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Original Paper

Vaccination with HPV16 Peptides of Patients with Advanced Cervical Carcinoma: Clinical Evaluation of a Phase I–II Trial

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A phase I–II clinical trial was performed involving vaccination with HPV16 E7 peptides of patients suffering from HPV16 positive cervical carcinoma which was refractory to conventional treatment. Patients receiving the vaccine were HLA-A*0201 positive with HPV16 positive cervical carcinoma. The clinical trial was designed as a dose-escalation study, in which successive groups of patients received 100 μg, 300 μg or 1000 μg of each peptide, respectively. The vaccine consisted of two HPV16 E7 peptides and one helper peptide emulsified in Montanide ISA 51 adjuvant. 19 patients were included in the study, no adverse side-effects were observed. 2 patients showed stable disease for 1 year after vaccination; 15 patients showed progressive disease of whom 1 died during the vaccination treatment due to progressive disease; and 2 patients showed tumour-regression after chemotherapy following vaccination. A relative low count of lymphocytes before and after vaccination was present in 11/19 patients indicating that these patients were immunocompromised. This study shows that HPV16 E7 peptide vaccination is feasible, even in a group of patients with terminal disease. This paves the way for vaccinating patients with less advanced disease, whose immune system is less compromised by progressive disease.

Key words: immunotherapy, cervix, neoplasm, peptide, vaccination, HPV, CTL
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INTRODUCTION

CERVICAL CANCER is preceded only by breast cancer as the leading cause of death from cancer in women worldwide [1]. Even after optimal primary treatment of low-risk early stage disease, recurrent disease is seen in 15% of these patients [2, 3]. Treatment results of recurrent disease are relatively poor [4, 5]. Therefore, patients with recurrent cervical cancer present a major clinical problem calling for additional therapeutic strategies.

Human papillomaviruses (HPV) are a heterogeneous group of papova viruses that infect epithelial tissues. HPV types 16 and 18 are often found in premalignant lesions of the cervix (cervical intra-epithelial neoplasia (CIN) I to III) which can progress to cervical carcinoma. HPV are also present in over 90% of cervical carcinoma (predominantly types 16 and 18) and are implicated in the aetiology of the disease [6]. Several findings indicate that cervical carcinoma may be immunogenic. In particular the cellular arm of the immune system has been shown to constitute a major defence against cervical carcinoma [7–9]. Early regions 6 and 7 of HPV are constitutively expressed by the majority of cervical tumour cells and are known to be involved in maintenance of the transformed state. Therefore, the HPV16 E6 and E7 proteins are attractive as tumour-specific targets for T cell based immunotherapy of cervical cancer.

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Nine HLA-A*0201-binding peptides have been identified within HPV16 E6 and E7 [10, 11]. Three of these binding peptides, encoded by HPV16 E7, have been shown to be immunogenic. These peptides elicited CTL (cytotoxic T-lymphocytes) responses both *in vitro* in primary response induction with human responding lymphocytes and *in vivo* in HLA-A2 transgenic mice [12].

In vitro studies have shown that patients with HPV16 positive lesions occasionally have memory CTL against one of these HPV16 E7 encoded peptides (sequence YMLDLQPETT), suggesting that there can be natural CTL immunity against HPV16 in patients with cervical lesions [13, 14]. *In vivo* studies in mice have shown that vaccination of mice with an HPV16 peptide ($E7_{49-57}$) with a high binding affinity for the H-$2D^b$ mouse MHC class I molecule completely prevented tumour growth in these mice upon subsequent challenge with HPV16 positive tumour cells. Concomitantly, a CTL response was elicited which lysed the tumour cells *in vitro* [15]. Moreover, adoptive transfer of a CTL clone obtained via peptide vaccination with the $E7_{49-57}$ peptide led to the eradication of established HPV16 tumours in T-cell deficient nude mice [16].

This study was designed to establish the clinical response and toxicity of such a form of vaccination in women with advanced cervical carcinoma. The clinical results, the occurrence of adverse side-effects, and a summary of the immunological findings are presented. In addition, future immunotherapeutic strategies are discussed.

PATIENTS AND METHODS

Patients

Patients from the German and Belgian hospitals were vaccinated in Freiburg and Antwerp, respectively. Dutch patients were vaccinated at the Leiden University Medical Centre. The study was approved by the local medical ethics committee of all participating hospitals. All patients had received optimal conventional therapy and were referred when residual or recurring tumour was considered untreatable. The criteria for eligibility were tumour HPV16 positive; patient HLA-A*0201 positive; presence of recurrent or residual disease; no immunocompromising therapy 3 months before vaccination; life expectancy sufficiently long for the duration and evaluation of the study; and Karnofsky scale ≥ 80. Before vaccination written or witnessed oral informed consent was obtained from all patients included in the study.

Vaccination

A total of four vaccinations were given subcutaneously (s.c.) every 3 weeks. Firstly in the right upper leg, then right upper arm, then left upper arm and then left upper leg, respectively. According to a dose-escalation scheme patients were vaccinated as follows: the first group (group I) of 5 patients received a low dosage (100 μg of each peptide). If no toxicity was observed, the second group (group II) of 5 patients was treated with a medium dose (300 μg of each peptide) which in the absence of toxicity was followed by the final group (group III) consisting of 9 patients who received a high dose (1000 μg of each peptide) per vaccination. Vaccination was given on an outpatient basis. Before each vaccination, history was taken and all patients underwent a routine physical examination. After each vaccination patients were observed for 4 h for occurrence of acute toxicity. During the vaccination scheme routine blood samples were taken every week for assessment of white blood cell count (WBC), electrolyte levels, liver enzyme levels and haemoglobin levels. Immediately before each vaccination a blood sample was taken in order to harvest lymphocytes for immunological evaluation. Before the first vaccination and after the last vaccination lymphocytes were sampled by leukapheresis in order to obtain sufficient cells to perform all immunological assays. After isolation and preservation the lymphocytes were stored until further use.

Vaccine

The vaccine consisted of two HPV16 E7 peptides (aa $E7_{11-20}$, sequence YMLDLQPETT and aa $E7_{86-93}$, sequence TLGIVCPI) representing 2 CTL epitopes with a high binding affinity for HLA-A*0201 molecules [12], and a pan-DR binding T helper peptide (sequence aKXVAAWTLKAAa in which X is cyclohexylalanine) [17] emulsified prior to use in Montanide ISA 51, a mineral oil-based adjuvant similar to incomplete Freund's adjuvant (Seppic, Paris, France). The peptides were manufactured under GMP conditions at the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

Study parameters

This clinical study was designed as a phase I–II trial with three main aims. First to assess adverse side-effect in order to determine safe dosage. Second, to evaluate the clinical response and third to evaluate the existence of a vaccine specific CTL response in relation to the dosage.

Two categories of adverse side-effects were evaluated according to their possible relationship to the vaccination. The first category consisted of the adverse physical changes noted during physical examination and interview. The second category consisted of abnormalities found by laboratory investigations. All adverse side-effects were defined according to the World Health Organisation (WHO) common toxicity criteria [18].

Clinical evaluation

To measure patient's clinical response we performed radiological examinations at two different times. One immediately before vaccination and the second 2 weeks after completion of the vaccination programme (short follow-up). Physical examination and history were taken at subsequent visits to evaluate long-term clinical response (long follow-up).

For the clinical evaluation, measurements at first and second radiological examination were compared. All patients were examined by either a computer tomography (CT) scan, magnetic resonance imaging (MRI) scan or ultrasound. The same radiological technique was used to establish tumour size before and after vaccination in all but 2 patients. Measurements in two axis were made of at least two lesions present before the start of the vaccination programme. These same lesions were examined at the end of the treatment by one of the radiologists at the hospital. The total sum of the products of the largest perpendicular diameters of the lesions before and after treatment were compared. A complete response was defined as the disappearance of all lesions. A partial response was defined as a 50% or greater decrease of all measurements without appearance of new lesions, and none should increase. Stable disease was defined as less than 50% decrease or less than 25% increase of all measurements and no appearance of new lesions. Any patient without CR,

Table 1. *Patients who were referred for the phase* I-II *study and reasons for exclusion*

HLA-A*0201 pos and HPV16 pos	31*
Included in the study	19
Excluded:	
Karnofsky scale < 80	8
No recurrence	2
Withdrawal	1
Other treatment	1
HLA-A*0201 neg and/or HPV16 neg	91
HLA-A*0201/other HPV	18
Other HLA/other HPV	15
Other HLA/HPV16 nt	17
Other HLA/HPV16	23
Other HPV/HLA nt	15
Not tested†	3

*Number of patients are provided. †Patients were too ill to be tested.

PR or SD were considered to have progressive disease. New lesions or an increase of greater than 25% of known lesions following a partial or complete response were considered relapses.

Immunohistochemistry

To evaluate the local effects of vaccination, biopsies were taken from 3 patients (patients 1, 5 and 6) at the vaccination sites 1 day after completion of the vaccination. These biopsies were snap frozen in isopentane and stored at -70°C until further use. Cryostat sections were stained with the following monoclonal antibodies: UCHT-1 (anti-CD3, DAKO), Leu 3A (anti-CD4, Becton and Dickinson) and DK 25 (anti-CD8, DAKO). Immunohistochemical staining was performed according to a three-step peroxidase staining as described elsewhere [19].

RESULTS

Patients

122 patients were referred to our hospital for the phase I–II study with HPV16 peptides from January 1995 to November 1997. Our criteria that patients should be HLA-A*0201 and HPV16 positive left 31 patients (25%) eligible for the study of whom 19 were included in the study. Reasons for exclusion are provided in Table 1. Patients who were included in the study had a mean age of 47 years (range 33–75 years) at the start of vaccination.

Of the 19 patients included, a leukapheresis was not performed in 1 case before start of the vaccination (patient 16) and in 5 cases after treatment for the following reason: 1 patient discontinued treatment due to deteriorating clinical condition (patient 3), 2 patients experienced a deterioration of their clinical condition after completion of vaccination therapy (patients 9 and 10); 1 patient receiving chemotherapy as primary therapy had obstructed peripheral veins precluding leukapheresis (patient 16) and in 1 patient the amount of lymphocytes obtained was insufficient to perform all immunological assays (patient 19). Thus, 15 vaccinated patients were also evaluated immunologically. All 19 patients were included for the clinical evaluation. Characteristics of those patients included in the study are shown in Table 2. Of the 19 patients included in this study, 17 patients originally had squamous cell carcinoma and 2 had adenosquamous carcinoma (patients 13 and 16).

Adverse side-effects

Clinical parameters. None of the vaccinated patients at any of the three doses experienced adverse side-effects based on the history taken or after physical examination before and after each successive vaccination. A temporary erythema was observed in 4 patients (patients 1, 2, 9 and 16) for all treatment groups. This erythema subsided after a few hours, was

Table 2. *Patients characteristics*

Patient	Age (years)	FIGO stage	Primary treatment	Interval between primary therapy to recurrence (months)	Site of recurrence or residual disease	Treatment of recurrent disease (before vaccination)
1	38	IIB	RT	3	Local	None
2	53	IB	Surgery + RT	35	Local and regional	CT
3	50	IVB	CT + RT	0	Regional and distant	None
4	60	IA	Surgery	1st 111 2nd 83	1st local 2nd locoregional	1st surgery 2nd RT
5	75	IIA	Surgery + RT	57	Distant	CT + RT
6	37	IB	Surgery	219	1st regional 2nd distant	1st RT 2nd RT
7	34	IA	RT	11	Local	Surgery
8	43	IIB	Surgery + RT	7	Locoregional	None
9	33	IB	RT	8	Local	Surgery
10	36	III	CT + RT	9	Local	None
11	40	IIB	Surgery + RT	19	Distant	CT
12	38	IIA	Surgery + RT	7	Regional	Surgery + CT
13	38	IIIB	RT	6	Local	None
14	75	IB	Surgery + RT	64	Regional	None
15	47	IA	Surgery + RT	84	Distant	CT + RT
16	40	IIB	RT + surgery	15	Locoregional	None
17	46	III	CT + RT	0	Locoregional	None
18	75	IB	Surgery	37	Regional	RT
19	47	IIB	RT	12	Distant	None

Age, age at first vaccination; RT, radiotherapy; CT, chemotherapy. The dosages are divided as follows: patients 1–5 low-dose; patients 6–10, moderate-dose; and patients 11–19 high-dose (see text).

Table 3. Leucocyte (white blood cell, WBC) counts pre- and post vaccination

	Pre vaccination			Post vaccination		
Patient	WBC$\times 10^9$/l	Lymphocytes (%)$\times 10^7$/l	% Granulocytes	WBC	Lymphocytes (%)	% Granulocytes
1	6	54 (8.9)	85	6.2	80 (12.9)	79.9
2	2.7	42 (15.5)	72.8	4	28 (7.0)	80
3	12.1	29 (2.4)	89	n.a.	n.a.	n.a.
4	7.2	58 (8.0)	73.9	7.2	58 (8.0)	76
5	6.2	97 (15.6)	75	5.3	51 (9.7)	82.5
6	5.5	73 (13.3)	78	10.8	94 (8.7)	85.1
7	5.9	110 (18. 7)	71.9	8.1	104 (12.9)	77.3
8	6.8	109 (16. 1)	70.5	8.9	107 (12.0)	76
9	9.9	105 (10. 6)	82.2	12.9	148 (11.5)	88
10	6.2	37 (5.9)	88.2	9.8	66 (6.7)	85.1
11	4.5	66 (14.6)	72	3.1	65 (21.0)	60
12	10.5	69 (6.6)	86.5	10.5	37 (3.5)	90.9
13	5.3	79 (14.9)	72.9	4.8	84 (17.6)	65.4
14	7.4	124 (16.7)	78	11.6	106 (9.1)	85
15	9	122 (13.5)	78	10.7	121 (11.3)	79.3
16	3.4	70 (20.6)	73.5	3.9	80 (20.5)	74.4
17	5.6	129 (23.1)	68.2	5.2	94 (18.0)	82
18	6.1	60 (10.0)	72	6	60 (10.0)	78
19	7.4	104 (14.0)	86	7.7	61 (7.9)	82.6

n.a., not available; normal values: leucocytes: 4.5–10.0 10^9/l, lymphocytes 90-500$\times 10^7$/l (20-50%), granulocytes 36–89%.

not painful or itchy, nor did this reaction worsen during the vaccination programme. A local induration was seen in 2 patients (patients 1 and 2) which was not painful and subsided over the following 6–8 weeks. One patient (patient 3) refused further treatment after the third vaccination because of progressive disease and deterioration of her condition. No adverse side-effects attributable to the vaccination were recorded.

Laboratory results. WBC count and lymphocyte count was determined in all patients at various time-points in the vaccination programme. As the values during vaccination showed no gross abnormalities when related to the first and last values, we present the values before the first vaccination (pre-treatment value) and after the last vaccination (post-treatment value). In 11 of the 19 patients, a low absolute lymphocyte count was found (patients 1, 2, 3, 4, 6, 10, 11, 12, 13, 16 and 18). These patients were from all three dosage groups (Table 3). 2 patients with a normal lymphocyte count before vaccination had a low lymphocyte count following completion of the vaccination treatment (patients 5 and 19) and both patients had progressive disease at short-term follow-up.

There was no significant difference in the mean time interval between last treatment and start of the vaccination, tumour size, and the mean increase in tumour size during the vaccination programme for patients with a normal or a low count of lymphocytes (Table 4). Although no significant correlations were found, patients with a low lymphocyte count tended to have a greater increase in tumour size during the vaccination programme. No correlations could be found between lymphocyte count and the appearance of new lesions.

Evaluation of the results of all routine blood tests during the course of the vaccination programme revealed no changes which were attributable to the vaccination programme (data not shown).

Clinical evaluation

A summary of clinical evaluation determined by radiological findings is shown in Table 5. No correlations could be made between the dosage of peptide applied and clinical outcome. On an individual basis, 2 patients who were treated in Freiburg (patients 11 and 13) received chemotherapy after completion of the vaccination which resulted in regression of the lesions in both patients. Patient 11, who was treated with chemotherapy (carboplatin and ifosfamide) before vaccination, received 6 cycles of cisplatin, ifosfamide and paclitaxel following the last vaccination. At the end of vaccination therapy this patient suffered from 6 lesions in total of which all but one completely regressed after chemotherapy as seen by radiological examination, leaving one palpable, left supraclavicular lymph node with a diameter of 2.5 cm. This patient is still alive with disease 11 months after the last chemotherapy. Patient 13, who was not treated by chemotherapy prior to vaccination, also received 6 cycles of the same triple chemotherapy after the last vaccination and showed complete clinical regression with normalisation of a tumour marker (SCC) for 1 month. Unfortunately, she progressed and died of disease 6 months after the last chemotherapy.

Table 4. Relationship between clinical characteristics and patients with a low or normal lymphocyte count

	Normal	Low	*P* value
Mean time (range) interval between last treatment and start vaccination	15.3 (3–64) months	7.8 (3–19) months	0.351*
Tumour size (range) before treatment (cm^2)	12.4 (4.4–28.8)	16.9 (1–40)	0.422†
Mean increase (range) in tumour size (cm^2)	12.9 (2.6–4.3)	17.4 (0–121)	0.071†

*Mann–Whitney test. †Unpaired *t*–test.

Table 5. *Clinical evaluation of treated patients*

Patient	Interval between last therapy and vaccination (mo)	Mode of evaluation	No. of lesions	Size (cm^2)		Follow-up (short)	Follow-up (long)	Time to last follow-up (months)
				Before vaccination	After vaccination			
1	9	CT scan	2	26.2	28.6	SD	DOD	22
2	4	CT scan	3	6.5	7.5	SD	DOD	18
3	3	MRI	2	39.6	nt	DOD	n.a.	0
4	12	MRI	1	5	126	PD	DOD	9
5	7	CT thorax	2	4.4	7.0*	PD	DOD	3
6	3	MRI	2	5.3	5.7	SD	DOD	6
7	9	MRI	1	28.8	36.3*	PD	DOD	10
8	10	MRI	2	17.4	31.5*	PD	DOD	8
9	10	MRI	1	15	nt	DOD	n.a.	0.5
10	9	MRI	1	27.6	nt	PD	DOD	1.5
11	3	CT scan	2	12.4	18.2*	PD	AWD†	14
12	4	MRI/CT	1	1	6	PD	DOD	4
13	6	MRI	2	16.7	17.4*	PD‡	DOD	11
14	64	CT abd	2	7.8	11.1	PD	DOD	2
15	4	MRI/CT	1	6	19.2*	PD	DOD	3
16	19	MRI	1	40	40	SD	PD	9
17	3	CT abd	1	10	53*	PD	PD	3
18	14	CT abd	2	6.5	29	PD	PD	2
19	15	CT thorax	2	9.8	17	PD	PD	2

SD, stable disease; PD, progressive disease; AWD, alive with disease; DOD, dead of disease. *New lesions. †After vaccination this patient received chemotherapy as explained in the text after which a regression of all but one lesion and a decrease in the serum marker SCC was seen. ‡Progressive disease was present after vaccination, but following chemotherapy complete remission for a period of 1 month was achieved. abd, abdominal; nt, not taken; n.a., not available; CT, computer tomography; MRI, magnetic resonance imaging.

The mean survival time of all patients included in the study was 7 months (range: 0–22 months) which is consistent with the survival of patients with advanced recurrent cervical cancer [20].

Immunohistochemical staining

To assess a local T cell response at the vaccination site, skin biopsies were taken from 3 patients, 1 day after the last vaccination (patients 1, 5 and 6). In general, $CD8^+$, $CD4^+$ and $CD3^+$ T cells were not present in normal skin biopsies. However, immunohistochemical stainings of these biopsies indicated an influx of $CD8^+$, $CD4^+$ and $CD3^+$ T cells in all 3 patients analysed (Figure 1). It cannot be formally concluded that this is due to the vaccine since T cells from the site of the vaccination were not isolated for functional analysis.

DISCUSSION

The present phase I–II study describes a new approach in immunotherapy for women suffering from recurrent or residual cervical carcinoma with HPV16 E7 peptides in an attempt to enhance the patients' HPV-specific CTL response. A total of 19 patients with recurrent or residual cervical carcinoma who had failed conventional treatment were treated by vaccination with HPV16 E7 encoded peptides. All but 1 patient completed the study and could be evaluated for safety, toxicity and clinical effects. No correlation between vaccine dose and clinical outcome was observed.

Administering HPV16 E7 peptides combined with a pan-DR binding helper peptide in Montanide ISA 51 apparently did not result in adverse side-effects nor were any of the patients incapacitated because of the vaccination. Some patients experienced a temporary rash not correlated with dosage of the vaccine and for which no treatment was necessary.

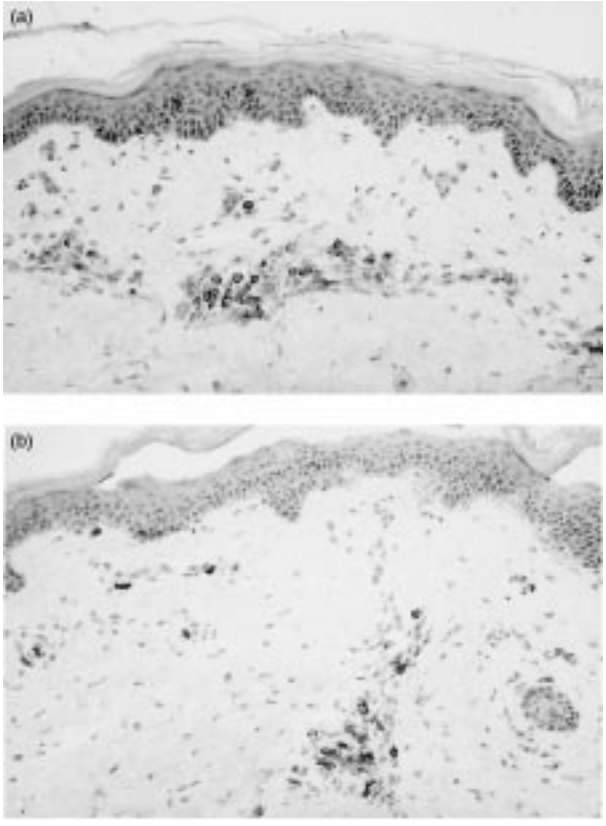

Figure 1. Skin biopsy 1 day after the last vaccination at the vaccination site, stained by immunohistochemistry. The stained cells were positive for CD4 (a) or CD8 (b).

No abnormal laboratory values were found in association with the vaccination programme apart from an absolute low lymphocyte count, which a majority of the patients experienced at the start of the vaccination programme. This absolute lymphopenia has been described in relation to the state of disease in cervical carcinoma and melanoma [21, 22]. Treatments such as radiotherapy, with or without chemotherapy prior to the first vaccination, are known to cause immunosuppression [23, 24]. The degree of immunosuppression in the patients in our study emphasises the need for clinical trials in an earlier stage of disease. In addition, immunisation with dendritic cells pulsed with E7 peptides could overcome the immunocompromised state of this group of patients and could augment the peptide specific responses [25, 26]. *In vivo* studies in mice demonstrated both *in vitro* and *in vivo* specific CTL responses following vaccination of dendritic cells with HPV16 E7 peptides resulting in protection against inoculation with syngeneic HPV16 induced tumour cells [27].

In biopsies taken at vaccination sites, we found infiltration of T lymphocytes. This may be a specific reaction to the vaccine or a general reaction to the injection. Normally, CD3, CD4 or CD8 positive T lymphocytes are not present in healthy skin. Since only small biopsies could be taken from the vaccination site we could not isolate these T lymphocytes from the biopsies nor was it possible to assess whether these cells show a specific reaction against the inoculated HPV16 E7 peptides. The group of patients treated in our study all had recurrent or residual disease but, as is often seen in such patients, these tumours were at inaccessible sites. Taking a biopsy would have caused unnecessary suffering. Treating patients at an earlier stage of disease makes it feasable to obtain information on HPV status and level of HLA expression.

The results of extensive immunological evaluation of general immunocompetence and HPV16 specific immune responses will be presented elsewhere (data not shown).

Besides peptide based vaccines, other immunotherapeutical approaches are currently under investigation such as adoptive transfer of CTL; using the recombinant *vaccinia* virus as a vector or vaccines with virus like particles (VLP's). Adoptive transfer of CTL raised against a subdominant HPV 16 E7 CTL epitope has been applied successfully in mice against HPV 16 induced tumours, resulting in eradication of the tumours [16]. However, this approach in a human setting may be a costly and time-consuming procedure since CTLs are required to be patient derived to avoid allogeneic rejection of CTLs because of MHC polymorphism. A recombinant *vaccinia* virus expressing the E6 and E7 protein of HPV 16 and 18 has been shown to induce vaccine-specific CTL activity and production of antibodies [28]. Whether these antibodies also have preventive activity against subsequent inoculation remains to be confirmed. Although this study also stressed the difficulty in detecting HPV specific CTL activity, the use of *vaccinia* virus seems promising and needs further evaluation to establish its significance. Other vaccines make use of virus-like particles (VLPs) and have been shown to generate humoral protection [29]. Cellular mediated protection against tumour injection in mice has been obtained using chimeric VLPs [30].

The present study reports the clinical results of HPV16 E7 peptide vaccination in patients with advanced cervical cancer. We recorded no unacceptable adverse side-effects after vaccinating patients with HPV16 E7 peptides. Therefore, future clinical studies in patients with a lower tumour burden seem warranted, using peptide vaccination as adjuvant therapy after surgery. Moreover, this approach will allow immunohistochemical determination of HLA expression, HPV detection in primary tumour or recurrence and detection of cytokines at the tumour site. The present study shows that treatment with HPV16 E7 peptides with a high binding affinity for HLA-A*0201 may be suitable for only a limited number of patients. Longer peptides, with binding affinities for other HLA tissue types including HLA class II binding epitopes, are currently produced in order to extend the applicability of this kind of vaccination to a larger group of patients. A phase I–II trial is currently in preparation addressing the aforementioned issues. These forth coming studies will make use of the longer peptides administered in either Montanide ISA 51 adjuvant or pulsed on to dendritic cells. At first patients with either recurrent or advanced cervical carcinoma will be treated. Subsequently, patients with early stage disease will be treated after initial surgical treatment. We hope these trials will provide us with more detailed knowledge and a clearer picture of how HPV peptide vaccination can contribute to the treatment and ultimately prevention of cervical cancer.

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