Abstract

Purpose: The study aims to evaluate the effect of a human papillomavirus type 16 (HPV16) E6 and E7 synthetic long peptides vaccine on the antigen-specific T-cell response in cervical cancer patients.

Experimental Design: Patients with resected HPV16-positive cervical cancer were vaccinated with an overlapping set of long peptides comprising the sequences of the HPV16 E6 and E7 oncoproteins emulsified in Montanide ISA-51. HPV16-specific T-cell immune responses were analyzed by evaluating the magnitude, breadth, type, and polarization by proliferation assays, IFN-γ-ELISPOT, and cytokine production and phenotyped by the T-cell markers CD4, CD8, CD25, and Foxp3.

Results: Vaccine-induced T-cell responses against HPV16 E6 and E7 were detected in six of six and five of six patients, respectively. These responses were broad, involved both CD4+ and CD8+ T cells, and could be detected up to 12 months after the last vaccination. The vaccine-induced responses were dominated by effector type CD4+CD25-Foxp3+ type 1 cytokine IFN-γ-producing T cells but also included the expansion of T cells with a CD4+CD25+Foxp3+ phenotype.

Conclusions: The HPV16 E6 and E7 synthetic long peptides vaccine is highly immunogenic, in that it increases the number and activity of HPV16-specific CD4+ and CD8+ T cells to a broad array of epitopes in all patients. The expansion of CD4+ and CD8+ tumor-specific T cells, both considered to be important in the antitumor response, indicates the immunotherapeutic potential of this vaccine. Notably, part of the vaccine-induced T cells display a CD4+CD25+Foxp3+ phenotype that is frequently associated with regulatory T-cell function, suggesting that strategies to disarm this subset of T cells should be considered as components of immunotherapeutic modalities against HPV-induced cancers.

Cervical cancer is the second most common cancer in women worldwide (1), and it is the first cancer recognized by the WHO to be 100% attributable to the infection with a high-risk type of human papillomavirus (HPV; ref. 2). HPV type 16 (HPV16) is the most common carcinogenic type and is found in around 50% of invasive cervical tumors worldwide (3, 4). The occurrence of HPV-induced cancer is strongly associated with failure to mount a strong HPV-specific type 1 T-helper and CTL response (5–7), the lack of CD8+ T cells migrating into the tumor, the induction of HPV16-specific regulatory T cells, and the influx of regulatory T cells into the tumor (8, 9).

Currently, a preventive vaccine, providing protection against HPV16 and HPV18, has been registered. This vaccine is fully protective against persistent infection and the associated development of high-grade genital lesions (reviewed in ref. 10). Notably, the prophylactic vaccine showed no benefit in women who were already infected with the HPV types covered by this vaccine (reviewed in ref. 10). Therefore, there is still a need for a so-called therapeutic vaccine that is able to protect those who are already infected. Not only because the lack of resources among the nations and individuals that have the greatest need will limit the benefits of prophylactic vaccination (reviewed in ref. 10), but also because an estimated 5 million cervical cancer deaths will occur in the next 20 years due to existing HPV infections (11).

All HPV16-induced cervical cancer cells expresses the HPV16 E6 and E7 oncoproteins (3), which makes these proteins excellent target antigens for T cell–mediated immunotherapeutic strategies (12). We have developed a long E6 and E7 synthetic long peptides vaccine.
peptides vaccine, containing all potential CTL and T-helper epitopes, which may elicit an effective HPV16-specific response able to eradicate existing infections and lesions in human individuals. In a prime boost vaccination scheme, a HPV16 E7 long peptide vaccine induced strong HPV16-specific CD4+ and CD8+ T-cell immunity in mice. In addition, therapeutic vaccination of mice with this vaccine mediated the eradication of established HPV16-positive tumors (13). Subsequently, the long peptide vaccine concept was tested in the cottontail rabbit papillomavirus outbred rabbit model. Therapeutic vaccination of cottontail rabbit papillomavirus–infected rabbits with a vaccine consisting of long overlapping peptides covering the entire sequence of the cottontail rabbit papillomavirus E6 and E7 proteins in Montanide was able to significantly control wart growth and abrogate latent cottontail rabbit papillomavirus infection compared with controls (14). Based on these results a clinical grade synthetic long peptides vaccine, consisting of 13 long peptides covering the HPV16 E6 and E7 antigens, was produced. In a phase I trial, four s.c. injections with the pool of peptides in Montanide ISA-51 in patients with advanced cervical cancer revealed that this vaccine was safe and immunogenic (15).

To delineate the T-cell response, induced by the HPV16 synthetic long peptides vaccine in human beings, we have assessed the magnitude, breadth, type, and polarization of vaccine-induced HPV16-specific T cells in a group of six patients, who were vaccinated after surgical removal of their HPV16-positive cervical tumor.

**Patients, Materials, and Methods**

**HPV16 E6 and E7 synthetic long peptides vaccine**

The HPV16 vaccine is a long peptide–based vaccine that consists of a pool of nine overlapping E6 peptides and four overlapping E7 peptides, which are 25 to 35 amino acids long and show an overlap of 10 to 14 amino acids. These clinical grade peptides cover the complete sequence of HPV16 E6 and E7 proteins (human papillomavirus 1997, Los Alamos National Laboratory6) and were synthesized at the interdepartmental GMP facility of by the Department of Clinical Pharmacy and Toxicology. Synthesis was done using a CS Bio CS536 solid-phase peptide synthesizer (CS Bio Co.) according to the Fmoc protocol. After purification by high-performance liquid chromatography, the peptide integrity was checked by mass spectrometry on a VOYAGER DE-PRO MALDI-TOF (PE Biosystems) and on a QTOF mass spectrometry (Micromass UK Ltd.) and Edman degradation. The peptides were also sequenced on an HP gas-phase sequencer on a VOYAGER DE-PRO MALDI-TOF (PE Biosystems) and on a QTOF matrix 1 protein of A/PR/8/34 (M1) were used for comparison of the T-cell reaction to a common virus. The peptides were synthesized and dissolved as described previously (16).

Memory response mix consisted of tetanus toxoid (0.75 limus flocculentis/mL; National Institute of Public Health and the Environment), sonicated *Mycobacterium tuberculosis* (5 μg/mL; kind gift from Dr. P. Klutser, Royal Tropical Institute), and Candida (0.015%, HAL Allergenen Lab.). The response to the pools of influenza peptides and/or MRM was used as positive control in the assays (5).

**Immunomonitoring**

**Analysis of antigen-specific T cells by IFN-γ-ELISPOT.** IFN-γ-producing HPV-specific T cells were quantified using ELISPOT that was done as described previously (17–20). Briefly, peripheral blood mononuclear cells (PBMC) were seeded at a density of 2 × 10^6 cells per well in a 24-well plate (Costar) in 1 mL of ISCOVE’s medium (Bio-Whittaker) enriched with 10% human AB serum (Sigma), in the presence or absence of 5 μg/mL of indicated HPV16 E6-derived or E7-derived 22-mers peptides combined in pools. As a positive control, PBMC were cultured in the presence of indicated pools of influenza A/PR/8/34 M1 protein-derived peptides and/or memory response mix. After 4 days of incubation at 37°C, PBMC were harvested, washed, and seeded in four replicate wells at a density of 10^6 cells per well in a Multiscreen 96-well plate (Millipore) coated with an IFN-γ-catching antibody (Mabtech AB). Further antibody incubations and development of the ELISPOT was done according to the manufacturer’s instructions (Mabtech). Spots were counted with a fully automated computer-assisted video-imaging analysis system (BioSys 4000). Specific spots were calculated by subtracting the mean number of spots plus 2×SD of the medium only control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were considered to be increased compared with nonresponders when specific T-cell frequencies were ≥1 of 10,000 (17). T-cell frequencies were considered to be boosted by the vaccine when they were at least 3-fold higher than the baseline sample (18–20).

6 http://hpv-web.lanl.gov
Proliferative capacity of HPV16-specific T cells by lymphocyte stimulation test. The capacity of T cells to proliferate upon stimulation with the antigen was determined by short-time proliferation assay as described earlier (5, 21). Briefly, freshly isolated PBMCs (1.5 × 10^6) were seeded into eight-replicate wells of a 96-well U-bottomed plate (Costar) to which the indicated peptide pools were added at a final concentration of 10 μg/mL. No antigen served as background control, and memory response mix was taken along as a positive control. The test was conducted in ISCOVE’s medium containing 10% autologous serum. On day 6, supernatant was harvested for cytokine analysis, and subsequently, the cells were pulsed with 0.5 μCi [3H]thymidine per well and incubated for an additional 18 h. Then, the cells were harvested onto filters (Wallac) using the Micro Cell Skatron Harvester (Skatron Instruments AS) and counted on the 1205 Betaplate counter (Wallac). The average and SD of the eight medium only control wells were calculated, and the cutoff was defined as this average plus 3 × SD. The stimulation index was calculated as the average of tested eight wells divided by the average of the medium control eight wells. A positive proliferative response was defined as a stimulation index of at least 3, and the counts of at least six of the eight wells must be above the cutoff value (6). A vaccine-induced response was defined as a stimulation index, which is at least 3-fold higher than the baseline sample.

Cytokine polarization analysis. The supernatants isolated on day 6 of the proliferation assay were subjected to a Th1/Th2 inflammation cytokine bead array kit (BD Biosciences). In this array, the levels of IFNγ, tumor necrosis factor-α, interleukin 10 (IL-10), IL-5, IL-4, and IL-2 were determined. According to firm prescription, the proposed detection limit was 20 ng/mL. However, for IFNγ, the cutoff value was set to 10 ng/mL because the standard curve showed linearity starting at a concentration of 100 pg/mL. Positive antigen-specific cytokine production was defined as a cytokine concentration above the cutoff value and >2 × the concentration of the medium control (6). A vaccine-induced response was defined as at least a 3-fold increase in the antigen-specific cytokine production in the baseline sample.

Identification of T-cell type using intracellular IFNγ and IL-5 cytokine staining. PBMCs were presensitized for 10 days with a pool of all peptides of HPV16 E6, HPV16 E7, or influenza A/PR/8/34 M1 protein at a concentration of 2.5 μg/mL per peptide. T-cell growth factor (Zetopetrix) and recombinant human IL-15 (PeproTech) was added at a final concentration of 10% and 5 ng/mL, respectively, on day 1. On day 4, autologous PBMCs were thawed, and monocytes were allowed to adhere in X-vivo medium (Cambrex) to 24-well plates (Costar) for 2 h at 37°C before removal of the nonadhered cells. The monocytes were cultured for 6 days in x-vivo medium with 800 IU/mL recombinant human GM-CSF (Invitrogen), washed, and then pulsed with pools of peptide at a concentration of 5 μg/mL per peptide or pulsed with 10 μg/mL of indicated protein overnight. The next day, these antigen-loaded monocytes were washed and used as APC for the presensitized PBMC in the intracellular cytokine staining. After 1 h of coincubation at 37°C, the Golgi-mediated secretion of cytokines was inhibited by the addition of Brefeldin A (Sigma) to a final concentration of 10 μg/mL, after which the cells were incubated for an additional 5 h at 37°C. Then, the cells were harvested, transferred into a V-bottomed 96-well plate, washed twice with ice-cold PBS, and fixed with 50 μL of 4% paraformaldehyde on ice for 4 min. After fixation, the cells were washed once with cold PBS and once with PBS/sodium azide 0.02%/bovine serum albumin 0.5%/saponin 0.1% for permeabilization of the cell membrane. This was followed by incubation in 50 μL PBS/sodium azide 0.02%/bovine serum albumin 0.5%/saponin 0.1%/FCS 10% for 10 min on ice. Cells were washed twice with PBS/sodium azide 0.02%/bovine serum albumin 0.5%/saponin 0.1%, and supernatant was removed before 25 μL of PBS/sodium azide 0.02%/bovine serum albumin 0.5%/saponin 0.1% containing anti-IFNγ FITC (clone 4S.B3, BD Pharmingen), anti–IL-5 PE (clone JES1-39D10, BD Pharmingen), anti-CD4 APC (clone RPA-T4, BD Pharmingen), and anti–CD4-APC (clone RPA-T4, BD Pharmingen) was added. After 30 min on ice, the cells were washed, suspended in 50 μL of 1% paraformaldehyde, and analyzed by flow cytometry (22). A positive response was defined as at least twice the percentage of IFNγ-producing CD4+ or CD8+ T cells than 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. A vaccine-induced reaction was defined as at least a 3-fold increase in the percentage of antigen-specific IFNγ-producing T cells of the baseline sample.

Detection of HPV16-specific CD4+ Foxp3+ T cells. PBMCs (1-2 × 10^6) were cultured for 10 days in medium only, in the presence of 5 μg/mL HPV16 E6 pooled peptides or HPV16 E7 peptides. Then the cells were harvested, and 2 × 10^5 cells were stained for Foxp3 and CD25 according to the manufacturer (Bioscience). Briefly, the stimulated cells were first stained for surface markers CD25 (anti-CD25 FITC; clone M-A251, BD Pharmingen), CD4 (anti-CD4-APC; clone RPA-T4, BD Pharmingen), and CD8 (anti-CD8 PerCP, clone SK1, BD Pharmingen) before the cells were fixed and permeabilized. Blocking was done with 2% normal rat serum followed by the addition of anti-human Foxp3 (PCH101) antibody or rat isotype IgG2a control. Then the cells were washed and analyzed on the cytometer. As a positive control, a previously isolated HPV16-specific CD4+Foxp3+ regulatory T-cell clone (C148.31) and, as negative control, a HPV16-specific CD4+Foxp3+ T-cell clone (C271.9; ref. 8) were used. The fluorescence intensity of these two control clones was used to set the gates for the other samples in which the Foxp3 positivity of the stimulated polyclonal T-cell populations were analyzed. An antigen-induced up-regulation of Foxp3 or CD25 was defined as at least twice the percentages of Foxp3-positive or CD25-positive cells in the medium only control, and a vaccine-induced increase in Foxp3-positive cells was defined as at least a 3-fold increase compared with the percentages of the baseline sample for the same condition.

Results

Safety of the vaccine. In a phase II trial, a total of six patients were vaccinated on average of 4 months (median, 4 months; range, 3-42 months) after surgical dissection of their histologically proved HPV16-positive cervical carcinoma. The patient characteristics are shown in Table 1. Four of the patients had completed the vaccination regimen of four injections, and two patients stopped earlier because of death due to local recurrence (ID103) and the other (ID106) due to inconvenience after the vaccination. Two patients did not meet the inclusion criteria and were withdrawn before first vaccination. Similarly to what we observed in our phase I studies (15), the adverse events did not exceed grade II toxicity and were temporary. All patients experienced the vaccination as mildly painful. The pain vanished within 10 to 15 min. The local pain was graded lower than grade II according to the common toxicity criteria. Fever (during the first night, not exceeding 40°C and effectively treatable with paracetamol was scored as grade I toxicity), flu-like symptoms (lasting 1-2 days), swelling of the injection site and itching of other skin sites (such as neck, breast, and back, as well as fingers and feet) were observed but did not exceed grade II toxicity. The time of follow-up and the clinical status is given in Table 1. Patient ID100 displayed a local recurrence at 7 months after the last vaccination, and patient ID103 displayed a local recurrence after the third vaccination. In the other patients, no signs of recurrence were found during the time of follow-up.

Clin Cancer Res 2008;14(1) January 1, 2008 180 www.aacrjournals.org
Downloaded from clincancerres.aacrjournals.org on April 13, 2017. © 2008 American Association for Cancer Research.
Vaccine-Induced HPV16-Specific T-Cell Immunity in Cervical Cancer

The synthetic long peptides vaccine induces IFN-γ-producing circulating T cells. To study the effect of the vaccine on the immune system, PBMC isolated before vaccination, after two and four vaccinations were analyzed for the presence of HPV16-specific T cells by IFN-γ-ELISPOT analysis. All patients displayed an HPV16-specific T-cell response after vaccination (Fig. 1). In five of the six patients, HPV16 E6-specific IFN-γ-producing T cells were already detectable after 2 vaccinations and in patient ID105 IFN-γ-producing HPV16 E6-specific T cells were detected after four vaccinations had been completed. Figure 1A shows two typical examples (ID102 and ID107) of the results obtained in this analysis. No HPV16 E6-specific or E7-specific IFN-γ-producing T cells were detected in the baseline samples, whereas up to 250 specific spots per 10⁵ PBMC were detected after vaccination. The magnitude of the vaccine-induced T-cell response in this patient group is depicted in Fig. 1B. The overall response against HPV16 E6 was stronger than against HPV16 E7. T-cell frequencies up to 1 per 500 PBMC (i.e., 200 specific spots) were found against HPV16 E6, whereas the HPV16 E7-specific response did not exceed 1 per 2000 PBMC (i.e., 50 specific spots). Furthermore, whereas the HPV16 E6-specific IFN-γ-associated immune response already peaked after two vaccinations, the IFN-γ-associated immune response against HPV16 E7 required more vaccinations. After two vaccinations only, two of the six patients responded (ID106 and ID107), whereas after four vaccinations, also patients ID100 and ID105 displayed an E7-specific response. In all patients, an influenza-specific T-cell response was detected in the baseline and postvaccination sample (data not shown). Only four patients (ID100, ID103, ID106, and ID107) displayed an IFN-γ-associated T-cell response to the positive control antigen mixture memory response mix, whereas the T cells of the other two patients failed to produce IFN-γ at both time points (data not shown). In conclusion, in all six patients, the synthetic long peptides vaccine was able to mount an HPV16 E6-specific T-cell response and, in four patients, the HPV16 E7-specific T cells were enhanced.

Vaccine-induced HPV16-specific T cells proliferate and produce IFN-γ and IL-5. To analyze the proliferative capacity of HPV16-specific T cells before and after vaccination as well as during follow-up, PBMC were tested in a lymphocyte stimulation test in eight replicate wells. Based on our cutoff value (stimulation index ≥ 3), the PBMC of patients ID102 and ID105 showed a proliferative response against HPV16 E6 peptide pools 1 to 3 (ID102) and/or HPV16 E7 pool 2 (ID102 and ID105) before vaccination (Fig. 1C). The proliferative response of PBMC from patient ID100 to HPV16 E6 peptide pool 1 in the baseline sample was just below cutoff.

After the vaccinations all six patients responded to at least three of the four peptide pools of HPV16 E6 and to one or both peptide pools of HPV16 E7 (Fig. 1C). The preexisting proliferative responses of ID102 were boosted by the vaccine, whereas this was not the case for the E7-specific proliferative response of ID105. A close examination of the proliferative response after each vaccination showed that vaccine-induced T-cell responses were already measurable after one vaccination, but it also revealed that the magnitude of these responses was subject to variation (Fig. 2). T cells to some peptide pools proliferated stronger after one vaccination than after multiple vaccinations, whereas the proliferative response to other peptide pools was relatively constant in time (e.g., ID102, pool E6.1 and ID107 E6.4, respectively; Fig. 2). In three cases (ID100, ID105, and ID107), we could obtain a blood sample 6 to 12 months after the last vaccination to measure the HPV16 E6-specific and E7-specific proliferative response. Even then, strong proliferative T-cell responses were observed in patients ID100 and ID107, whereas in patient ID105 the

### Table 1. Patient characteristics and adverse events observed

<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>T.N.M</th>
<th>Diagnose</th>
<th>Invasion depth</th>
<th>HPV type</th>
<th>Time (mo)</th>
<th>N vacc</th>
<th>Adverse events</th>
<th>Study status</th>
<th>FU (mo)</th>
<th>Clinical status (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>33</td>
<td>1B1.0.0</td>
<td>SCC</td>
<td>9 mm</td>
<td></td>
<td>16</td>
<td>42</td>
<td>4 Fever, flu-like symptoms, itching injection site, swelling injection site; burning eyes</td>
<td>Completed</td>
<td>27</td>
<td>Recurrence (7)</td>
</tr>
<tr>
<td>101</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>Fever, flu-like symptoms, itching injection site, swelling injection site, itching neck, back, breast</td>
<td>Withdrawn</td>
<td>24</td>
<td>Free of disease</td>
</tr>
<tr>
<td>102</td>
<td>59</td>
<td>1B1.0.0</td>
<td>SCC</td>
<td>6 mm</td>
<td></td>
<td>16</td>
<td>4</td>
<td>4 Fever, flu-like symptoms, itching injection site, swelling injection site, itching fingers and feet</td>
<td>Completed</td>
<td>9</td>
<td>Recurrence (0)</td>
</tr>
<tr>
<td>103</td>
<td>35</td>
<td>1B1.0.0</td>
<td>SCC</td>
<td>13 mm</td>
<td></td>
<td>16</td>
<td>3</td>
<td>3 Fever, swelling injection site, itching fingers and feet</td>
<td>Not completed</td>
<td>9</td>
<td>Recurrence (0)</td>
</tr>
<tr>
<td>104</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td>Withdrawn</td>
<td>13</td>
<td>Free of disease</td>
</tr>
<tr>
<td>105</td>
<td>34</td>
<td>1B1.0.0</td>
<td>SCC</td>
<td>8 mm</td>
<td></td>
<td>16</td>
<td>6</td>
<td>4 Swelling injection site, itching injection site</td>
<td>Withdrawn</td>
<td>10</td>
<td>Free of disease</td>
</tr>
<tr>
<td>106</td>
<td>58</td>
<td>1B1.0.0</td>
<td>ADC</td>
<td>not determined</td>
<td></td>
<td>16</td>
<td>4</td>
<td>3 Flu-like symptoms, swelling injection site</td>
<td>Not completed</td>
<td>13</td>
<td>Free of disease</td>
</tr>
<tr>
<td>107</td>
<td>33</td>
<td>1B1.0.0</td>
<td>ADC</td>
<td>4.5 mm</td>
<td></td>
<td>16</td>
<td>4</td>
<td>4 Swelling injection site</td>
<td>Completed</td>
<td>10</td>
<td>Free of disease</td>
</tr>
</tbody>
</table>

NOTE: The cervical cancer tumor was histologically diagnosed as squamous cell carcinoma or adenocarcinoma. The time period (in months) between the treatment of cervical cancer and the first vaccination is given. Patients were scheduled to receive a total of four vaccinations; the number of vaccinations is given. All adverse events of the vaccine are temporarily, and systemic side effects like fever and flu-like symptoms were readily treated with paracetamol. The swelling at the injection site was not painful and probably due to the adjuvant Montanide ISA-51 in the vaccine. The follow-up of these patients after the last vaccination is indicated in months. Patient ID103 died 9 mo after the last vaccination because of local recurrence. The clinical status is given, and the time period (in months) after the last vaccination between brackets. Abbreviations: SCC, squamous cell carcinoma; ADC, adenocarcinoma; N vacc, number of vaccinations; FU, follow-up.
vaccine-induced response had contracted (Fig. 1C). In all cases a proliferative response against memory response mix could be detected in the baseline and postvaccination samples (data not shown).

Supernatants of all samples tested in the lymphocyte stimulation test were isolated at day 6 for the analysis of antigen-specific production of cytokines by cytometric bead array (6). In all six patients, a vaccine-induced HPV16 E6-specific and/or E7-specific production of IFNγ was found. The highest detected levels — as measured for a single peptide pool — ranged from 250 to >5,000 pg/mL. The production of IL-5 frequently coincided with that of IFNγ, but not in all cases. The levels of IL-5 were usually lower, and the highest detected levels did not exceed 1,000 pg/mL. The production of IL-10 always coincided with IFNγ production, but not the other way round, and peaked at 100 pg/mL. Typical examples (ID102 and ID107) of the proliferative response of PBMC, as well as the associated production of cytokines against every peptide pool before the first vaccination and after every following vaccination, are depicted in Fig. 2. It was noted that the amount of cytokine produced did not always parallel the magnitude of the proliferative response. Importantly, based on the amounts of cytokines detected, the overall response was mainly tipped toward a type 1 (IFNγ) and not a type 2 (IL-5 and IL-10) cytokine profile.

Both HPV16-specific CD4+ and CD8+ T cells were induced by vaccination. The production of mainly IFNγ and IL-5 allowed us to use intracellular cytokine analysis to determine the frequency of HPV16-specific CD4+ and/or CD8+ T cells in 10-day presensitized bulk cultures of PBMC isolated either before or after the last vaccination. To analyze HPV16-specific CD4+ T-cell responses, these bulk cultures were stimulated with pools of overlapping 22-mer peptides pulsed APC, whereas pools of overlapping 10-mer peptides pulsed APC were used to analyze the HPV16-specific CD8+ T-cell response. After vaccination, all six patients displayed circulating HPV16-specific CD4+ T cells that were directed against HPV16 E6. In five of these six patients, the circulating HPV16-specific CD4+ T cells also responded to APC pulsed with HPV16 E6 protein, indicating that these HPV16-specific CD4+ T cells recognized their cognate antigen when processed from the context of whole protein (Fig. 3 and Table 2A). In three of the six patients, HPV16 E7-specific CD4+ T cells were also detected; however, only in one patient did these cells also recognized the E7 protein (ID106). In all cases, an influenza-specific CD4+ T cell could be detected in the postvaccination sample (data not shown).

HPV16 E6-specific CD8+ T cells were observed in all six patients against one to three pools of 10-mer peptides, whereas HPV16 E7-specific CD8+ T cells were detectable in PBMC of three patients (Fig. 3 and Table 2A). Both the CD4+ and CD8+ T-cell responses directed at HPV16 E6 were stronger and broader than against E7. Notably, whereas in three patients (ID102, ID103, and ID106) HPV16-specific IFNγ-producing CD4+ T cells could be detected in the baseline samples, HPV16-specific CD8+ T cells were only detected after vaccination (Table 2A). Note that preexisting CD4+ T-cell responses increased after vaccination (Table 2A).

In conclusion, the synthetic long peptides vaccine is capable of eliciting HPV16-specific CD4+ and CD8+ T-cell responses in all six postoperative cervical cancer patients.

Synthetic long peptides vaccination enhances the HPV16-specific CD4+Foxp3+ T-cell subset. We recently reported the existence of HPV16 E6-specific CD4+CD25+Foxp3+ regulatory T cells in patients with HPV16 cervical cancer (8). Although the synthetic long peptides vaccine was injected with the purpose to enhance CD4+ and CD8+ T-cell effector immunity against the E6 and E7 oncoproteins of HPV16, the presence of such preexisting E6-specific CD4+CD25+Foxp3+ regulatory
T cells in cervical cancer patients (8) brings forward the possibility that vaccination might also result in activation and expansion of this regulatory T-cell subset.

Therefore, PBMC isolated before the first and after the last vaccination were tested for the presence of HPV16-specific CD4+CD25+Foxp3+ T cells. PBMC were stimulated with either HPV16 E6 or E7 peptides and rested for 10 days, as this allows the measurement of stably Foxp3-expressing T cells (23), which are specific for HPV16. As a control, PBMC were cultured without antigen. HPV16-specific CD4+CD25+Foxp3+ T cells were found in five patients before vaccination (Fig. 4 and Table 2B). These responses ranged from 0.1% to 1.94% and were predominantly reactive to E6 (four of the five patients). In all cases, E6-specific and/or E7-specific CD4+CD25+Foxp3+ T-cell responses of similar magnitude were detected. After vaccination, both the HPV16 E6-specific subset of Foxp3− and Foxp3+CD4+ T cells were expanded. In four of the six patients, the Foxp3− subset was significantly larger than the Foxp3+ subset of CD4+ T cells (Fig. 4 and Table 2B), whereas in two patients (ID100 and ID103), the percentage of HPV16 E6-specific CD4+CD25+Foxp3− and CD4+CD25+Foxp3+ T cells was similar after vaccination. In case of HPV16 E7, only small increases in both the percentage of CD4+CD25+Foxp3− and Foxp3+ T cells were found after vaccination. Moreover, the balance between these two subsets of E7-specific T cells was not tilted toward one or the other. These data suggest that therapeutic vaccination not only results in the induction of HPV16-specific CD4+ T-helper cells (CD4+CD25+Foxp3−) but can also induce/boost HPV16-specific CD4+CD25+Foxp3+ T-cell populations.

**Discussion**

We have assessed the cellular immune response of six patients who were treated for HPV16-induced cervical cancer and subsequently vaccinated four times with a synthetic long peptides vaccine covering the complete sequence of the oncoproteins of HPV16 E6 and E7 emulsified in the mineral oil–based adjuvant Montanide ISA-51. Utilization of several complementary immunologic assays showed that this vaccine induced broad HPV16 E6-specific T-cell responses in six of six patients and HPV16 E7-specific T-cell responses in five of six patients. Upon antigenic stimulation, these HPV16-specific T cells were able to proliferate and to produce predominantly the T-helper type 1 cytokine IFNγ, which is a hallmark for the HPV16-specific immune response in healthy subjects (6).
Notably, vaccination resulted in the enhancement of HPV16-specific CD4+ and CD8+ T-cell reactivity in all cases. Both types of HPV16-specific T cells have been implicated in the protection against progressive disease (5–7). Furthermore, vaccine-induced HPV16-specific T-cell reactivity could be measured up to 12 months after the last vaccination.

Previously, we have developed a number of assays to measure, quantify, and type spontaneously induced HPV16-specific T-cell responses (5, 6, 8, 17–21) and used these techniques to measure the HPV16-specific T-cell response upon vaccination. In general, the results of these different assay techniques were comparable. However, in a number of cases, slightly different results were obtained. IFNγ-ELISPOT revealed no preexisting HPV16-specific immunity, whereas the short-term proliferation assay did. However, in the latter assay, proliferation was accompanied by the production of either no or very low levels of IFNγ, explaining why no reactivity was detected by IFNγ-ELISPOT. Furthermore, the proliferation assay detected more responses associated with IFNγ production in the postvaccination samples when compared with ELISPOT. This can be explained in several ways. First, the levels of cytokines detected in the supernatant can be either the net result of the production by many cells producing low levels or that of a few cells producing high levels, the latter resulting in only few spots and thus a negative score in the ELISPOT analysis. Furthermore, the prolonged incubation time (7 days proliferation versus 4 days ELISPOT) is likely to contribute to the enhanced sensitivity of the proliferation assay. The use of fresh PBMC over thawed PBMC is not likely to affect the analyses (24). This was also shown by the assessment of HPV16-specific T-cell responses by intracellular cytokine analysis. This assay, set up to reveal whether CD4+ and/or CD8+ T cells responded to the vaccine, required a 10-day expansion period in vitro before the analysis was done. As a result both preexisting and broad T-cell responses after vaccination were observed similar to what was seen in the proliferation assay. However, the fact that not all of the preexisting HPV16-specific T-cell reactivities were detected by

![Fig. 3. Both CD4+ and CD8+ IFNγ-producing T cells are enhanced after vaccination. Examples of the flow cytometric dot plots used to determine the percentage of IFNγ-producing T cells on the x axis and IL-5 producing HPV16-specific T cells on the y axis present in 10-d presensitized PBMC isolated after vaccination by intracellular cytokine analysis are depicted. For each patient, the medium control, the strongest HPV16 E6-specific peptide response (see Table 2), and the reactivity against the natural protein of HPV16 E6 are shown. In addition, the fluorescence-activated cell sorting plots of the strongest HPV16-specific CD8+ T-cell response present in each patient (see Table 2) are shown.](image-url)
either one of the assays indicates that the detection of this low frequency preexisting T cells is less accurate.

Although high amounts of both HPV16 E6 and E7 peptides were injected, a more pronounced immune response was mounted against HPV16 E6. Previous studies in which subjects were vaccinated with either HPV16 L2E6E7-fusion protein (TA-CIN; ref. 18) or recombinant vaccinia viruses expressing the two oncoproteins of both HPV16 and HPV18 (TA-HPV; refs. 19, 20) also resulted in more T cells against E6 than to E7. This suggests that antigenic competition can affect the magnitude of vaccine-induced T-cell responses to some of the injected antigens. One study reported no differences in the response rate against E6 and E7 (11), but here the number of responding T cells were not quantified, excluding the possibility to reveal differences in the magnitude of the response. Importantly, the number of E7-specific T cells can be increased to the level of the E6-specific response by injecting the E6 and E7 antigens in two different locations (15).

All six patients in our study showed a proliferative response to E6 and E7 after vaccination. Based on the results of the IFN-γ-ELISPOT and intracellular IFNγ analysis, the response to E6 was always associated with the production of IFNγ, whereas in four of six cases INFγ was produced when PBMC were stimulated with E7. When compared with other studies with similar or larger group sizes, in which, based on IFNγ production, a response rate between 40% and 75% is observed (11, 18–20), the long peptides vaccine is somewhat more effective in inducing HPV16-specific immunity (six of six responding patients). Not only the response rate but also the breadth of the response is greater after peptide vaccination when compared with vaccine trials in which either TA-CIN, TA-HPV, or both were injected. In these trials we analyzed, similar to the analysis of the current study, the HPV16-specific immune response by stimulating PBMC with four different pools of E6 peptides and two pools of E7 peptides by IFNγ-ELISPOT (18–20). Of the total of 23 subjects that responded in the former trials, 16 displayed a response to one peptide pool and only five patients (~21%) responded to three or more peptide pools. In comparison, in the current study all patients reacted to three or more peptide pools.

In addition to the HPV16-specific CD4+ T-cell responses, all six patients also displayed circulating HPV16 E6-specific IFNγ-producing CD8+ T cells after vaccination. Furthermore, we detected HPV16 E7-specific CD8+ T cells in three of the six

Table 2. CD4+ T cells and CD8+ T-cell responses upon stimulation with peptide pools of HPV16

A. Percentages of IFNγ producing CD4+ and CD8+ T cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4</th>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E6.1</td>
<td>E6.2</td>
<td>E6.3</td>
<td>E6.4</td>
</tr>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>ID100</td>
<td>0.09</td>
<td>0.06</td>
<td>0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>ID102</td>
<td>0.09</td>
<td>0.21</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>ID103</td>
<td>0.10</td>
<td>0.39</td>
<td>0.55</td>
<td>0.46</td>
</tr>
<tr>
<td>ID105</td>
<td>0.05</td>
<td>0.05</td>
<td>0.02</td>
<td>0.46</td>
</tr>
<tr>
<td>ID106</td>
<td>0.45</td>
<td>0.27</td>
<td>1.00</td>
<td>0.64</td>
</tr>
<tr>
<td>ID107</td>
<td>0.16</td>
<td>1.59</td>
<td>0.22</td>
<td>0.94</td>
</tr>
</tbody>
</table>

B. Percentages of CD4+CD25+ Foxp3-negative and Foxp3-positive cells

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD4+CD25+</th>
<th>Foxp3-</th>
<th>Foxp3+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>ID100</td>
<td>E6</td>
<td>0.32</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>E7</td>
<td>0.68</td>
<td>0.48</td>
</tr>
<tr>
<td>ID102</td>
<td>E6</td>
<td>—</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>E7</td>
<td>0.69</td>
<td>1.81</td>
</tr>
<tr>
<td>ID103</td>
<td>E6</td>
<td>0.29</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>E7</td>
<td>0.36</td>
<td>0.28</td>
</tr>
<tr>
<td>ID105</td>
<td>E6</td>
<td>0.22</td>
<td>35.4</td>
</tr>
<tr>
<td></td>
<td>E7</td>
<td>—</td>
<td>1.61</td>
</tr>
<tr>
<td>ID106</td>
<td>E6</td>
<td>0.39</td>
<td>39.4</td>
</tr>
<tr>
<td></td>
<td>E7</td>
<td>—</td>
<td>1.99</td>
</tr>
<tr>
<td>ID107</td>
<td>E6</td>
<td>1.18</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>E7</td>
<td>0.28</td>
<td>0.62</td>
</tr>
</tbody>
</table>

NOTE: (A) The percentage of IFNγ-producing T cells upon stimulation with pools of 22-mer peptides (CD4) or pools of 10-mer peptides (CD8) is indicated. For the CD8+ T-cell responses, the number of the first and last amino acid of the HPV16 antigen that is covered by the peptide pool recognized is given. The results of both before vaccination and only the positive responses after the last vaccination are shown.

(B) The percentages of HPV16 peptide stimulated CD4+CD25+ T cells which do not (Foxp3−) or do express Foxp3 (Foxp3+) after subtraction of the percentage of cells in nonstimulated (medium only) cultures are given.

Abbreviations: Pre, before vaccination; Post, after the last vaccination; aa, amino acid.
patients (Table 2A). An HPV16-specific CD8+ T-cell response to E6 or E7 was only detected in case of a concomitant CD4+ T-cell response to the same antigen (Table 2A). In contrast, only 5 of 32 analyzed patients injected with TA-HPV showed evidence of vaccine-induced HPV16/18-specific CD8+ T-cell immunity (25, 26). The injection of E6E7 fusion protein in ISCOMATRIX resulted in the induction of HPV16-specific CD8+ T-cell responses in 5 of 15 patients (11). It is not clear whether the CD8+ T-cell responses of these earlier reported trials were directed against E6 or E7. In two other trials with TA-CIN or TA-HPV, the CD8+ T-cell response was not measured to all possible CD8+ T-cell epitopes but to three predefined HPV16 E7 HLA-A*0201-restricted peptides. Only 1 of the 17 patients analyzed showed a vaccine-induced response to one of the E7 peptides (19, 27). In these studies, the lack of a good response rate of CD8+ T cells against E7 is possibly related to the failure to induce E7-specific CD4+ T-cell immunity (19, 20). Other vaccines specifically aiming at the induction of HPV16-specific CD8+ T cells to one or two predefined HLA-A2–restricted E7 peptides reported higher response rates (28–30).

We observed that vaccination not only resulted in the boost of HPV16-specific CD4+CD25+Foxp3+ T cells but also enhanced the HPV16 E6-specific CD4+CD25+Foxp3+ T-cell population (Table 2B). Based on our recent study, in which we isolated

![Flow cytometric dot plots depicting the CD4+CD25+Foxp3- and CD4+CD25+Foxp3+ T-cell population in PBMC after 10 d of stimulation with E6 or E7. As a control, 10-d cultured nonstimulated PBMC are shown. The percentages of positive cells, before and after vaccination, are given in the quadrants. ND, not done.](image-url)

**Fig. 4.** Vaccination results in the enhancement of HPV16-specific CD4+CD25+Foxp3+ T-cells. The flow cytometric dot plots depict the CD4+CD25+Foxp3- and CD4+CD25+Foxp3+ T-cell population in PBMC after 10 d of stimulation with E6 or E7. As a control, 10-d cultured nonstimulated PBMC are shown. The percentages of positive cells, before and after vaccination, are given in the quadrants. ND, not done.
HPV16 E6-specific CD4+CD25+Foxp3− T cells from cervical cancer patients and showed that these T cells were able to suppress the proliferation and cytokine production of other T-helper type 1 cells after stimulation with their cognate HPV16 E6 antigen (8). Although no formal proof is given in this way, one may assume that also the vaccine-induced HPV16 E6-specific Foxp3+ T cells are able to exert suppression on other T cells. Interestingly, although the percentage of HPV16-specific CD4+CD25+Foxp3+ T cells readily outnumbered their Foxp3− counterparts in four patients, two patients (ID100 and ID103) displayed a lower number of HPV16-specific CD4+CD25+Foxp3− T cell subset. These latter two patients were the ones who progressed during (ID103) and after vaccination (ID100). It is highly likely that also the other therapeutic HPV vaccines (11, 18–20) will stimulate HPV-specific CD4+CD25+Foxp3+ T cells. The presence and vaccine-induced increase of HPV16-specific regulatory T cells indicate that strategies to eliminate or disarm regulatory T cells should be considered for immunotherapeutic strategies against HPV-induced cancers.

In conclusion, the HPV16 E6 and E7 synthetic long peptides vaccine is able to vigorously enhance the number and activity of HPV16-specific CD4+ and CD8+ T cells to a broad array of epitopes in all vaccinated patients. The expansion of both types of HPV16 (tumor)–specific T cells indicates the potential of this vaccine for the immunotherapy of HPV16-induced progressive infections, lesions, and malignancies.

Acknowledgments

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

References

Clinical Cancer Research

Induction of Tumor-Specific CD4+ and CD8+ T-Cell Immunity in Cervical Cancer Patients by a Human Papillomavirus Type 16 E6 and E7 Long Peptides Vaccine


Updated version  Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/14/1/178

Cited articles  This article cites 29 articles, 16 of which you can access for free at: http://clincancerres.aacrjournals.org/content/14/1/178.full.html#ref-list-1

Citing articles  This article has been cited by 26 HighWire-hosted articles. Access the articles at: /content/14/1/178.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.