Cell-mediated Immunological Responses in Cervical and Vaginal Cancer Patients Immunized with a Lipidated Epitope of Human Papillomavirus Type 16 E7


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ABSTRACT

Human papillomavirus (HPV) infection has been causally associated with cervical cancer. We tested the effectiveness of an HLA-A*0201-restricted, HPV-16 E7 lipopeptide vaccine in eliciting cellular immune responses in vivo in women with refractory cervical cancer. In a nonrandomized Phase I clinical trial, 12 women expressing the HLA-A2 allele with refractory cervical or vaginal cancer were vaccinated with four E7_93 lipopeptide inoculations at 3-week intervals. HLA-A2 subtyping was also performed, and HPV typing was assessed on tumor specimens. Induction of epitope-specific CD8+ T-lymphocyte (CTL) responses was analyzed using peripheral blood leukapheresis specimens obtained before and after vaccination. CTL specificity was measured by IFN-γ release assay using HLA-A*0201 matched target cells. Clinical responses were assessed by physical examination and radiographic images. All HLA-A*0201 patients were able to mount a cellular immune response to a control peptide. E7_93-specific CTLs were elicited in 4 of 10 evaluable HLA-A*0201 subjects before vaccination, 5 of 7 evaluable HLA-A*0201 patients after two vaccinations, and 2 of 3 evaluable HLA-A*0201 cultures after all four inoculations. Two of three evaluable patients’ CTLs converted from unreactive to reactive after administration of all four inoculations. There were no clinical responses or treatment toxicities. The ability to generate specific cellular immune responses is retained in patients with advanced cervical cancer. Vaccination with a lipidated HPV peptide epitope appears capable of safely augmenting CTL reactivity. Although enhancements of cellular immune responses are needed to achieve therapeutic utility in advanced cervical cancer, this approach might prove useful in treating preinvasive disease.

INTRODUCTION

Cervical cancer is a leading cause of cancer-related death in women worldwide and accounts for over 200,000 deaths annually (1). Based on extensive epidemiological data, the HPV2 is strongly associated with a spectrum of anogenital neoplasias, including condyloma, cervical dysplasia, and cervical cancer. Greater than 90% of invasive cervical tumors harbor HPV DNA, and genotype 16 can be detected in ~50% of squamous cell carcinomas of the cervix (2).

Recent research has shown that the E6 and E7 genes of HPV-16 and -18 encode for nucleoproteins that can transform human keratinocytes by mechanisms that involve, respectively, the inactivation of the tumor suppressor proteins p53 and retinoblastoma (pRB; Ref. 3). In addition, continued expression of these transforming nucleoproteins appears necessary to maintain neoplasia in vitro (4). Several lines of evidence suggest the importance of the host’s immune response in the pathogenesis of HPV-associated cervical lesions. For example, >60% of HPV positive, mildly dysplastic lesions resolve spontaneously (5), and such factors as exposure to immunosuppressive agents and HIV infection have been strongly associated with HPV-induced carcinogenesis (6, 7). Thus, these observations provide the impetus for the development of novel immunotherapies targeted at HPV.

Various investigations have shown that MHC class I-restricted CTLs can play a central role in the prevention, control, and cure of infectious diseases as well as cancer (8–11). Presentation to T cells of endogenously synthesized antigens involves degradation of the protein to small epitope peptides that are transported via the endoplasmic reticulum to the cell surface in association with class I MHC molecules (12–15). CTLs recognize antigens by the binding of their clonotypic T-cell receptors to the processed endogenous peptides associated with class I molecules. Class I-restricted CTL peptide epitopes have been generated from HPV-16 E7 (16), and recent studies have demonstrated that these peptides are capable of eliciting an immune response in animals and humans (17, 18). Because of the consistent expression of E7 in human cervical tumors asso-

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2 The abbreviations used are: HPV, human papillomavirus; HBV, hepatitis B virus; ECOG, Eastern Cooperative Oncology Group; PBMC, peripheral blood mononuclear cell; DC, dendritic cell.
associated with HPV-16 (19) and the recognition that E7 contains peptide epitopes that are processed intracellularly and presented in association with the class I molecule HLA-A2, it is an attractive target in the development of novel immunotherapies.

Nevertheless, a major obstacle in the development of peptide-based vaccines has been the finding that when the optimal peptides are administered alone, they are extremely inefficient at inducing a CTL response (8–11). Helper T cells are required in the production of the humoral immune response and are necessary for the optimal generation of cytolytic T-cell responses in vivo. Incomplete Freund’s adjuvant has been used clinically for several years, but it remains a rather nonspecific adjuvant and often induces local inflammatory responses. Several T-helper peptides have been identified recently (20, 21), and when covalently attached to a lipitated HBV core antigen peptide, primary HBV-specific CTL responses have been elicited in human subjects (22).

On the basis of these immunological observations, we have conducted a Phase I study to assess the toxicity, immunological effects, and potential therapeutic role of repeated doses of a lipitated HPV-16 E7 peptide epitope linked to PADRE (21), a non-specific helper peptide, in patients with recurrent or refractory cervical cancer.

PATIENTS, MATERIALS, AND METHODS

Peptide and Lipopeptides. The HPV-16 E786–93 (TL-GIVCPI) peptide and the control influenza matrix M158–66 peptide (GILGFVFTL) used for the in vitro analysis were synthesized by a solid-phase method and purified by high-pressure liquid chromatography (>95% pure). The binding affinity of E786–93 and M158–66 to HLA-A*0201 has been reported previously (17). Lipopeptides were prepared by coupling the preformed symmetrical anhydride of palmitic acid to the NH2 terminus of the resin-bound KSS-elongated peptide with a purity of >85%, as determined by high-pressure liquid chromatography. All peptides were diluted from aliquots dissolved in 100% DMSO and stored at −70°C. E786–93 was used at a final concentration of 20 μM; M158–66 was used at a final concentration of 1 μM.

Patient Population and Clinical Protocol. The patient population consisted of women with biopsy-diagnosed recurrent or persistent cervical cancer not amenable to surgery or radiation therapy. Other eligibility criteria included: (a) patients ages 18–65 years; (b) HLA-A2 positive; (c) an expected survival of greater than 3 months; (d) evaluable disease on pelvic exam or CT scan; and (e) a performance status of ECOG 0 or 1. In this Phase I dose-escalating protocol, 12 HLA-A*02 patients with recurrent or refractory cervical or vaginal cancer were divided into four dosage groups. Each group consisted of three subjects. Groups were immunized sequentially with the lipopeptide at escalating doses of 0.1, 0.3, 1.0, and 2.0 mg. A vaccination course included four sequential inoculation dosages of lipopeptide at 3-week intervals. Subjects were clinically monitored for adverse reactions after each immunization, and toxicities were graded using the National Cancer Institute’s Common Toxicity Criteria (23). During treatment, patients had a complete blood count, serum electrolytes, renal and hepatic function tests, and mineral panels every 3 weeks. Patients were monitored after each injection with vital signs for at least 1 h. Escalations of vaccine dosages were made in groups of up to three patients. If grade III or IV major organ toxicity was attained at any dose in an individual patient, up to six patients were to be treated at that dose level to determine the incidence of toxicity at that dose. The protocol was to be completed when dose-limiting, i.e., grade III or IV major organ toxicity in two of six patients was attained at an individual dose of lipopeptide. The maximum tolerated dose was defined as the dose level immediately below that causing dose-limiting toxicity.

To determine CTL responses in immunized subjects, PBMCs were collected by leukapheresis before vaccination (condition A) and 3 weeks after the second vaccination (condition B) and fourth vaccination (condition C). HPV-16 E786–93-specific CTLs induced from these PBMCs were analyzed in vitro for their ability to secrete IFN-γ. For this purpose, autologous DCs were prepared from the PBMCs and used as antigen-presenting cells in a peptide stimulation protocol (see below). The protocol was approved by the Clinical Research Committee of the National Cancer Institute. Patients signed a written informed consent before enrollment in the protocol.

HLA Typing and Subtyping. HLA class I was established on PBMCs as described previously (24), and all patients were HLA-A2 subtyped using a high-resolution nested sequence PCR set to resolve the HLA-A*0201 through the HLA-A*0217 alleles (25).

HPV Typing. HPV typing was performed on paraffin section samples from the patients’ tumors using a PCR amplification protocol described elsewhere (26).

Preparation of PBMCs and Lymphocytes. We obtained 1–4 x 10⁶ PBMCs from all patients by leukapheresis and separated them in Ficoll-Hyapaque gradients (LSM; Organon Teknika, Durham, NC). All PBMC preparations were frozen in human AB serum with 10% DMSO (Sigma Chemical Co., St. Louis, MO) and stored in liquid nitrogen.

Preparation of DCs. After Ficoll-Hyapaque separation, 1–3 x 10⁶ PBMCs were processed for preparation of DCs as described previously (27). The PBMCs were cultured in 75-cm² culture flasks for 3 h at 37°C. The nonadherent cells were removed, and the adherent cells were cultured for 5–7 days in sterile conditions in 10 ml of complete medium consisting of Iscove’s (Biofluids, Rockville, MD) plus 0.03% L-glutamine, 100 units/ml penicillin (both from NIH media unit), 10% heat-inactivated human AB serum (Biofluids), and 25 mm HEPES (Biofluids). Human recombinant granulocyte/macrophage colony-stimulating factor (2000 IU/ml; Pepro Tech, Inc., Rocky Hill, NJ) and human recombinant interleukin 4 (2000 IU/ml; Pepro Tech, Inc.) were added every 2–3 days from day 0.

T2 Cell Line. T2 cells were used in cytokine release assays for the HLA-A*0201-restricted E786–93 and M158–66 epitopes. The T2 cell line was selected as a target because it expresses only the HLA-A*0201 allele, which was the restriction element for this vaccination. This cell line is also defective in endogenous processing, which enhances the effectiveness of exogenous peptide loading (28, 29).

Peptide-Pulsing of DCs and T2 Cells. The recovered DCs or T2 cells were pulsed with 20 μg/ml of E786–93 peptide or 1 μg/ml M158–66 peptide for 2 h in 15-ml conical tubes at 37°C for a concentration of 1 x 10⁶ cells/ml.
In Vitro Sensitization of Peripheral Blood Lymphocytes with DCs. CD8+ enrichment of T cells was achieved by positive selection on biomagnetic separation beads (Dynal Corp., New York, NY). In all experiments, the T-cell population was >95% CD8+ and included <5% contamination with CD4+ cells by fluorescence-activated cell sorter analysis. CD8+ lymphocyte cells (4–5 × 10^6/well) were coincubated with 1 × 10^6 peptide-pulsed (E786_93 or M158) DCs in 24-well plates and were restimulated after 1 week with 1 × 10^6 peptide-pulsed DCs. Interleukin 2 (300 IU/ml) was added 24 h after each stimulation and every 2–3 days thereafter. The effectors were tested for specificity 7–9 days after the restimulation.

Assessment of CTL Reactivity using Cytokine Release Assay. Effector cells (1 × 10^5) were coincubated with 1 × 10^5 stimulator cells for 24 h at 37°C in 200 μl of complete medium (5 × 10^5 effector cells/ml). Supernatants from these cocultures were tested for specific secretion of IFN-γ by human IFN-γ Quantikine enzyme-linked immunosorbent assay kits (R & D Systems, Minneapolis, MN). Data are presented as picograms of IFN-γ released by 5 × 10^5 effectors/24 h.

Statistical Analysis. Nonparametric Fisher's Exact test was used to compare the frequency of in vitro CTL induction between prevaccination and postvaccination cultures. Specific release of IFN-γ by a PBMC culture was arbitrarily defined as: (a) 2-fold or higher difference in IFN-γ production in response to relevant (T2 + E786–93 or T2 + M158–64) versus irrelevant (T2 alone) stimulation; and (b) at least 100 pg/5 × 10^5 cells/24-h production of IFN-γ. A 2-fold increase in specific release between prevaccination and postvaccination cultures was arbitrarily chosen as evidence of differences in CTL reactivity and statistically compared by Fisher's Exact test.

RESULTS

Clinical Summary. Fifty-three patients were screened for entry into the study. The majority were excluded on the basis of complete blood count or serum chemistry analyses. No short-term or medium-term toxicities, including physical exam findings or mild swelling but required no therapy, typically lasted less than 24 h, and were not associated with regional adenopathy or systemic symptoms. These local reactions consisted of erythema or mild swelling and were not associated with regional adenopathy or systemic symptoms. These inoculation-site local reactions were mild in all subjects, regardless of the dose of lipopeptide administered. There were no laboratory toxicities identified in any patient on the basis of complete blood count or serum chemistry analyses. No no objective clinical responses observed in any of these 12 patients. Six patients were unable to complete the full course of vaccinations consisting of four inoculations due to rapidly progressive disease (patients 2, 4, 6, 8, 9, and 12), and two patients declined the follow-up leukapheresis (patients 5 and 11); however, three of these eight patients underwent leukapheresis prior to the third vaccination, which allowed assessment of CTL responsiveness to two prior inoculations. Of the six patients who were unable to complete the full course of inoculations, there was no clinical or laboratory evidence to refractory cervical cancer and a measurable lesion satisfied all protocol eligibility criteria and were entered into the study (Table 1). Patients ranged in age from 32 to 65 years; 4 patients had previously undergone radical hysterectomy; 7 patients had received previous chemotherapy; and all 12 patients underwent pelvic radiation therapy before entering this study. All patients had a performance status of ECOG 0 or 1. No correlation was noted between any of these variables and toxicity, response, or T-cell activation. Analysis of tumor specimens detected HPV-16 in 8 of 10 evaluable patients. Two patients had no detectable HPV in their tumors, and two others did not have samples available to permit satisfactory analysis.

No vaccination-related systemic toxicities were noted in any patient. The first patient in each group underwent in-patient observation after the initial inoculation, and no serious systemic side effects attributable to vaccination were noted. Adverse events related to the lipopeptide were mild, reversible, and generally related to skin discomfort at the site of the inoculations. These local reactions consisted of erythema or mild swelling but required no therapy, typically lasted less than 24 h, and were not associated with regional adenopathy or systemic symptoms. These inoculation-site local reactions were mild in all subjects, regardless of the dose of lipopeptide administered. There were no laboratory toxicities identified in any patient on the basis of complete blood count or serum chemistry analyses. No objective clinical responses observed in any of these 12 patients. Six patients were unable to complete the full course of vaccinations consisting of four inoculations due to rapidly progressive disease (patients 2, 4, 6, 8, 9, and 12), and two patients declined the follow-up leukapheresis (patients 5 and 11); however, three of these eight patients underwent leukapheresis prior to the third vaccination, which allowed assessment of CTL responsiveness to two prior inoculations. Of the six patients who were unable to complete the full course of inoculations, there was no clinical or laboratory evidence to

Table 1. Clinical characteristics of patients receiving HPV lipopeptide vaccine

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age at diagnosis</th>
<th>Primary tumor site</th>
<th>FIGO stage</th>
<th>Histological type</th>
<th>HPV type</th>
<th>HLA-A genotype</th>
<th>Follow-up status</th>
<th>Clinical outcome</th>
<th>Post-vax (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43</td>
<td>Cervix</td>
<td>IB1</td>
<td>Squamous cell</td>
<td>16</td>
<td>0205,23</td>
<td>Progression</td>
<td>Alive with disease</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>Cervix</td>
<td>IB2</td>
<td>Squamous cell</td>
<td>16</td>
<td>0201,24</td>
<td>Dead of disease</td>
<td>Dead of disease</td>
<td>8</td>
</tr>
<tr>
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<td>60</td>
<td>Cervix</td>
<td>IIB</td>
<td>Squamous cell</td>
<td>16</td>
<td>0201,01</td>
<td>Stable</td>
<td>Alive with disease</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>Cervix</td>
<td>IB1</td>
<td>Squamous cell</td>
<td>16</td>
<td>0201,28</td>
<td>Dead of disease</td>
<td>Dead of disease</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>31</td>
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<td>IIB</td>
<td>Squamous cell</td>
<td>16</td>
<td>0201,01</td>
<td>Not evaluable</td>
<td>Alive with disease</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>38</td>
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<td>IB1</td>
<td>Squamous cell</td>
<td>16</td>
<td>0201,11</td>
<td>Dead of disease</td>
<td>Dead of disease</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>43</td>
<td>Cervix</td>
<td>IVA</td>
<td>Squamous cell</td>
<td>16</td>
<td>0201,03</td>
<td>Stable</td>
<td>Alive with disease</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>44</td>
<td>Cervix</td>
<td>IIB</td>
<td>Squamous cell</td>
<td>16</td>
<td>0201,02</td>
<td>Dead of disease</td>
<td>Dead of disease</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td>Vagina</td>
<td>III</td>
<td>Squamous cell</td>
<td>16</td>
<td>0201,03</td>
<td>Progression</td>
<td>Alive with disease</td>
<td>4</td>
</tr>
<tr>
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<td>IIB</td>
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<td>16</td>
<td>0201,26</td>
<td>Progression</td>
<td>Alive with disease</td>
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<tr>
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<td>Adenocarcinoma</td>
<td>16</td>
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<td>Alive with disease</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>47</td>
<td>Cervix</td>
<td>IB2</td>
<td>Squamous cell</td>
<td>16</td>
<td>0206,29</td>
<td>Progression</td>
<td>Alive with disease</td>
<td>2</td>
</tr>
</tbody>
</table>

* Fédération Internationale des Gynécologistes et Obstétristes.

† The genotype of both HLA-A alleles is presented, separated by a comma.

‡ After vaccination.
2106 Cellular Immune Responses to a HPV Lipopeptide Vaccine

Effecter cells (1 × 10⁵) were coincubated with 1 × 10⁵ stimulator cells for 24 h, and supernatants from these cocultures were tested for specific secretion of IFN-γ. Data are presented as picograms of IFN-γ released by 5 × 10⁵ effectors per 24 h. Specific release of IFN-γ by a peripheral blood mononuclear cell culture was arbitrarily defined as: (a) 2-fold or higher difference in IFN-γ production in response to relevant (T2 + E7⁸₆₋⁹₃ or T2 + M₅₈₋₆₆) versus irrelevant (T2 alone) stimulation; and (b) at least 100 pg/ml × 10⁵ cells/24-h production of IFN-γ. Numbers in parentheses represent nonspecific IFN-γ production when CTLs were reacted with irrelevant (T2 alone) target cells. A 2-fold increase in specific release between prevaccination and postvaccination cultures was arbitrarily chosen as evidence of differences in CTL reactivity.

### Table 2 Immunological responses of patients receiving HPV lipopeptide vaccine

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>HLA-A</th>
<th>HPV Dose (µg)</th>
<th>IFN-γ release (pg/ml per 24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prevaccination</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>T2 + E7⁸₆₋₉₃</td>
</tr>
<tr>
<td>1</td>
<td>0205</td>
<td>16 100</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0201</td>
<td>16 100</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0201</td>
<td>16 100</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0201</td>
<td>Neg 300</td>
<td>476 (71)</td>
</tr>
<tr>
<td>5</td>
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<td>194 (2)</td>
</tr>
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<td>16 300</td>
<td>32 (0)</td>
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<td>78 (108)</td>
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<td>9</td>
<td>0201</td>
<td>16 1000</td>
<td>180 (16)</td>
</tr>
<tr>
<td>10</td>
<td>0201</td>
<td>Unsat 2000</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>0201</td>
<td>Neg 2000</td>
<td>138 (48)</td>
</tr>
<tr>
<td>12</td>
<td>0206</td>
<td>16 2000</td>
<td>0</td>
</tr>
</tbody>
</table>

* NT, not tested; Neg, HPV negative; NA, not available; Unsat, unsatisfactory sample.

suggest that an inappropiate immune response contributed to their clinical deterioration. Indeed, all of these patients had advanced metastatic disease upon enrollment in the trial. One patient was lost to follow-up following the fourth vaccination (patient 5). After receiving all four vaccinations, two patients are alive with stable disease on no additional therapy (patients 3 and 7), whereas three others are alive with progression of disease (patients 1, 10, and 11).

**Induction Peptide-specific CTLs.** PBMCs obtained before and at various times after the primary and booster immunizations were stimulated in vitro and tested for peptide-specific CTL reactivity. CD8⁺ T-cell reactivity was tested for IFN-γ release in an HLA-A*0201-restricted assay by pulsing M₅₈₋₆₆ or E7⁸₆₋₉₃ on T2 cells expressing HLA-A*0201 molecules (Table 2). This assay excludes non-HLA-A*0201-restricted secretion of IFN-γ, because the T2 cell line does not express any other HLA class I or class II alleles. Therefore, this assay is aimed at analyzing specifically HLA-A*0201-restricted secretion of IFN-γ. In all patients expressing the HLA-A*0201 allele, CTL cultures raised against the M₅₈₋₆₆ control peptide demonstrated specific cytokine release, indicating that the capacity to generate specific cellular immune responses is retained in cervical cancer patients, even after extensive previous treatment. Anti-M₅₈₋₆₆ cultures from one patient (patient 12 expressing the HLA-A*0206 allele also demonstrated specific cytokine release, whereas cultures from another patient expressing the HLA-A*0205 allele (patient 1) did not. These results suggest that CTL responses to M₅₈₋₆₆, an HLA-A*0201-restricted peptide, vary depending on peptide and the degree of HLA-A2 molecule binding site similarity among HLA-A2 subtypes. For patients expressing the HLA-A*0201 allele, anti-E7⁸₆₋₉₃-specific cytokine release was noted in 4 of 10 prevaccination cultures, 5 of 7 cultures after two inoculations, and 2 of 3 cultures after all four inoculations. The two patients with positive cultures after four vaccinations (patients 3 and 7) represent primary CTL responses because their initial CTL cultures were negative. Another patient’s cultures (patient 10) converted from negative to positive after two inoculations but reverted to negative after all four inoculations were administered. In a two-tailed paired analysis, there were no statistically significant differences in the amount of IFN-γ secreted when the prevaccination (three of seven) and postvaccination (five of seven) cultures from the seven evaluable HLA-A*0201 patients were compared (paired sample Fisher’s Exact test, P₉ < 0.56). Cultures from the two patients not possessing the HLA-A*0201 subtype failed to show any E7⁸₆₋₉₃-specific cytokine release.

**DISCUSSION**

The causal linkage of HPV infection and the development of cervical carcinoma has been firmly established and serves as compelling rationale for the development of alternative treatment modalities, including immunological ones. We have studied the use of an HPV-16 E7⁸₆₋₉₃ lipopeptide vaccine in the treatment of patients with advanced cervical cancer in a Phase I protocol to evaluate the safety and immunogenicity of this vaccine preparation. No clinically significant toxicities were observed, and none of the patients vaccinated demonstrated an objective clinical response to treatment.

The lipidated E7⁸₆₋₉₃ peptide, along with the covalently linked PADRE helper epitope, was selected for clinical use to treat HLA-A2⁺ cervical cancer based on several factors. To date, HLA-A*0201-restricted CTL responses represent the majority of those described, reflecting the prevalence of this class I allele among humans in general and Caucasians in particular (30). The E7⁸₆₋₉₃ peptide was selected on the basis of experiments performed using HLA-A*0201 transgenic mice in which 15 of 15 mice receiving this peptide were protected from subsequent challenge with a lethal dose HPV-16 E7-induced tumor; this peptide was also successful in eliciting specific CTL re-
responses in six of nine human donors and has been shown to be immunologically relevant for the recognition and lysis of cervical cancer cells harboring HPV-16 DNA (17). However, the possibility that human cervical tumor cells may not always naturally present this epitope has been suggested (31, 32). The advantage of using the potent helper peptide PADRE to augment CD8+ responses has been described recently (20) and permits more specific CD4+ immunostimulation, providing a theoretic advantage over nonspecific immunostimulants such as incomplete Freund’s adjuvant.

Although it has been reported that 93% of cervical tumors harbor HPV DNA and that HPV-16 is associated with nearly 60% of cases, the ability to detect HPV in clinical specimens is problematic (2). The rate limiting factor in detecting HPV in tissues lies primarily with the quality of the tissue available for analysis; the yield when fresh tissues are used is considerably higher than when formalin-fixed, paraffin-embedded specimens are studied (33). However, in clinical practice, the majority of patients eligible for this trial would not have fresh tissue available for analysis. Because of this limitation, we elected not to include HPV typing of tumors as an eligibility criteria. We believe that this practice is justified because: (a) HPV-16, which is the target of the lipopeptide vaccine, is the HPV type that is by far the one associated most frequently with cervical cancers (2); (b) only HLA-A2+ patients with cervical cancer who have failed all other effective treatment modalities were eligible to enroll in this trial; and (c) the primary objective of this Phase I study was to assess toxicity to the lipopeptide.

Although the clinical effectiveness of the HPV lipopeptide vaccine cannot be determined in a small study of this type, we have attempted to assess the direct effect of the vaccine on its immunological target. Accordingly, we have used a method able to quantitate epitope-specific sensitization against E786-93. In another viral system, T-cell sensitization to the in vitro administration of HBV vaccine (HBVC18-27) lipopeptide was monitored by comparing cytotoxic activity in prevaccination and postvaccination PBMC cultures induced in vitro by stimulation with HBVC18-27 (22). Analysis of the frequency of CTL precursor cells using limiting dilution assays has also been advocated, but this generally requires that the immunogen induces relatively robust CTL responses. We have therefore monitored the effects of the HPV lipopeptide vaccine on CTL activity by comparing epitope-specific reactivity between CD8+ T-cell cultures using a cytokine release assay. With this assay, we were able to identify a significant proportion of patients in whom specific anti-E786-93 reactivity was enhanced by the vaccination. The results using this assay correlate well with other standard measures of CTL function, such as chromium release cytotoxicity assays, and have proven to be more sensitive (34, 35). For example, specific anti-M158-66 CTL responses were detected in all HLA-A*0201 patients tested using this assay, whereas a lack of such responses to the same peptide in a similar group of patients has been reported when chromium release assays were used (36).

The results of the cytokine release assays also suggest that anti-E786-93 CTL precursors are variably present in cervical cancer patients. It is of interest to note that a remarkably robust cellular immune response to E786-93, even before receiving the vaccine, occurred in a patient whose tumor did not contain HPV DNA, suggesting that this patient was capable of successfully clearing a challenge with this virus. A total of four HLA-A*0201 patients demonstrated CTL reactivity to the E786-93 peptide before receiving the vaccine, whereas six other HLA-A*0201 patients did not, suggesting that a memory response may also be involved in the generation of epitope-specific CTL in some patients. In other studies performed recently in our laboratory, we have found that the frequency of CTL responses to the E786-93 epitope is similar for both healthy women and those with cervical cancer. Therefore, immunological deficits other than insufficient CTL precursor frequencies, such as defects in T-cell signaling or differences in the mechanisms HPV-infected cells use to escape immunological recognition, may have important roles in the pathogenesis of HPV-associated cervical cancer.

The conversion of CTL cultures from unreactive to reactive in patients 3, 7, and 10 is encouraging evidence that specific CTL responses can be augmented as a consequence of vaccination. Clearly, enhancements of cellular immune responses are needed to achieve therapeutic utility in advanced cervical cancer; however, this approach might prove useful in treating patients with less extensive disease, such as preinvasive dysplastic lesions. Established tumors are usually a heterogeneous mixture of different malignant cell populations; therefore, it seems likely that variant tumor clones within a tumor may not express the target antigen or will possess defects in their antigen-presenting mechanism. Because less advanced lesions such as high-grade dysplasia or carcinoma in situ are generally much smaller lesions that are more stable genetically, this type of vaccine therapy may ultimately be better suited for the treatment of patients with preinvasive disease.

The exquisite specificity of HLA-restricted T-cell recognition is emphasized by the inability of the two cultures from the non HLA-A*0201 patients to secrete any measurable amount of cytokine after stimulation with the E786-93 peptide. This finding is consistent with recently published reports indicating that HLA-A2 CTL responses are variably restricted by the HLA-A2 subtype (37, 38). Interestingly, a potent response to M158-66, which is HLA-A*0201-restricted, was elicited from patient 12, who possesses the HLA-A*0201 subtype, whereas no specific M158-66 reactivity was observed in cultures from patient 1, who possesses the HLA-A*0205 subtype. The ability of HLA-A*0206 (which varies from HLA-A*0201 by only one amino acid residue compared with four amino acid residue differences between HLA-A*0205 and HLA-A*0201) to successfully present an HLA-A*0201-restricted peptide was partially predicted by a previous analysis, which showed recognition of the M158-66 peptide pulsed on HLA-A*0206 B-cell targets by HLA-A*0201-restricted CTLs (37). Such cross-reactivity between HLA-A*0206 and HLA-A*0201 was not observed in the context of other HLA-A*0201-restricted epitopes, suggesting
that the cross-reactivity of peptides among different members of the HLA-A2 superfamily is dependent on the relationship between each peptide and the HLA-A2 allele (37). Functional heterogeneity of HLA-A2 subtypes was also reported in another recent study of a melanoma-specific tumor antigen (38). In this analysis, functional cross-reactivity between HLA-A*0201 CTL and HLA-A*0206, but not HLA-A*0205, B-cell targets was observed. Therefore, HLA-A2-restricted CTL responses appear to be further restricted, but not excluded, by the HLA-A2 subtype, with the interaction between both the peptide and the precise HLA-A2 subtype molecule determining the nature and specificity of the cellular immune response. For the purpose of monitoring immune responses in patients enrolling in HLA-A2-restricted, peptide-based vaccination protocols, these observations emphasize the importance of matching patient effector cells (CTLs) with appropriate subtype-matched target cells. This will permit a direct analysis of immunogenicity rather than cross-reactivity of peptide-induced CTL responses.

It is also noteworthy that two of twelve patients had a subtype different from HLA-A*0201. The frequency of this allele is related to ethnic heterogeneity of cervical cancer patients. It is predictable that subtype analysis will be particularly important in patients of non-Caucasian ancestry (especially patients of Asian and Mediterranean ancestry) because the frequency of HLA-A*0201 has been noted to be significantly decreased in certain ethnic groups (39).

By using autologous DCs to generate CTL responses, this study may provide a basis by which to compare CTL activation and clinical results in future HPV vaccine trials. Our results with the HPV lipopeptide vaccine indicate that it may induce specific CTL reactivity in the peripheral circulation of patients with cervical cancer. Although the enhancement of CTL reactivity did not occur in tumor regression, further investigations of therapeutic HPV vaccines seem justified. Another therapeutic HPV vaccine approach has been reported recently using a recombinant vaccinia virus vaccine encoding the E6 and E7 oncoproteins from HPV-16 and HPV-18 to treat patients with advanced cervical cancer, but no objective clinical responses were observed (40). It is possible that the CTL reactivity stimulated by HPV-specific vaccines is not quantitatively sufficient to cause tumor regression in patients with advanced disease, and studies in patients with preinvasive lesions, who may be capable of rejecting a considerably smaller burden of HPV-infected cells, are in progress.

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