ORIGINAL ARTICLE

Vaccination against HPV-16 Oncoproteins for Vulvar Intraepithelial Neoplasia

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ABSTRACT

BACKGROUND

Vulvar intraepithelial neoplasia is a chronic disorder caused by high-risk types of human papillomavirus (HPV), most commonly HPV type 16 (HPV-16). Spontaneous regression occurs in less than 1.5% of patients, and the rate of recurrence after treatment is high.

METHODS

We investigated the immunogenicity and efficacy of a synthetic long-peptide vaccine in women with HPV-16–positive, high-grade vulvar intraepithelial neoplasia. Twenty women with HPV-16–positive, grade 3 vulvar intraepithelial neoplasia were vaccinated three or four times with a mix of long peptides from the HPV-16 viral oncoproteins E6 and E7 in incomplete Freund's adjuvant. The end points were clinical and HPV-16–specific T-cell responses.

RESULTS

The most common adverse events were local swelling in 100% of the patients and fever in 64% of the patients; none of these events exceeded grade 2 of the Common Terminology Criteria for Adverse Events of the National Cancer Institute. At 3 months after the last vaccination, 12 of 20 patients (60%; 95% confidence interval [CI], 36 to 81) had clinical responses and reported relief of symptoms. Five women had complete regression of the lesions, and HPV-16 was no longer detectable in four of them. At 12 months of follow-up, 15 of 19 patients had clinical responses (79%; 95% CI, 54 to 94), with a complete response in 9 of 19 patients (47%; 95% CI, 24 to 71). The complete-response rate was maintained at 24 months of follow-up. All patients had vaccine-induced T-cell responses, and post hoc analyses suggested that patients with a complete response at 3 months had a significantly stronger interferon-γ–associated proliferative CD4+ T-cell response and a broad response of CD8+ interferon-γ T cells than did patients without a complete response.

CONCLUSIONS

Clinical responses in women with HPV-16–positive, grade 3 vulvar intraepithelial neoplasia can be achieved by vaccination with a synthetic long-peptide vaccine against the HPV-16 oncoproteins E6 and E7. Complete responses appear to be correlated with induction of HPV-16–specific immunity.

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N Engl J Med 2009;361:1838-47.

Vulvar intraepithelial neoplasia is a chronic premalignant disorder of the vulvar skin that is caused by high-risk types of human papillomavirus (HPV); HPV type 16 (HPV-16) is involved in more than 75% of cases.[1-5] Spontaneous regression occurs in less than 1.5% of patients, and the rate of recurrence after surgical treatment is high.[6] Expression of the viral oncoproteins E6 and E7 contributes to the development of these genital lesions and their progression to invasive carcinoma.[6,7]

Virus-specific, interferon-γ–producing CD4+ T cells and CD8+ cytotoxic T lymphocytes are essential components in controlling chronic viral infections.[8,9] In the blood of patients with high-grade (grade 3) vulvar intraepithelial neoplasia lesions, these T cells, directed against HPV-16 viral oncoproteins E6 and E7, are not detectable or are present in low numbers.[10-13] Vaccination might overcome this inertia of the immune system.

Recently, we reported that in patients with cervical cancer, vaccination with synthetic long peptides, spanning the complete sequence of the two oncogenic proteins E6 and E7 of HPV-16, induces an HPV-16–specific CD4+ and CD8+ T-cell response.[14,15] To assess the clinical efficacy of this vaccine, we performed a phase 2 study involving women with HPV-16–positive, grade 3 vulvar intraepithelial neoplasia.

Methods

Patients

Patients with histologically confirmed HPV-16–positive, grade 3 vulvar intraepithelial neoplasia were eligible for the study. Additional criteria for eligibility were a good performance status (World Health Organization performance status score of 1 or 2 [on a scale of 0 to 5, with lower scores indicating better performance status], a Karnofsky performance score of ≥60 [on a scale of 0 to 100, with higher scores indicating better performance status], or both), normal pretreatment laboratory blood values as described previously,[15] and no pregnancy, immunosuppressive medication or viral diseases, or diseases associated with immunodeficiency.

Study Design

In view of the low rate of spontaneous regression of lesions in patients with grade 3 vulvar intraepithelial neoplasia,[6] a single-center, single-group (noncontrolled), observational phase 2 study was designed. The end points were clinical efficacy and HPV-16–specific T-cell responses. If no clinical response was observed in the first 10 patients, the trial would be discontinued; otherwise, more patients would be enrolled, for a total of 20 patients who could be evaluated. Patients received three or four vaccinations. The vaccine was administered subcutaneously at 3-week intervals, each time in a different arm or leg. All vaccinations were performed between October 2004 and May 2007.

The study was sponsored by the Dutch Cancer Society, the European Union, and ISA Pharmaceuticals and approved by the medical ethics committee of the Leiden University Medical Center, and all patients gave written informed consent. All drafts of the manuscript were prepared by the writing committee, which consisted of three academic authors and one academic author who was also employed by the sponsor; the final draft was submitted for publication with approval by the coauthors. All authors vouch for the completeness and accuracy of the data presented.

Composition of the Vaccine

The vaccine contained nine HPV-16 E6 and four HPV-16 E7 synthetic peptides (see the Supplementary Appendix, available with the full text of this article at NEJM.org) dissolved in dimethylsulfoxide in 20 mM of phosphate-buffered saline (pH 7.5) and emulsified with incomplete Freund's adjuvant (Montanide ISA-51, Seppic); the final ratio of dimethylsulfoxide to phosphate-buffered saline to adjuvant by volume was 20:30:50. One dose of each vaccine contained 0.3 mg of each peptide in a total volume of 2.8 ml.

Assessment of Clinical Efficacy

The variables for evaluation of clinical responses were symptoms, lesion size, histologic features, and the presence or absence of HPV-16 DNA. The lesions were described in detail and measured bidimensionally by a single investigator, with the largest diameters in two dimensions recorded. In the case of a multifocal lesion, the total lesion size was determined. Symptoms were classified as none, mild to moderate (not interfering with daily life), or severe (interfering with daily life). All lesions were monitored by means of digital photography. A partial response was defined as a decrease in lesion size of 50% or more after the vaccinations. A complete clinical response was defined as complete disappearance of the lesions and symptoms of vulvar intraepithelial neoplasia.

No response was defined as a reduction of less than 50% in lesion size.

Biopsy specimens of the same lesion were obtained before and 3 months after the last vaccination. For histologic analysis, the specimens were prepared in formalin and stained with hematoxylin and eosin. Grading of the lesions was performed by one experienced pathologist according to the criteria of the Armed Forces Institute of Pathology, with consensus review by two pathologists. HPV typing of paraffin-embedded sections of biopsy specimens was performed with the use of three general HPV primer sets (CPI/II, MY 9/11, and GP 5+/6+), followed by sequencing and analysis by the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information. Beta-globin polymerase-chain-reaction product and a blank sample were included as controls.[16-18] All histologic and virologic results were collected in a blinded fashion with respect to clinical outcome.

SAFETY AND TOLERABILITY

Monitoring for spontaneous adverse events and injection-site reactions, clinical assessments, and laboratory tests were performed after each vaccination and thereafter every 3 months for a total of 24 months of follow-up, as described previously.[14] Adverse events were graded according to version 3.0 of the Common Terminology Criteria for Adverse Events (CTCAE), which grades events on a scale of 1 to 5, with higher grades indicating greater severity.[14]

IMMUNOLOGIC MONITORING OF HPV-16–SPECIFIC T-CELL RESPONSES

Peripheral-blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood samples by means of Ficoll density-gradient centrifugation, with a portion of the cells subjected immediately to lymphocyte stimulation tests and the remaining cells cryopreserved. HPV-16–specific T-cell responses were determined with the use of a previously described set of complementary assays according to standard-operating-procedure protocols with predefined criteria for positive and vaccine-induced responses.[15] These tests were performed by trained personnel (see the Supplementary Appendix).

STATISTICAL ANALYSIS

Clinical responses are reported as the percentage of patients with a given response and the 95% confidence interval. Prespecified analyses were used for the measurement of vaccine-induced HPV-16–specific T-cell responses (see the Supplementary Appendix). A response rate of 30% was considered promising. In total, five post hoc subgroup analyses were performed and are reported here. The clinical response at 24 months was assessed according to the size of the lesion at study entry. For this analysis, the patients were divided into two groups (those with either no response or a partial response at 12 months, and those with a complete response at 12 months), and the between-group difference in the average lesion size at baseline was calculated with the use of Welch's corrected unpaired t-test. The strength of the different types of immune responses and the number of different CD8+ T-cell epitopes were compared between the group with no clinical response and the group with a complete clinical response 3 months after the last vaccination, with the use of the nonparametric Mann–Whitney test and GraphPad InStat software, version 3.00. For each type of immune assay, the strength of the immune response for no clinical response, partial clinical response, or complete clinical response was operationally defined as a combination of the magnitude and breadth (i.e., the number of different peptide pools recognized by an individual patient's T cells) of the T-cell response by taking the median number of HPV-16–specific spots (interferon-γ enzyme-linked immunosorbent spot [ELISPOT] assay), the median stimulation index (lymphocyte stimulation test), or the median amount of interferon-γ production (cytometric bead array) obtained for all six peptide pools per patient in the group (e.g., the median of 6 peptide pools × 5 patients = 30 data points in the complete-response group and 6 peptide pools × 8 patients = 48 data points in the no-response group). All reported P values are two-sided and have not been adjusted for multiple comparisons. A P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

STUDY POPULATION

Characteristics of the enrolled patients are summarized in Table 1. Thirty patients were screened for the trial. Two patients withdrew (Patients 15 and 20), and one patient (Patient 24) had vaginal intraepithelial neoplasia. Five patients did not meet other inclusion criteria: in one patient (Pa-

Table 1. Baseline Characteristics of the Patients.

Patient No.	Age	Duration of Neoplasia	Lesion Size	Type of Neoplasia	Symptoms*
	yr	*mo*	*cm²*		
1	50	123	10	Multifocal	Mild to moderate
2	48	155	3	Unifocal	Severe
3	46	142	34	Multifocal	Severe
6	61	16	8	Unifocal	None
7	43	26	9	Unifocal	Mild to moderate
8	56	62	12	Unifocal	Severe
9	39	121	4	Unifocal	Mild to moderate
10	35	34	10	Multifocal	Mild to moderate
11	23	9	2	Multifocal	Mild to moderate
12	30	8	18	Multifocal	Mild to moderate
13	51	43	16	Multifocal	Mild to moderate
16	42	14	6	Multifocal	Mild to moderate
18	44	133	12	Unifocal	Severe
22	36	4	24	Multifocal	Severe
23	46	185	15	Unifocal	Severe
26	37	97	35	Multifocal	None
27	49	4	4	Multifocal	Mild to moderate
28	47	4	5	Unifocal	Mild to moderate
29	36	7	5	Multifocal	Mild to moderate
30	52	3	8	Multifocal	Mild to moderate

* Symptoms were classified as mild to moderate if they did not interfere with daily life and as severe if they interfered with daily life.

tient 19), the lesion was not grade 3, in three patients (Patients 5, 17, and 25) the grade 3 lesion was not HPV-16–positive, and one patient (Patient 14) received immunosuppressive medication. The remaining 22 patients with histologically confirmed HPV-16–positive, grade 3 vulvar intraepithelial neoplasia were included in the study; 20 of these patients received three or four vaccinations and were evaluated. The two patients (Patients 9 and 27) who received three vaccinations declined to receive the last vaccination because of inconvenience. One patient (Patient 4) received only two vaccinations because of symptoms associated with the vaccination site in her leg. The fact that she worked as a swimming teacher and stood in water most of the day may have been associated with these symptoms. Another patient (Patient 21) received only one vaccination because she subsequently moved abroad.

ADVERSE EVENTS

Table 2 lists all systemic adverse events assessed as possibly being associated with the vaccine in 22 patients who received at least one vaccination. All patients reported local swelling, redness, and increased skin temperature, and 95% of the patients reported local pain at the vaccination site (see the Table in the Supplementary Appendix). These symptoms and fever at night after the vaccination were the most common adverse events, as observed previously.[14] Systemic adverse events, including influenza-like symptoms, chills, tiredness, or all of these symptoms, within 24 to 48 hours after vaccination, typically started after the second vaccination. No vaccine-related events exceeded grade 2 according to the CTCAE. One patient (Patient 10) died 12 months after the fourth vaccination as a result of sudden heart failure. No postmortem investigation was performed. This event was considered to be unrelated to the vaccination.

Table 2. Systemic Adverse Events in 22 Patients Who Received at Least One Vaccination.

Event	Patients (N=22)*			Vaccinations (N=81)		
	CTCAE Grade 1	CTCAE Grade 2	CTCAE Grade 1 or 2	CTCAE Grade 1	CTCAE Grade 2	CTCAE Grade 1 or 2
	no.		*total no. (%)*	*no.*		*total no. (%)*
Fever	9	5	14 (64)	10	8	18 (22)
Chills	4	0	4 (18)	5	0	5 (6)
Malaise	3	2	5 (23)	3	2	5 (6)
Nausea	2	1	3 (14)	2	1	3 (4)
Vomiting	2	0	2 (9)	2	0	2 (2)
Dizziness	1	0	1 (5)	1	0	1 (1)
Rash	4	0	4 (18)	5	0	5 (6)
Inability to concentrate	0	1	1 (5)	0	1	1 (1)
Palmar erythema	1	0	1 (5)	1	0	1 (1)
Swelling in arms	0	1	1 (5)	0	1	1 (1)
Tiredness	3	0	3 (14)	3	0	3 (4)
Pain in arms and legs	0	2	2 (9)	1	2	3 (4)

* If an event occurred more than once in a patient, it was counted only once. CTCAE denotes Common Terminology Criteria for Adverse Events.

CLINICAL EFFICACY

At 3 months of follow-up (range, 2.5 to 4.0), symptom relief was reported by 11 patients; 5 of 20 patients had a complete clinical and histologic response (25%; 95% confidence interval [CI], 9 to 49) (Fig. 1), and 7 other patients (35%; 95% CI, 15 to 59) had a partial response. Concomitant loss of HPV-16 from the original lesion sites was observed in four of the patients who had a complete response (Table 3).

At 12 months of follow-up, 15 of 19 patients (79%; 95% CI, 54 to 94) had an objective clinical response: the number of patients with a complete response increased to 9 (47%; 95% CI, 24 to 71), a partial response was seen in 6 patients (32%; 95% CI, 13 to 57), and no response was seen in 3 patients (16%; 95% CI, 3 to 40). Invasive carcinoma developed in one patient (Patient 2) at 6 months of follow-up. One patient died. Symptom relief was reported by 12 patients (63%; 95% CI, 38 to 84) (Table 3).

All patients with a complete response were still free of disease at 24 months of follow-up, whereas microinvasion was observed in one patient with a partial response (Patient 23). A carcinoma developed in two other patients (Patients 1 and 3) 3.5 and 2.5 years, respectively, after the last vaccination.

Post hoc analysis of the mean (±SD) lesion size at study entry and clinical outcome at 12 months suggested that, on average, the lesions were smaller in the complete-response group than in the group with no response or a partial response (6.4±3.1 cm^2 vs. 17.2±11 cm^2, (P=0.01).

IMMUNOLOGIC RESPONSES

After they received the last vaccination, 85% of the patients had circulating vaccine-induced HPV-16–specific T cells, which proliferated when stimulated with E6, E7, or both. Proliferation was accompanied by the production of both T-helper type 1 (Th1) and T-helper type 2 (Th2) cytokines, mostly interferon-γ and interleukin-5 (Table 4) and, to a lesser extent, by tumor necrosis factor α, interleukin-4, interleukin-10, or all of these cytokines. In addition, 83% of the patients had a T-cell response against HPV-16, as determined by interferon-γ ELISPOT assay. Some preexisting HPV-16 T-cell immunity that was E6-specific, E7-specific, or both was detected in six patients (including three patients who had a complete response); this immunity was boosted by vaccination and coincided with enhanced interferon-γ production. No boost was observed in one patient, and she did not have an immunologic response to the vaccine. We were able to obtain blood samples

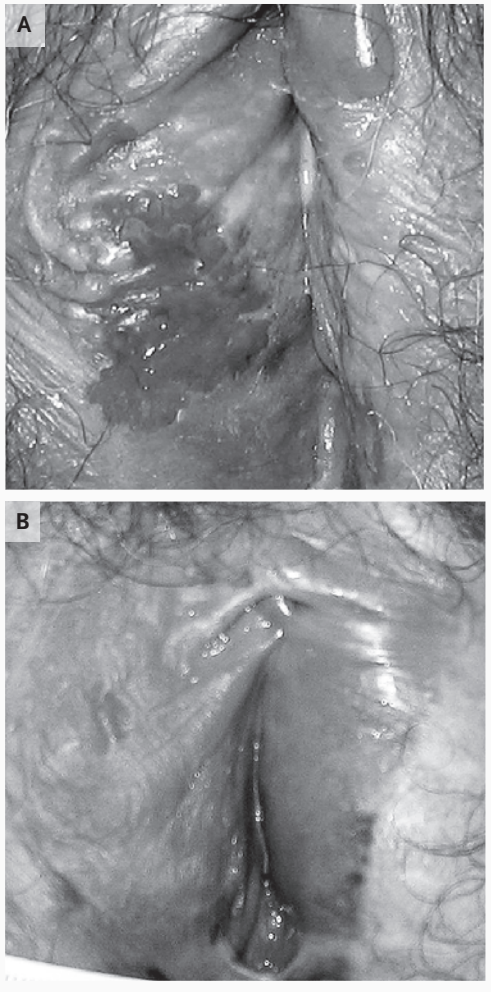

Figure 1. Complete Clinical Response to Vaccination in a Patient with Grade 3 Vulvar Intraepithelial Neoplasia.

Panel A shows a typical lesion in Patient 16 before the start of the treatment, and Panel B shows complete regression of the lesion in the same patient 12 months after four vaccinations with a mix of synthetic long peptides from the human papillomavirus type 16 oncoproteins E6 and E7 in incomplete Freund's adjuvant.

from a number of patients 1 to 2 years after the last vaccination. In 12 of 14 patients, strong HPV-16–specific proliferative responses were still detectable. Analyses of PBMCs after one round of in vitro stimulation by multiparameter flow cytometry revealed the presence of vaccine-induced HPV-16–specific CD4+ T cells in all 20 patients and of CD8+ T cells in 83% of the patients. The CD8+ T-cell responses were predominantly against the HPV-16 oncoprotein E6 (Table 4).

The immunologic analysis in which PBMCs were tested against four different pools of overlapping E6 peptides and two pools of E7 peptides not only measured the presence of a vaccine-induced T-cell response but also allowed the calculation of the strength of the immune response, operationally defined as a combination of the magnitude and breadth of the T-cell response to all six peptide pools. To assess the correlation between the strength of the vaccine-induced immune response and the clinical outcome, the patients were classified as having no response, a partial response, or a complete response according to their clinical status at the predetermined clinical-evaluation point of 3 months of follow-up. We focused on the eight patients with no response and the five patients with a complete response, since these groups were clinically well segregated and a comparison of them was therefore likely to reveal any potential correlation with immunity. Our post hoc subgroup analyses showed that as compared with the patients who had no clinical response, the group of patients with a complete response had a significantly higher number of interferon-γ–producing CD4+ T cells ($P=0.001$) (Fig. 2A) and stronger proliferative responses ($P=0.005$) (Fig. 2B), which were associated with the production of larger amounts of interferon-γ ($P=0.02$) (Fig. 2C). Moreover, all patients with a complete response had an HPV-16–specific CD8+ T-cell response, although the mean number of T-cell epitopes detected in this group was not significantly higher than in patients with no response ($P=0.17$) (Fig. 2D). Patient 2, in whom an invasive carcinoma developed, had an HPV-16–specific proliferative response that was associated with the production of only low amounts of interferon-γ (mean, 160 pg per milliliter) and no other Th1 and Th2 cytokines. These results suggest that the clinical efficacy of a therapeutic HPV-16 vaccine may be determined by its capacity to induce a strong and broad multifunctional immune response to the HPV-16 oncoproteins E6 and E7.

DISCUSSION

This study shows that vaccination with synthetic long peptides that represent the entire length of the two oncoproteins E6 and E7 of HPV-16 is effective over a period of 12 to 24 months for the treatment of high-grade vulvar intraepithelial

Table 3. Clinical Results at 3, 12, and 24 Months after the Last Vaccination.*

Patient No.	No. of Vaccinations	At 3 Months				At 12 Mo		At 24 Mo
		Symptoms	Lesion Response	Histologic Findings	Type of HPV Infection	Symptoms	Lesion Response	Lesion Response
1	4	Mild to moderate	Partial	VIN 2	16	Mild to moderate	Partial	Partial†
2	4	Severe	None	VIN 3	16		Carcinoma	
3	4	Severe	None	VIN 3	16	None	Partial	Partial‡
6	4	None	Complete	Normal	16	None	Complete	Complete
7	4	None	Complete	Normal	None	None	Complete	Complete
8	4	Mild to moderate	Complete	Normal	6b	None	Complete§	Complete
9	3	None	Complete	Normal	None	None	Complete	Complete
10	4	None	Partial	VIN 3	16	Lost to follow-up¶		
11	4	None	None	VIN 3	16	None	Complete	Complete
12	4	Mild to moderate	None	VIN 3	16	Mild to moderate	Partial	None‖
13	4	Mild to moderate	Partial	VIN 3	16	Mild to moderate	Partial	Partial
16	4	Mild to moderate	Partial	VIN 1	16	Mild to moderate	Complete	Complete
18	4	Severe	None	VIN 3	16	Severe	None	None
22	4	Mild to moderate	None	VIN 3	16	Severe	Partial	Partial
23	4	Mild to moderate	Partial	VIN 2	16	None	Partial	Microinvasive carcinoma**‖
26	4	None	None	VIN 3	16	None	None	None
27	3	None	Partial	VIN 3	16	None	Complete	Complete
28	4	None	None	VIN 3	16	None	None	None
29	4	None	Complete	Normal	None	None	Complete	Complete
30	4	Mild to moderate	Partial	VIN 2	16	None	Complete	Complete

* Symptoms were classified as mild to moderate if they did not interfere with daily life and as severe if they interfered with daily life. HPV denotes human papillomavirus, VIN 2 grade 2 vulvar intraepithelial neoplasia, and VIN 3 grade 3 vulvar intraepithelial neoplasia.
† Carcinoma developed in this patient 3.5 years after the last vaccination.
‡ Carcinoma developed in this patient 2.5 years after the last vaccination.
§ A condyloma was removed from this patient 6 months after the last vaccination.
¶ This patient died of sudden heart failure before the 12-month follow-up.
‖ There was no lesion response as compared with the lesion before vaccination.
** Microinvasive carcinoma developed in this patient 13 months after the last vaccination.

neoplasia lesions. This clinical efficacy is probably related to a vaccine-induced HPV-16–specific T-cell response.

Therapeutic vaccination targeting an established, persistent viral infection had a clinical benefit in a number of our patients with grade 3 vulvar intraepithelial neoplasia. Regression of HPV-16–positive lesions was noted in the majority of patients (79%), and complete regression was observed in 47% of patients 1 year after the last dose of vaccine. The number of complete responses increased from five to nine between 3 and 12 months after the last vaccination, and this response was maintained at 24 months. Since the rate of spontaneous regression of lesions in patients with grade 3 vulvar intraepithelial neoplasia is low (<1.5%),[6,19] the high response rate observed in the current trial is probably related to the vaccination.

Topical treatment with imiquimod cream was shown to induce complete regression of lesions in 9 of 26 patients with grade 3 vulvar intraepithelial neoplasia (35%).[19] Subgroup evaluations of these patients revealed a significant correlation between the presence of circulating, preexisting HPV-16–specific, interferon-γ–producing

Table 4. Immunologic Response Rate on Stimulation with HPV-16 Oncoproteins E6, E7, or Both, According to Immunologic Assay.*

Response	E6, E7, or Both	E6	E7	E6 and E7
		percent of patients		
Proliferation of HPV-16–specific T cells	85	80	70	65
Interferon-γ production	95	95	85	85
Interleukin-5 production	90	90	80	80
Interferon-γ ELISPOT	83	83	61	61
CD4+ T-cell reactivity	100	94	94	89
CD8+ T-cell reactivity	83	78	11	6

* ELISPOT denotes enzyme-linked immunosorbent spot, and HPV-16 human papillomavirus type 16.

T cells and regression of the HPV-16–positive lesions[13]; this correlation suggests that the clinical benefit of topical imiquimod cream may depend on the presence of interferon-γ–producing, HPV-16–specific T cells and that the use of imiquimod cream after vaccination may further increase the complete-response rate.

Like cervical intraepithelial neoplasia, vulvar intraepithelial neoplasia is caused by persistent infection with a high-risk type of HPV and can progress to invasive carcinoma.[20-24] Virus-specific T cells are essential components in the immune response to chronic viral infection.[8,9] As compared with patients, healthy subjects with evidence of previous exposure to HPV-16 have relatively strong recall T-cell responses against the early viral proteins; these responses are characterized by CD4+ T cells producing mixed cytokines, including interferon-γ and interleukin-5.[25-28] In addition, most patients clearing HPV-16 have a CD8+ cytotoxic T-lymphocyte response that is specific for the HPV-16 oncoprotein E6.[29,30] The notion that the lack of generation of interferon-γ–producing T cells allows persistent virus infection is strengthened by our finding that such T-cell responses are weak or lacking in patients with cervical cancer,[28] cervical intraepithelial neoplasia,[28,31,32] or grade 3 vulvar intraepithelial neoplasia.[10-13] The idea that these interferon-γ–producing T cells exert therapeutic activity is supported by our post hoc analyses of subgroups of patients with grade 3 vulvar intraepithelial neoplasia, which showed a significant correlation between vaccine-induced complete regression of lesions and higher numbers of HPV-specific CD4+ T cells producing large amounts of interferon-γ in patients with a complete response than in patients with no response. Furthermore, vaccine-induced CD8+ T-cell responses were predominantly directed against HPV-16 oncoprotein E6. This finding is consistent with the association between spontaneous clearance of HPV infection and detection of CD8+ T-cell responses that are specific for HPV-16 oncoprotein E6.[29,30,33]

Our results underscore the importance of studying immunity to the antigen of interest in protected and nonprotected persons to identify assays that are likely to yield correlates of protection after vaccination — in our case, the interferon-γ ELISPOT assay, proliferation assays, and interferon-γ enzyme-linked immunosorbent assays.[10-13,25-28] Although vaccination with HPV peptides did not prevent the development of carcinoma in all patients, no carcinomas were seen in the group of patients who had a complete response. Moreover, all the patients in whom carcinomas developed had had vulvar intraepithelial neoplasia for more than 10 years (Table 1). In studies of other vaccines against HPV-16 oncoproteins E6 and E7, some clinical benefit was observed, but a durable complete response was observed only occasionally.[10-12] We ascribe the potent T-cell response induced by the vaccine to efficient dendritic-cell targeting, the absence of antigenic competition from viral vectors, and the high dose of specific antigen.[34] The therapeutic activity of this vaccine is consistent with our findings in rodent models, in which vaccination with long peptides induced strong, long-lasting, virus-specific T-cell immunity that was associ-

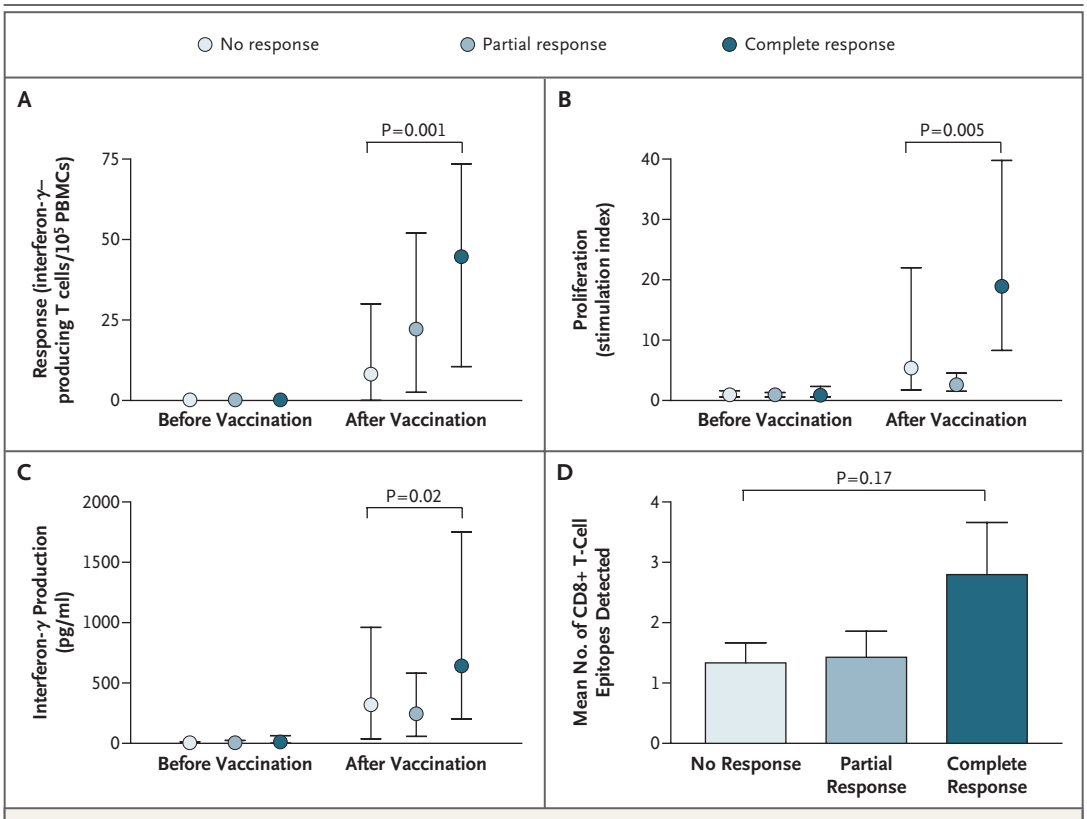

Figure 2. Immune Response before and after Vaccination.

The immune response was analyzed in blood samples obtained from eight patients with no clinical response, seven patients with a partial response, and five patients with a complete response, as observed at 3 months after the last vaccination. Panels A, B, and C show the immune response before the first vaccination and 2 weeks after the last vaccination. These panels also show the strength of the T-cell response that was specific for human papillomavirus type 16 (HPV-16) oncoproteins E6 and E7. The median number of interferon-γ–producing T cells per 100,000 peripheral-blood mononuclear cells (PBMCs) is shown, with the interquartile range (I bars). Panel B shows the strength of proliferation (median stimulation index and interquartile range [I bars]). Panel C shows interferon-γ production (median and interquartile range [I bars]) by proliferating PBMCs that were specific for HPV-16 oncoproteins E6 and E7. The strength of the immune response was determined by calculating the median immune response in all patients in the group on the basis of data on six different peptide pools per patient. Panel D shows the mean number of CD8+ T-cell epitopes detected in the three groups of patients 2 weeks after the last vaccination. The T bars indicate 95% confidence intervals. P values in all four panels were calculated with the Mann–Whitney test.

ated with regression of lesions[35,36] and suppression of persistent viral infection.[35]

Supported by grants from the Dutch Cancer Society (RUL 2000-2200), the European Union Integrated Project Cancer Immunotherapy (LSHC-CT-2006-518234), the European Union Network of Excellence DC-THERA (LSHB-CT-2004-512074), and ISA Pharmaceuticals. This study was conducted by the Leiden University Medical Center (LUMC), which holds a patent on the use of synthetic long peptides as vaccine (U.S. patent number 7,202,034). The LUMC does not share the financial benefit from this patent with its employees. ISA Pharmaceuticals, a biotechnology company, has licensed the patent from the LUMC.

Dr. Melief reports having a stock-appreciation right that is the equivalent of a stock option in 1% of the issued share capital of ISA Pharmaceuticals and being named as an inventor on the patent for the use of synthetic long peptides as vaccine and serving as a member of the steering committee of ISA Pharmaceuticals; Drs. Kenter, Oostendorp, and Drijfhout, serving as nonpaid members of the strategy team of ISA Pharmaceuticals and holding no financial interest in the company; Dr. Offringa, being named as one of the inventors on the patent for the use of synthetic long peptides as vaccine but holding no financial interest and being involved in the study before he was employed by Genentech; and Dr. van der Burg, being named as one of the inventors on the patent for the use of synthetic long peptides as vaccine but holding no financial interest and serving as a nonpaid member of the steering committee of ISA Pharmaceuticals. No other potential conflict of interest relevant to this article was reported.

We thank the patients who participated in the study as well as their referring physicians; C.L.M.C. Franken (Leiden University Medical Center, the Netherlands) for preparing the recombinant human proteins used; and Dr. V.T.B.H.M. Smit for kindly reviewing the pathological samples.

REFERENCES

1. Buscema J, Naghashfar Z, Sawada E, Daniel R, Woodruff JD, Shah K. The predominance of human papillomavirus type 16 in vulvar neoplasia. Obstet Gynecol 1988;71:601-6.
2. Hording U, Junge J, Poulsen H, Lundvall F. Vulvar intraepithelial neoplasia III: a viral disease of undetermined progressive potential. Gynecol Oncol 1995;56:276-9.
3. van Beurden M, ten Kate FJ, Smits HL, et al. Multifocal vulvar intraepithelial neoplasia grade III and multicentric lower genital tract neoplasia is associated with transcriptionally active human papillomavirus. Cancer 1995;75:2879-84.
4. Insinga RP, Liaw KL, Johnson LG, Madeleine MM. A systematic review of the prevalence and attribution of human papillomavirus types among cervical, vaginal, and vulvar precancers and cancers in the United States. Cancer Epidemiol Biomarkers Prev 2008;17:1611-22.
5. Srodon M, Stoler MH, Baber GB, Kurman RJ. The distribution of low and high-risk HPV types in vulvar and vaginal intraepithelial neoplasia (VIN and VaIN). Am J Surg Pathol 2006;30:1513-8.
6. van Seters M, van Beurden M, de Craen AJ. Is the assumed natural history of vulvar intraepithelial neoplasia III based on enough evidence? A systematic review of 3322 published patients. Gynecol Oncol 2005;97:645-51.
7. Daling JR, Madeleine MM, Schwartz SM, et al. A population-based study of squamous cell vaginal cancer: HPV and cofactors. Gynecol Oncol 2002;84:263-70.
8. Matloubian M, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. J Virol 1994;68:8056-63.
9. Zajac AJ, Murali-Krishna K, Blattman JN, Ahmed R. Therapeutic vaccination against chronic viral infection: the importance of cooperation between CD4+ and CD8+ T cells. Curr Opin Immunol 1998;10:444-9.
10. 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.
11. 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.
12. Smyth LJ, van Poelgeest MI, 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.
13. van Poelgeest MI, van Seters M, van Beurden M, et al. Detection of human papillomavirus (HPV) 16-specific CD4+ T-cell immunity in patients with persistent HPV16-induced vulvar intraepithelial neoplasia in relation to clinical impact of imiquimod treatment. Clin Cancer Res 2005;11:5273-80.
14. Kenter GG, Welters MJ, Valentijn AR, et al. 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. Clin Cancer Res 2008;14:169-77.
15. Welters MJ, 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;14:178-87.
16. de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ. The use of general primers GP5 and GP6 elongated at their 3′ ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 1995;76:1057-62.
17. Tieben LM, ter Schegget J, Minnaar RP, et al. Detection of cutaneous and genital HPV types in clinical samples by PCR using consensus primers. J Virol Methods 1993;42:265-79.
18. van den Brule AJ, Pol R, Fransen-Daalmeijer N, Schouls LM, Meijer CJ, Snijders PJ. GP5+/6+ PCR followed by reverse line blot analysis enables rapid and high-throughput identification of human papillomavirus genotypes. J Clin Microbiol 2002;40:779-87.
19. van Seters M, van Beurden M, ten Kate FJ, et al. Treatment of vulvar intraepithelial neoplasia with topical imiquimod. N Engl J Med 2008;358:1465-73.
20. zur Hausen H. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000;92:690-8.
21. Bosch FX, Lorincz A, Muñoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 2002;55:244-65.
22. Ostör AG. Natural history of cervical intraepithelial neoplasia: a critical review. Int J Gynecol Pathol 1993;12:186-92.
23. Herod JJ, Shafi MI, Rollason TP, Jordan JA, Luesley DM. Vulvar intraepithelial neoplasia: long term follow up of treated and untreated women. Br J Obstet Gynaecol 1996;103:446-52.
24. Jones RW, Rowan DM, Stewart AW. Vulvar intraepithelial neoplasia: aspects of the natural history and outcome in 405 women. Obstet Gynecol 2005;106:1319-26.
25. van der Burg SH, Ressing ME, Kwappenberg KM, et al. Natural T-helper immunity against human papillomavirus type 16 (HPV16) E7-derived peptide epitopes in patients with HPV16-positive cervical lesions: identification of 3 human leukocyte antigen class II-restricted epitopes. Int J Cancer 2001;91:612-8.
26. 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.
27. Welters MJ, de Jong A, van den Eeden SJ, 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.
28. de Jong A, van Poelgeest MI, 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.
29. Nakagawa M, Stites DP, Patel S, et al. Persistence of human papillomavirus type 16 infection is associated with lack of cytotoxic T lymphocyte response to the E6 antigens. J Infect Dis 2000;182:595-8.
30. Nakagawa M, Stites DP, Farhat S, et al. Cytotoxic T lymphocyte responses to E6 and E7 proteins of human papillomavirus type 16: relationship to cervical intraepithelial neoplasia. J Infect Dis 1997;175:927-31.
31. Frazer IH, Quinn M, Nicklin JL, et al. Phase 1 study of HPV16-specific immunotherapy with E6E7 fusion protein and ISCOMATRIX adjuvant in women with cervical intraepithelial neoplasia. Vaccine 2004;23:172-81.
32. de Vos van Steenwijk PJ, Piersma SJ, Welters MJ, et al. Surgery followed by persistence of high-grade squamous intraepithelial lesions is associated with the induction of a dysfunctional HPV16 specific T-cell response. Clin Cancer Res 2008;14:7188-95.
33. Nimako M, Fiander AN, Wilkinson GW, Borysiewicz LK, Man S. Human papillomavirus-specific cytotoxic T lymphocytes in patients with cervical intraepithelial neoplasia grade III. Cancer Res 1997;57:4855-61.
34. Melief CJ, van der Burg SH. Immunotherapy of established (pre)malignant disease by synthetic long peptide vaccines. Nat Rev Cancer 2008;8:351-60.
35. 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.
36. 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.