
34. Pasquetto V, Bui H-H, Giannino R, et al. HLA-A*0201, HLA-A*1101 and HLA-B*0702 transgenic mice recognize numerous poxvirus determinants from a wide variety of viral gene products. J Immunol 2005;175:5504–15.
35. Jing L, Chong TM, McClurkan CL, et al. Diversity in the acute CD8 T cell response to vaccinia virus in humans. J Immunol 2005;175:7550–9.
36. McShane H, Hill A. Prime-boost immunization strategies for tuberculosis. Microbes Infect 2005;7:962–7.
37. Ferrara A, Nonn M, Sehr P, et al. Dendritic cell-based tumor vaccine for cervical cancer II: results of a clinical pilot study in 15 individual patients. J Cancer Res Clin Oncol 2003;129:521–30.
38. Santin AD, Hermonat PL, Ravaggi A, et al. Induction of human papillomavirus-specific CD4(+) and CD8(+) lymphocytes by E7-pulsed autologous dendritic cells in patients with human papillomavirus type 16- and 18-positive cervical cancer. J Virol 1999;73:5401–10.
39. Borysiewicz LK, Fiander A, Nimako M, et al. A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. Lancet 1996;347:1523–7.
40. Piersma SJ, Jordanova ES, van Poelgeest MIE, et al. High number of intraepithelial $CD8^+$ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. Cancer Res 2007;67:354–61.
41. Van der Burg SH, Piersma SJ, de Jong A, et al. Association of cervical cancer with the presence of $CD4^+$ regulatory T cells specific for human papillomavirus antigens. Proc Natl Acad Sci U S A 2007;104:12087–92.
42. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. Nat Rev Immunol 2006;6:836–48.
43. Mehta AM, Jordanova ES, Van Wezel T, et al. Genetic variation of antigen processing machinery components and association with cervical carcinoma. Genes Chromosomes Cancer 2007;46:577–86.
44. Algarra I, Garcia-Lora A, Cabrera T, et al. The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol Immunother 2004;53:904–10.
45. Cao ZA, Daniel D, Hanahan D. Sub-lethal radiation enhances anti-tumor immunotherapy in a transgenic mouse model of pancreatic cancer. BMC Cancer 2002;2:1–11.
46. Bracci L, Moschella F, Sestili P. Cyclophosphamide enhances the antitumor efficacy of adoptively transferred immune cells through the induction of cytokine expression, B cell and T cell homeostatic proliferation and specific tumor infiltration. Clin Cancer Res 2007;13:644–53.
47. Lake RA, Robinson BW. Immunotherapy and chemotherapy—a practical partnership. Nat Rev Cancer 2005;5:397–405.
48. Nowak AK, Lake RA, Robinson BW. Combined chemoimmunotherapy of solid tumours: improving vaccines? Adv Drug Deliv Rev 2006;58:975–90.
49. Antonia SJ, Mirza N, Fricke I, et al. Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. Clin Cancer Res 2006;12:878–87.
50. Gabrilovich DI. Combination of chemotherapy and immunotherapy for cancer: a paradigm revisited. Lancet Oncol 2007;8:2–3.
51. Kaufmann AM, Stern PL, Rankin EM, et al. Safety and immunogenicity of TA-HPV, a recombinant vaccinia virus expressing modified human papillomavirus (HPV)-16 and HPV-18 E6 and E7 genes, in women with progressive cervical cancer. Clin Cancer Res 2002;8:3676–85.
52. Davidson EJ, Boswell CM, Sehr P, et al. Immunological and clinical responses in women with vulval intraepithelial neoplasia vaccinated with a vaccinia virus encoding human papillomavirus 16/18 oncoproteins. Cancer Res 2003;63:6032–41.

Clinical Cancer Research

Phase I Immunotherapeutic Trial with Long Peptides Spanning the E6 and E7 Sequences of High-Risk Human Papillomavirus 16 in End-Stage Cervical Cancer Patients Shows Low Toxicity and Robust Immunogenicity

Gemma G. Kenter, Marij J.P. Welters, A.Rob P.M. Valentijn, et al.

Clin Cancer Res 2008;14:169-177.