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Human Papillomavirus Type 16 and 18 E7-Pulsed Dendritic Cell Vaccination of Stage IB or IIA Cervical Cancer Patients: a Phase I Escalating-Dose Trial[▽]

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The safety and immunogenicity of the human papillomavirus type 16 (HPV16) or HPV18 (HPV16/18) E7 antigen-pulsed mature dendritic cell (DC) vaccination were evaluated for patients with stage IB or IIA cervical cancer. Escalating doses of autologous DC (5, 10, and 15 × 10^6 cells for injection) were pulsed with recombinant HPV16/18 E7 antigens and keyhole limpet hemocyanin (KLH; an immunological tracer molecule) and delivered in five subcutaneous injections at 21-day intervals to 10 cervical cancer patients with no evidence of disease after they underwent radical surgery. Safety, toxicity, delayed-type hypersensitivity (DTH) reaction, and induction of serological and cellular immunity against HPV16/18 E7 and KLH were monitored. DC vaccination was well tolerated, and no significant toxicities were recorded. All patients developed CD4$^+$ T-cell and antibody responses to DC vaccination, as detected by enzyme-linked immunosorbent spot (ELISpot) and enzyme-linked immunosorbent assays (ELISA), respectively, and 8 out of 10 patients demonstrated levels of E7-specific CD8$^+$ T-cell counts, detected by ELISpot during or immediately after immunization, that were increased compared to prevaccination baseline levels. The vaccine dose did not predict the magnitude of the antibody or T-cell response or the time to detection of HPV16/18 E7-specific immunity. DTH responses to intradermal injections of HPV E7 antigen and KLH were detected for all patients after vaccination. We conclude that HPV E7-loaded DC vaccination is safe and immunogenic for stage IB or IIA cervical cancer patients. Phase II E7-pulsed DC-based vaccination trials with cervical cancer patients harboring a limited tumor burden, or who are at significant risk of tumor recurrence, are warranted.

Cervical cancer is the second most common cause of cancer-related deaths of women worldwide, with about 450,000 new cases diagnosed each year (13). In the United States and other industrialized countries, cervical cancer remains an important health problem for women, especially in underserved and minority groups (13, 8). Although early-stage cervical tumors (stage IB or IIA) can be cured by radical surgery or radiotherapy with similar effectiveness, up to 20% of patients with negative lymph nodes and up to 50% of patients with positive lymph nodes may develop recurrent disease for which treatment results are poor (8). Novel therapeutic strategies that are effective in reducing the risk of recurrence in cervical cancer patients remain desperately needed.

In the last few years, a multitude of epidemiological studies (29) have shown a strong and specific association, beyond reasonable doubt, between human papillomavirus (HPV) infection and cervical cancer. Accumulating evidence suggests that the majority of cervical squamous cell carcinomas and a large proportion of adenocarcinomas share a common pathogenesis involving infection with the oncogenic HPV type 16 (HPV16) and HPV18 (4, 29). The E6 and E7 transforming oncoproteins of these high-risk HPV genotypes play a crucial role in both the transformation and the maintenance of the malignant phenotype and are detected in a large majority of HPV-positive cancer biopsy specimens and almost all HPV-containing cell lines (4, 29). Hence, these viral proteins represent ideal candidates as potential tumor-specific target antigens for cervical cancer immunotherapy.

Dendritic cells (DC) are rare but extremely potent antigen-presenting cells (APC) that function in vitro and in vivo to initiate T-lymphocyte responses to antigens. A wealth of evidence has established the ability of monocyte-derived DC to stimulate naive CD4$^+$ and CD8$^+$ T cells in vitro and in vivo (1, 23, 24). Immature DC (i.e., monocytes cultured for 5 to 7 days in granulocyte-macrophage colony-stimulating factor [GM-CSF] and interleukin-4 [IL-4]) effectively capture antigens but lack full T-cell-stimulatory activity and are sensitive to the immunosuppressive effects of immunoregulatory cytokines (e.g., IL-10) that can be produced by tumors. In contrast, when DC are completely mature, they demonstrate a reduced level of phagocytic activity but higher production of some key cytokines (e.g., IL-12), an increased level of antigen presentation and T-cell costimulatory activity, a decreased sensitivity to the immunosuppressive effects of IL-10, and an up-regulated ex-

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TABLE 1. Clinical characteristics of vaccinated patients

Patient	Age (yr)	Race	HPV type	Cancer stage	Histology	Primary treatment	Lymph node assay	Follow-up (mo)	Status	No. of vaccinations
1	31	Black	16	IB	Squamous cell carcinoma	Radical hysterectomy	Negative	31	NED[a]	5
2	37	White	18	IB	Squamous cell carcinoma	Radical hysterectomy	Negative	30	NED	5
3	32	White	16	IB	Squamous cell carcinoma	Radical hysterectomy	Negative	30	NED	5
4	34	White	16	IB	Squamous cell carcinoma	Radical hysterectomy	Negative	26	NED	5
5	43	White	16	IB	Adenocarcinoma	Radical hysterectomy	Negative	26	NED	5
6	72	White	16	IB	Squamous cell carcinoma	Radical hysterectomy	Negative	25	NED	5
7	39	White	16	IB	Adenocarcinoma	Radical hysterectomy	Negative	25	NED	5
8	23	White	16	IB	Squamous cell carcinoma	Radical hysterectomy	Negative	20	NED	5
9	32	White	16	IB	Squamous cell carcinoma	Radical hysterectomy	Negative	19	NED	5
10	23	White	16	IB	Squamous cell carcinoma	Radical trachelectomy	Negative	17	NED	5

[a] NED, no evidence of disease.

pression level of selected chemokine receptors that guide their migration to secondary lymphoid organs for priming antigen-specific T cells (1, 25). Preliminary clinical studies have shown that DC-based vaccinations can rapidly generate broad T-cell immunity in healthy subjects (6, 7) and are able to induce regression of tumor metastases without significant side effects for some patients harboring human malignancies, including lymphoma and melanoma (12, 16, 26). These observations support the concept that immunization with mature, monocyte-derived DC loaded with the full-length E7 protein from HPV16 or HPV18 (HPV16/18) may represent a potentially powerful method for inducing antitumor immunity in patients at risk of developing recurrent/metastatic HPV16/18-positive tumors.

In this phase I study, we have used escalating doses of mature autologous DC pulsed with full-length HPV16/18 E7 oncoprotein and keyhole limpet hemocyanin (KLH) to evaluate the immunologic potential of a therapeutic vaccine for patients harboring HPV16/18-infected stage IB or IIA cervical carcinoma. The safety, toxicity, delayed-type hypersensitivity (DTH) reactions, and induction of serological and cellular immunity against HPV16/18 E7 and KLH were monitored.

MATERIALS AND METHODS

HPV16/18-infected stage IB or IIA cervical cancer patients were enrolled in the study at the University of Arkansas for Medical Sciences between 2004 and 2006. The characteristics of the patients are described in Table 1. The study was designed as a phase I dose escalation study of three DC dose level cohorts, with the first (low-dose) cohort consisting of 3 subjects, the second (medium-dose) cohort consisting of 3 subjects, and the final (high-dose) cohort consisting of 4 subjects, for a total of 10 subjects enrolled in the study. The protocol was approved by the local institutional review board and the Food and Drug Administration (no. BB-IND-11307). Written informed consent was obtained from all patients. Eligible patients had not received steroids, chemotherapy, or radiation therapy for at least 4 weeks prior to starting vaccination therapy. HPV16/18 had to be present in the tumor, as determined by PCR analysis. Figure 1 describes the timetable for the study. All patients included in the protocol underwent collection of peripheral blood leukocytes (PBL) by leukopheresis. HPV16/18 E7/KLH-pulsed DC were injected subcutaneously 10 cm inferior to the inguinal ligament of the anterior mid-thigh at doses of 5×10^6 cells for the low-dose cohort, 10×10^6 cells for the medium-dose cohort, and 15×10^6 cells for the high-dose cohort. Five DC vaccinations were performed at 21-day intervals (Fig. 1).

HPV typing. Sequence-specific primers for E7 oncoproteins were used to confirm or exclude, by PCR, the presence of the HPV16 and HPV18 genotypes in tumor sections obtained from formalin-fixed paraffin-embedded autologous cervical tumor tissue. Briefly, for HPV16/18 E7, 0.1 to 1 μg of each genomic DNA sample was amplified in a 50-μl reaction mixture containing 0.3 μM of each of the individual primers (HPV16 E7, F1 primer 5′-AGA TAC ACC TAC ATT GCA T-3′ and R1 primer 5′-CTT GCA ACA AAA GGT TACA-3′; or HPV18 E7 F1 primer 5′-GGT TGA CCT TCT ATG TGA CGA GCA-3′ and R1 primer 5′-GTT CAG AAA CAG CTG CTG GAA TGC-3′) in the presence of 1× PCR buffer, 2.5 μM MgCl2, 0.8 μM of each deoxynucleoside triphosphate, and 0.025 U/μl AmpliTaq DNA polymerase (Applied Biosystems). Amplifications were performed with an Applied Biosystems GeneAmp PCR system 2700 (Applied Biosystems) for HPV6 at 94°C for 5 min, followed by 35 cycles at 94°C for 55 seconds, and 50°C for 60 seconds; then 35 cycles at 72°C for 55 seconds and a final extension of 72°C for 10 min. For HPV18, amplification was performed at 95°C for 5 min, followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 35 cycles at 72°C for 60 seconds, and then a final extension of 72°C for 7 min. The PCR products were stored at 4°C before electrophoresis on a 2% agarose gel. Beta-tubulin gene amplification performed with the primers F (5′-CGC ATC AAC GTG TAC TAC AA-3′) and R (5′-TAC GAG CTG GTG GAC TGA GA-3′) (0.25 μM of each primer) was used as a positive internal control. SiHa (HPV16) and HeLa (HPV18) cervical carcinoma cell line DNAs were used as positive external controls. C33A (HPV-negative) cell line DNA and a water template were used as negative controls. PCR analysis of tumor tissue revealed that tumor cells harbored the HPV16/18 genotype.

Autologous DC vaccine preparation. PBL obtained by leukopheresis were either cryopreserved in RPMI 1640 medium (Invitrogen) plus 10% dimethyl sulfoxide and 30% autologous plasma or were used immediately for DC generation. Briefly, PBL were placed in T150 tissue culture flasks (Corning Corp.) at 3×10^8 to 5×10^8 cells in 30 ml of AIM-V medium (Invitrogen) per flask. After incubation for 2 h at 37°C, nonadherent cells were removed, and the adherent cells were cultured at 37°C in a humidified 5% CO_2/95% air incubator in AIM-V medium supplemented with recombinant human GM-CSF (800 U/ml; Immunex) and IL-4 (500 U/ml; R&D Systems) as described previously (23, 24). At day 4, overnight pulsing (12 to 16 h) with HPV16/18 E7 proteins and KLH (Intracell Resources) at a dose of 50 μg/ml was used to deliver antigens to the DC. Purified recombinant E7 proteins were produced using a plasmid carrying glutathione *S*-transferase E7 fusion protein as described previously (23). In this regard, preparations were typically >98% pure E7 protein with no detectable traces of glutathione *S*-transferase by high-performance liquid chromatography, scanning spectrometry, and multiple gel assays (i.e., sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting). At day 5, the final maturation of monocyte-derived DC was induced by exposure during the last 48 h of culture (day 5 to day 7) to tumor necrosis factor-alpha (1,000 U/ml), IL-1β (500 U/ml) (both from R&D Systems), and prostaglandin E_{2a} (0.5 μM) (Sigma) (20, 23). After the final maturation, DC were harvested by gentle pipetting, washed twice with phosphate-buffered saline (PBS) and AIM-V, counted to assess the cell number and viability (by trypan blue exclusion), and tested for safety before being used (see below) in the vaccination protocol. At least seven vials of E7/KLH-pulsed DC were cryopreserved at each dose, constituting the master vaccine bank for each patient. DC were frozen in RPMI 1640 medium, 30% autologous plasma, and 10% dimethyl sulfoxide. Vaccine production, blood drawing, vaccination, and measurement of immune responses in vivo against HPV16/18 and KLH (i.e., the DTH reaction), as well as in vitro assessments (by enzyme-linked immunosorbent assay [ELISA] and enzyme-linked immunosorbent spot [ELISpot] assay) were done at different time points as described in Fig. 1.

Clinical evaluation. Because clinical responses to treatment were not a goal of this phase I study, patients were monitored individually according to hospital standards and underwent routine follow-up monitoring for disease development. Pelvic examinations and Pap smear collections were performed every 3 months

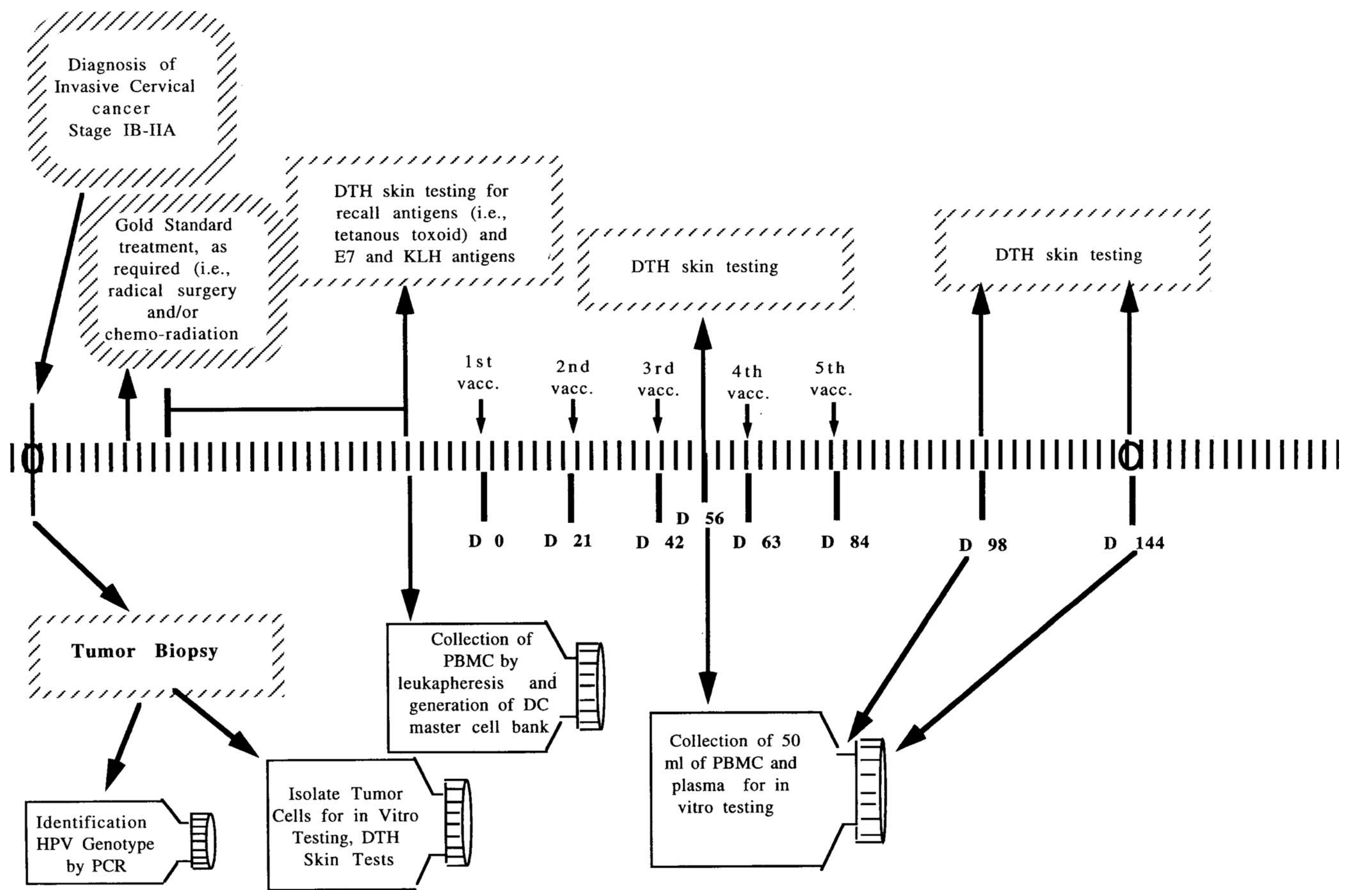

FIG. 1. Schematic timetable for the phase I escalating dose trial of HPV E7 antigen-pulsed DC vaccination for the treatment of patients with stage IB or IIA cervical cancer. Schematic timetable of the study protocol. Immunological parameters were studied at prevaccination (time zero), after three vaccinations (day 56, time 1), immediately after the last (fifth) vaccination (vaccination 5, day 98, time 2), and 2 months after the last vaccination (day 144, time 3).

for the first 2 years and every 6 months thereafter. Computed tomography scanning of subjects' abdomens and pelvises was performed annually or when clinically indicated. Toxicity was scored according to National Cancer Institute common toxicity criteria, but no vaccine-related toxicity was recorded.

Serology for KLH and HPV16/18 E7-specific antibodies. Serum samples were collected and frozen at −70°C. Samples were analyzed for antibodies to KLH and HPV E7 proteins by antigen-specific capture ELISA as previously described (17). Antigen biotinylation was performed by gently mixing 1 mg of E7 protein or KLH in 0.1 M bicarbonate buffer (pH 9.6) with 120 μg of biotin solution (AH-BIOTIN-NHS; Biospa) diluted to 1 mg/ml in *N*,*N*-dimethylformamide. After 4 h of incubation at room temperature, the excess free biotin was removed by overnight dialysis in PBS at 4°C. Maxisorp flat-bottom 96-well microtiter plates (Dako) were coated with 100 μl/well streptavidin (Biospa) diluted to 15 μg/ml in bicarbonate buffer overnight at 4°C. Plates were subsequently washed four times with PBS plus 0.05% Tween 20 (PBS-T) and over-coated with 100 μl/well KLH (0.3 μg/ml), HPV18 E7 (0.3 μg/ml), or HPV16 E7 (1.2 μg/ml) antigen conjugated to biotin in 1% (weight/volume) bovine serum albumin fraction V (BSA) in PBS. The plates were washed with PBS-T and blocked with 200 μl/well 1% nonfat dried skimmed milk and 1% BSA in PBS (blocking buffer) for 1 h at 37°C. Human sera were diluted 1:50 in blocking buffer, and 100-μl aliquots were added to KLH- or E7-coated wells and to noncoated wells (negative control). The plates were washed as described above and incubated with 100 μl of anti-human immunoglobulin G-peroxidase conjugate (Sigma) diluted 1:10,000 in PBS plus 1% BSA for 2 h at 37°C. The wells were washed four times with PBS-T, followed by the addition of substrate and a chromogen tetramethylbenzidine liquid substrate system (Sigma). The enzyme-catalyzed color development was stopped after 30 min by the addition of 0.2 M H_2SO_4, and plates were read at an optical density at 450 nm (OD_{450}) on an automatic plate reader (Spectramax). Each serum sample was tested in duplicate, and the specific reactivity to the KLH and E7 antigens was determined by subtracting the averaged OD due to streptavidin and coating buffer alone as described previously (17). All longitudinal sets of sera from each woman were analyzed simultaneously to determine if the antibody levels had increased significantly between the prevaccination samples and the postvaccination samples.

ELISpot assay for IFN-γ. Cryopreserved PBL harvested before and at different time points during the vaccination protocol period were thawed and restimulated once in vitro with HPV16/18 E7- or KLH-pulsed DC (ratios from 20:1 to 30:1, responder PBL:DC) in 6-well culture plates (Costar) in AIM-V medium. The cultures were initially supplemented with recombinant human GM-CSF (500 U/ml; R&D Systems), while 10 U/ml human recombinant IL-2 (rIL-2; Proleukin, Chiron Corp.) and 5% human AB serum were added to support T-cell survival and expansion after 7 days. Cells were incubated for 2 to 3 weeks to expand reactive T cells. $CD8^+$ and $CD4^+$ T cells were separated from the bulk cultures by positive selection with CD8 or CD4 Dynabeads (Dynal, Inc.) before specificity was investigated using ELISpot assays. Gamma interferon (IFN-γ) ELISpot kits (R&D Systems) were used to determine the frequency of cytokine-expressing in vitro-stimulated $CD8^+$ and $CD4^+$ T cells after overnight activation with KLH and E7-antigen-loaded and unloaded stimulator DC. Anti-CD3-stimulated T cells (0.2 μg/ml OKT3; Ortho Pharmaceutical) were used as internal positive controls, while T cells alone were analyzed to estimate background spot formation. Briefly, 96-well plates (Multiscreen-IP opaque plate; Millipore) were coated with diluted capture antibody (1:60 dilution in PBS) overnight at 4°C. The plates were then washed three times with 0.05% Tween 20 in PBS (wash buffer) and blocked at room temperature with 1% BSA in PBS for 2 h. After three additional washes, the plates were given a final wash with RPMI medium-10% human serum. T cells (2.5×10^4 and 5×10^4 cells/well) and DC (5×10^3 cells/well) were added to duplicate wells and left overnight for T-cell activation. The plates were washed as described above prior to the addition of the diluted

detection antibody (1:60 dilution in 1% BSA in PBS) and then incubated overnight at 4°C. After the plates were washed three times with wash buffer, streptavidin-AP (1:60 dilution in 1% BSA in PBS) was added and incubated at room temperature for 2 h. The plates were again washed three times with wash buffer and then once with deionized water. BCIP/NBT chromogen (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) solution was added to each well, and the plates were left in the dark for about 10 min at room temperature before deionized water was added to stop development. Plates were scanned and analyzed with an AID ELISpot reader system (Cell Technology, Inc.). Results were plotted as the number of IFN-γ-producing cells per 2.5×10^4 cells/well after the background responses were subtracted.

Flow cytometry for detecting FOXP3 and CD25 in peripheral blood CD4$^+$ T-cell lymphocytes before and after HPV16/18 E7-pulsed DC vaccination. Recent reports have suggested a potential increase in the number of CD4$^+$ FOXP3$^+$ regulatory T cells (Tregs) in the PBL of DC-vaccinated cancer patients (2). To evaluate this possibility, blood samples from all 10 cervical cancer patients were assessed before the beginning of vaccination and after the last immunization for FOXP3 and CD25 expression by CD4$^+$ T cells, using an APC anti-human FOXP3 staining kit (eBiosciences, San Diego, CA). Rat immunoglobulin G2a APC (BD Biosciences) was used as an isotype control. Samples were also simultaneously stained with CD25 phycoerythrin and CD4 fluorescein isothiocyanate (BD Biosciences).

DTH tests. Cellular immune responses to KLH and E7 proteins were measured in vivo by DTH testing. Mumps (viral), purified protein derivative, tetanus toxin (bacterial), *Trichophyton* spp., and candidal (fungal) antigens were used as positive control antigens for DTH responsiveness before initiation of the DC vaccination series. Purified KLH and E7 proteins (25 μg) in 0.1 ml of injection-grade saline were used as test antigens. Negative controls also included 0.1 ml of saline solution. Patients were observed for 1 h for the development of any adverse reactions. The diameter of induration was recorded at 48 ± 24 h following inoculation. Reactions were scored as negative (induration/swelling ≤ 0.5 cm and/or erythema ≤ 20 mm), 1+ (induration/swelling = 0.6 to 0.8 cm and/or erythema = 21 to 40 mm), 2+ (induration/swelling = 0.9 to 2.0 cm and/or erythema = 41 to 60 mm), 3+ (induration/swelling ≥ 2.1 cm and/or erythema ≥ 61 mm). DTH responses were assessed prior to treatment and after the third and fifth DC vaccinations (Fig. 1).

Safety tests. DC cultures were routinely tested before each injection to exclude bacterial and fungal contamination. Standard gram staining and culture analyses were performed. In addition, injection products were tested for mycoplasmal contamination and endotoxin levels. No culture was observed to be contaminated with mycoplasma, and the level of endotoxin did not exceed 1 endotoxin unit/ml of culture medium.

Statistics. To adjust for patient differences at baseline, ELISA titers and ELISpot counts at time 1 (day 56), time 2 (day 98), and time 3 (day 144) (Fig. 1) were converted to ratios relative to that at time zero. Ratios were further transformed to their base-10 logarithms (log ratios) for hypothesis testing to satisfy the symmetry assumption of the signed-rank tests, to stabilize the variance for the repeated measures analyses, and to facilitate statistical inferences about changes expressed as ratios. For the initial analyses at each time averaged across dose groups, one-sided signed-rank tests were conducted at a Bonferroni-adjusted 1.67% alpha to assess whether median log ratios at times 1, 2, and 3 were significantly greater than that at time zero among the 10 patients; this was equivalent to testing whether the median untransformed ratios were significantly greater than 1. To determine whether the dose of vaccine influenced the magnitude of response, analysis of variance (ANOVA) with repeated measures was conducted using mixed models with compound symmetry for the intrapatient correlations among log ratios at times 1, 2, and 3. Post hoc contrasts were conducted at a Bonferroni-adjusted 1.67% alpha to determine if significant dose-group differences existed at times 1, 2, and 3. One-sided location tests were conducted to assess whether the means of log ratios were significantly greater than zero; this was equivalent to testing whether the geometric means of untransformed ratios (equivalent increases [fold]) were significantly greater than 1. To maintain an overall 5% type I error rate per antigen and assay, the location tests were conducted at adjusted alphas of 0.33% for each dose group at each time point, and 0.66% for each dose group was averaged across time points.

For the DTH data, some combinations of time point and challenge yielded identical responses among all patients, and generalized estimation equations for the analysis of discrete repeated measures could not be applied. Instead, a cluster analysis approach was used in which DTH responses from the same patient were treated as clusters. First, DTH response scores were converted to ranks, with averaging for ties, and ranks were subjected to mixed-models analysis to estimate repeated-measures variance components. Variance components were used to calculate intracluster correlations and corresponding variance inflation factors (VIFs). Next, DTH responses to E7 oncoprotein or KLH were tested for trends across time via the correlation chi-square test. Finally, by following the method of Donner and Klar (9), correlation chi-square test statistics were divided by their VIF values to adjust for the clustering, and VIF-adjusted chi-square values were used to calculate *P* values.

RESULTS

Characterization of DC vaccine. Autologous DC were generated from an initial leukopheresis as described previously (18, 20, 22, 23). The phenotype of mature DC was determined by flow cytometric analysis of HLA-DR, CD86, CD80, and CD83 expression. Viability was determined by trypan blue exclusion. All vaccine preparations were highly enriched in mature DC with a large and veiled appearance, expressing HLA-DR(+++) CD86(+++) CD80(++) CD40(++). Most DC (i.e., 85%) also expressed CD83, a maturation marker. This phenotype was stable after it was pulsed with full-length E7 and/or KLH (not shown).

Demographics and clinical characteristics of the patients. A total of 14 patients were enrolled in the vaccination study. Two patients were ineligible for DC vaccination because the HPV genotypes identified in their tumors differed from that of HPV16/18 (screening failures), while two HPV16-positive (HPV16$^+$) patients withdrew from the study for personal reasons. A total of 10 patients, one harboring an HPV18$^+$ tumor and nine harboring HPV16$^+$ carcinomas, completed the DC vaccine protocol. The primary treatment was a radical hysterectomy in nine patients and a radical trachelectomy in one patient with bilateral pelvic lymphadenectomy (Table 1). One patient (PT2) received adjuvant radiation treatment (i.e., whole-pelvis radiation) because of the presence of poor prognostic factors at the time of pathology review. No patients had clinical evidence of recurrent disease at the time of vaccination.

Vaccination, safety, and toxicity. Five DC vaccinations were given to each of the 10 patients (Table 1). Immunological parameters were studied at prevaccination (time zero), after the third vaccination (day 56; time 1), immediately after the fifth and last vaccination (day 98; time 2), and 2 months after the last vaccination (day 144; time 3). Patients were allocated sequentially to the lowest and then to the higher doses, according to their order of recruitment to the study. No significant local or systemic reactions were detected at the time of or after DC vaccinations. There were no signs of any life-threatening reactions, such as allergic reactions or anaphylactic shock, to the vaccine.

Clinical response. Vaccination treatment was given as an adjuvant therapy to all patients within a phase I clinical trial setting. Therefore, the evaluation of the treatment was designed primarily to test for the feasibility and safety of the vaccine and immunological responses from the patients. Injections of KLH/E7-pulsed DC were well tolerated by the patients, and no adverse side effects were observed or reported by subjects following immunization, beyond the immediate discomfort associated with injection. We noticed, however, local reactions (mild erythema, swelling/induration, and pruritus) at the subcutaneous vaccination sites that increased with the number of vaccinations in most of the patients. We also clinically observed a slight enlargement in the draining lymph node in the groin after DC injections in some of the patients. The

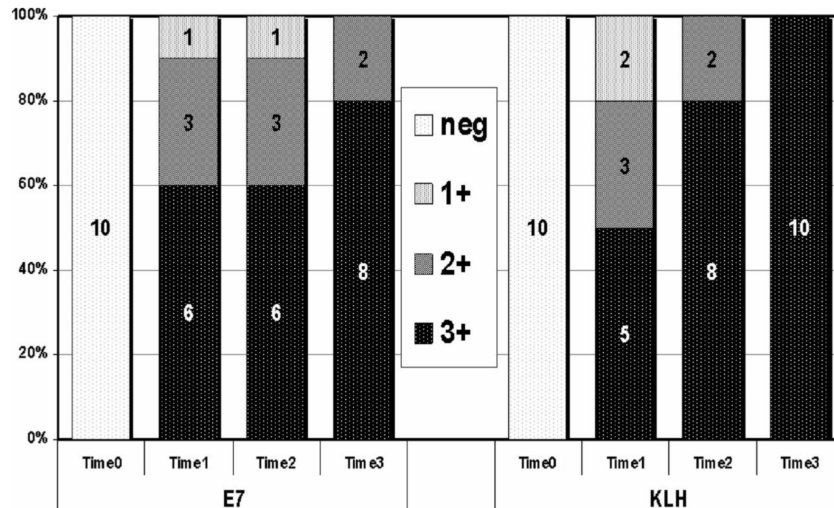

FIG. 2. Strength of DTH reactions over time in response to E7 and KLH. The stacked bars show the number and proportion of patients having reaction strengths as indicated by scores in the legend. Reaction strengths were scored as negative (induration/swelling ≤ 0.5 cm and/or erythema ≤ 20 mm), 1+ (induration/swelling = 0.6 to 0.8 cm and/or erythema = 21 to 40 mm), 2+ (induration/swelling = 0.9 to 2.0 cm and/or erythema = 41 to 60 mm), 3+ (induration/swelling ≥ 2.1 cm and/or erythema ≥ 61 mm). DTH responses were assessed prior to treatment and following the third and fifth DC vaccinations. Increasing trends in reaction strength with time are evident. The significance of time trends was assessed via the correlation chi-square test with adjustments for repeated-measures dependency through the VIF method described by Donner and Klar (9). VIFs were 2.12 for E7 and 1.26 for KLH, based on a cluster size of four DTH assessments per patient. VIF-adjusted correlation chi-square statistics (df = 1) for DTH reaction strength versus time were 10.48 (P = 0.0012) for E7 and 21.55 (P < 0.0001) for KLH.

patients were monitored during treatment with complete blood count and serum chemistry analyses that included liver and renal function and electrolyte tests. No alteration was detected in liver and renal function during the vaccine treatment (data not shown). Clinical investigation by routine diagnostic procedures including computer-automated tomography scan imaging and pelvic examinations during the clinical follow-up of the patients revealed no sign of tumor recurrence in any of the treated patients up to the time of writing (Table 1).

DTH responses. DTH responses against KLH and HPV16/18 E7 oncoproteins were assessed at different time points before, during, and after completion of the DC vaccination schedule (Fig. 1). Both antigens were delivered intradermally on the volar aspect of the forearms. All patients who received the DC vaccine responded to at least one out of five recall antigens before the beginning of the vaccination schedule (data not shown). Figure 2 shows the study subjects' DTH reaction strengths to a challenge with the E7 oncoprotein or the KLH at different assessment times during the study. At baseline (time zero), all 10 subjects showed a negative DTH response to both antigens. At day 56 (time 1, after the third DC vaccination), all 10 subjects showed some level of positive DTH response to both antigens, with patients 1, 3, and 6 showing reaction scores of 1+, 2+, and 3+, respectively, to E7 challenge, and patients 2, 3, and 5 showing reaction scores of 1+, 2+, and 3+, respectively, to KLH challenge. It is also evident in Fig. 2 that there was a tendency for the reaction scores to increase further by day 144 (time 3). Treating reaction scores numerically yielded mean scores for E7 reaction of 0.0 at time zero, 2.5 at time 1, 2.5 at time 2, and 2.8 at time 3, and mean scores for KLH reaction of 0.0 at time zero, 2.3 at time 1, 2.8 at time 2, and 3.0 at time 3. The mean scores thus quantify trends toward progressively stronger cellular immune responses to both E7 and KLH at later and later assessment times, and the VIF-adjusted correlation chi-square values (χ_1^2 1 = 10.5, P = 0.001 for E7; and χ_1^2 1 = 21.5, P < 0.001 for KLH) show that the trends in response strength with time were statistically significant for both antigens. As representatively shown in Fig. 3, an erythematous reaction with central swelling and ulceration was visible at the sites of injection of both the KLH and E7 antigens in the majority of the patients.

Humoral immunity against HPV16/18 E7 antigens. ELISAs for the detection of antibodies against KLH and HPV16 and HPV18 E7 antigens were performed with serum samples obtained from the 10 patients at the study entry and at different time points during the vaccination schedule (Fig. 1). Prior to their immunizations, low but measurable seroreactivity to E7, defined as an OD reactivity value of >3 standard deviations from the mean of a pool of "normal" sera (17), was observed with five cancer patients. After they were immunized, 10 out of 10 patients showed increases in reactivity to HPV16/18 E7 at times 1, 2, and 3, relative to their baseline values (Table 2). The medians (ranges) of anti-E7 titer ratios relative to baseline were 11.5 (1.0 to 121.5) at time 1 (signed-rank test, P = 0.002), 58.0 (2.0 to 260.0) at time 2 (signed-rank test, P = 0.001), and 93.0 (2.1 to 300.0) at time 3 (signed-rank test, P = 0.001); under a Bonferroni alpha value of 0.0167, all medians were considered significantly greater than 1.00. The upward trend in anti-E7 median ratios from time 1 to time 3 was consistent, with a statistically significant main effect for time ($F_{2,14}$ = 12.7; P = 0.0007) in the repeated-measures analysis of anti-E7 log-ratios. For KLH reactivity after immunization, all 10 patients showed increases over baseline at all time points (Table 2). The medians (ranges) of anti-KLH titer ratios relative to baseline were 187 (2 to 404) at time 1 (signed-rank test, P = 0.001), 1,270 (160 to 3,864) at time 2 (signed-rank test, P =

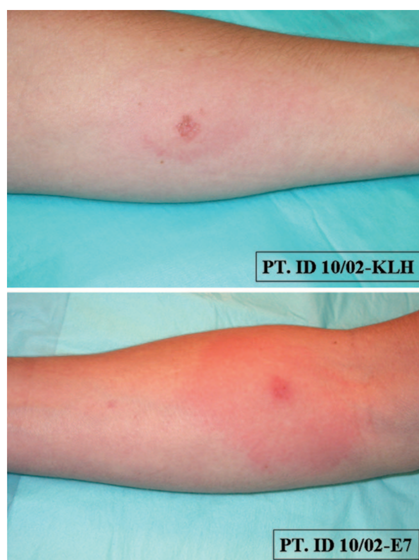

FIG. 3. Representative DTH reaction tests by intradermal injection of KLH antigen (upper panel) and HPV16 E7 antigen (lower panel) after the fifth subcutaneous vaccination of HPV16-E7/KLH-pulsed DC in patient 5 (PT.10/02). An erythematous reaction with central swelling and ulceration is clearly visible at the sites of injection of both the KLH and E7 antigens (i.e., 25 μg, each). No significant reaction is evident fof the saline control (lower panel).

0.001), and 1,498 (189 to 5,256) at time 3 (signed-rank test, $P = 0.001$); all medians were again significantly greater than 1.00. The upward trend in anti-KLH median ratios from time 1 to time 3 was consistent, with a statistically significant main effect for time ($F_{2,14} = 45.7$; $P < 0.0001$) in the repeated-measures analysis of anti-KLH log-ratios.

Figure 4 shows the relative titers for humoral reactivity to E7 and KLH by dose group at each time point, with statistically significant elevations over that at time zero. The previously mentioned significant main effects for time are visible as upward trends averaged over dose groups. Repeated-measures analyses of ELISA log ratios yielded mixed-model estimates of total variance (intrapatient correlation) of 0.52 (70%) for anti-E7 activity and 0.30 (68%) for anti-KLH activity. The time-by-dose interactions ($F_{4,14} = 0.87$, $P = 0.50$ for anti-E7; $F_{4,14} = 2.55$, $P = 0.09$ for anti-KLH) were statistically insignificant, showing that the individual dose groups did not differ much from each other with respect to their upward trends over time. For anti-E7, the differences among dose groups were statistically insignificant both overall ($F_{2,7} = 0.34$, $P = 0.72$) and at individual time points ($F_{2,14} = 0.01$, $P = 0.99$ for time 1; $F_{2,14} = 0.50$, $P = 0.62$ for time 2; and $F_{2,14} = 0.83$, $P = 0.46$ for time 3). For anti-KLH, the differences among dose groups were likewise statistically insignificant both overall ($F_{2,7} = 0.70$, $P = 0.53$) and at individual time points ($F_{2,14} = 2.59$, $P = 0.11$ for time 1; $F_{2,14} = 0.23$, $P = 0.80$ for time 2; and $F_{2,14} = 0.48$, $P = 0.63$ for time 3). One-sided location tests with adjusted alphas for anti-KLH log ratios yielded statistically significant increases for all dose groups both at each time point (Fig. 4) and overall, showing that all relative titers for anti-KLH were significantly greater than 1, regardless of dose group or time point. For anti-E7 log ratios, the one-sided location tests yielded statistically significant results (Fig. 4) for the low-dose and high-dose groups at time 2 and for all three dose groups at time 3 but not for any dose group at time 1 and not for medium-dose patients at time 2. At time 3 in particular, the increases from baseline were 68.0-fold ($P = 0.0003$) for the low-dose group, 21.9-fold ($P = 0.0031$) for the medium-dose group, and 110.4-fold ($P < 0.0001$) for the high-dose group, showing that all dose groups significantly increased their anti-E7 titers by the last evaluation. Moreover, when dose group ratios were averaged over the three postvaccination assessment times and evaluated via a location test against an adjusted 0.0067 alpha, the increases for the low-dose group (26.4-fold, $P = 0.0033$) and the high-dose group (38.5-fold, $P = 0.0009$) attained statistical significance, and the increase for the medium-dose group (15.1-fold, $P = 0.0078$) was almost statistically significant. Overall, these results demonstrated significant and progressively stronger humoral immune responses against the E7 oncoprotein and the KLH. However, the number of injected DC did not predict the magnitude of HPV16/18 humoral immunity elicited. No patient demonstrated cross-reactivity against both HPV16/18 E7 oncoproteins (data not shown).

Cellular immunity against HPV16/18 E7 and KLH. ELISpot analyses were performed for all patients to determine antigen-specific IFN-γ secretion as a hallmark of Th1 activation in $CD4^+$ and $CD8^+$ T cells collected before the beginning of vaccinations and at different time points during the study. To amplify the number of T cells available for ELISpot assays, the patients' PBL were restimulated once in vitro with either full-

TABLE 2. ELISA titers over time for individual patients

Dose group	Patient	Baseline titer	ELISA titer ratio relative to time zero		
			Time 1	Time 2	Time 3
Anti-E7 titer ratios relative to baseline					
05:Low	1	11	2.2	107.7	140.5
05:Low	2	5	1.2	4.4	10.8
05:Low	3	2	121.5	131.5	207.5
10:Med	4	1	16.0	42.0	28.0
10:Med	5	1	21.0	74.0	176.0
10:Med	6	7	1.9	2.0	2.1
15:High	7	1	19.0	76.0	72.0
15:High	8	1	31.0	34.0	114.0
15:High	9	1	7.0	260.0	300.0
15:High	10	3	1.0	26.0	60.3
Median ratio			11.5	58.0	93.0
Signed-rank test P value[a]			0.002	0.001	0.001
Anti-KLH titer ratios relative to baseline					
05:Low	1	11	2.2	279.1	1,435.5
05:Low	2	2	19.5	690.0	885.0
05:Low	3	1	215.0	1,850.0	2,850.0
10:Med	4	1	404.0	1,710.0	1,040.0
10:Med	5	1	306.0	1,590.0	1,920.0
10:Med	6	8	7.9	160.0	188.8
15:High	7	1	181.0	1,060.0	675.0
15:High	8	1	147.0	430.0	1,560.0
15:High	9	2	193.0	1,480.0	1,910.0
15:High	10	1	230.0	3,864.0	5,256.0
Median ratio			187.0	1,270.0	1,497.7
Signed-rank test P value[a]			0.001	0.001	0.001

[a] One-sided test of whether the median ratio is equal to (versus greater than) 1.00. Under a Bonferroni-adjusted alpha value of 0.0,167, all of the ELISA titer increases for both E7 and KLH are considered statistically significant.

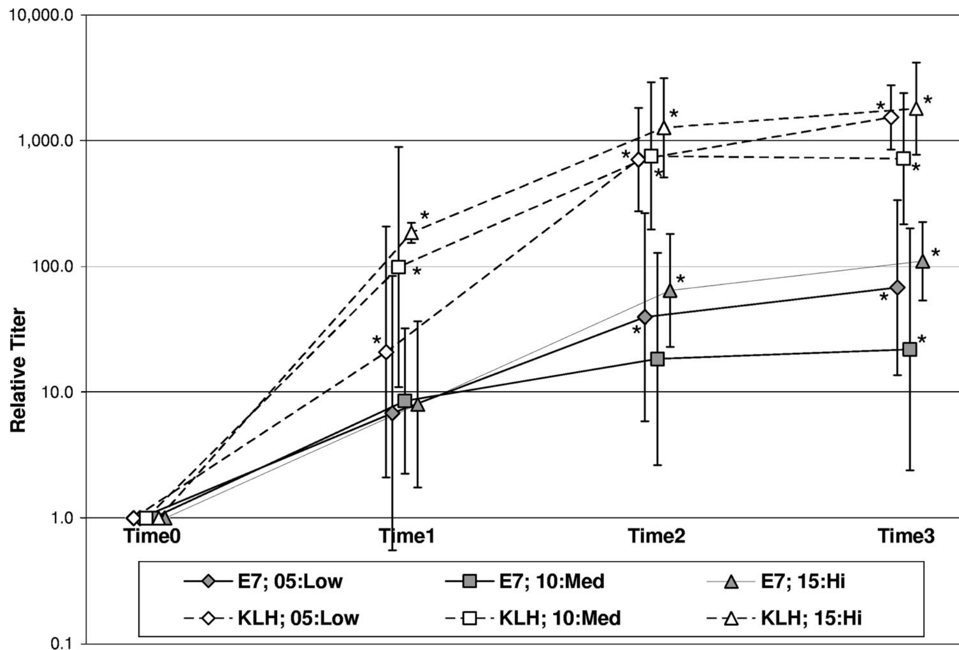

FIG. 4. ELISA antibody titers to E7 and KLH, ratios relative to time zero. Sera obtained before and after multiple vaccinations were analyzed by capture ELISA for E7- and KLH-specific antibodies from patients receiving the low, intermediate, and high dose of the vaccine. Gray symbols represent antibody responses to HPV E7; open symbols represent antibody responses to KLH. Antibody titers were measured for each patient at the indicated time points, converted to ratios (relative titers) relative to time zero to adjust for baseline differences, and analyzed as log10(ratios) with repeated-measures ANOVA. Symbols (error bars) represent means (SDs) based on log10(ratios); symbols marked with an asterisk show (at $P < 0.0033$) a statistically significant elevation above time zero.

length E7 protein or KLH-loaded autologous DC before T-cell subset separations with CD4 and CD8 Dynabeads, as described in Materials and Methods.

For both the E7 oncoprotein and the KLH, $CD4^+$ T cells collected from 10 out of 10 patients during or after the vaccinations showed a higher frequency of IFN-γ-positive cells compared to that of $CD4^+$ T cells collected before vaccination. E7-specific $CD4^+$ count ratios relative to that of the baseline (Table 3) had a median of 2.10 (range of 1.16 to 3.87) at time 1 (signed-rank test, $P = 0.001$), a median of 2.34 (range of 1.51 to 5.20) at time 2 (signed-rank test, $P = 0.001$), and a median of 2.15 (range of 1.24 to 4.28) at time 3 (signed-rank test, $P = 0.001$). KLH-specific $CD4^+$ count ratios relative to those at baseline (Table 3) had a median of 9.78 (range of 3.15 to 30.83) at time 1 (signed-rank test, $P = 0.001$), a median of 13.92 (range of 4.16 to 37.14) at time 2 (signed-rank test, $P = 0.001$), and a median of 14.04 (range of 5.17 to 52.86) at time 3 (signed-rank test, $P = 0.001$). Likewise, $CD8^+$ T cells reactive to KLH collected from 10 out of 10 patients at all three time points increased in frequency compared to those at baseline. KLH-reactive $CD8^+$ count ratios relative to that at baseline (Table 4) had a median of 5.54 (range of 3.47 to 31.00) at time 1 (signed-rank test, $P = 0.001$), a median of 7.04 (range of 4.17 to 40.00) at time 2 (signed-rank test, $P = 0.001$), and a median of 6.90 (range of 4.20 to 59.00) at time 3 (signed-rank test, $P = 0.001$). In contrast (Table 4), only patients 7 and 8 and 7 out of 10 patients showed increases from baseline in E7-reactive $CD8^+$ T-cell counts at times 1, 2, and 3, respectively; indeed, 2 patients (PT2 and PT7) showed decreases from baseline at all three time points. E7-reactive $CD8^+$ count ratios

TABLE 3. $CD4^+$ ELISpot counts over time for individual patients

Dose group	Patient	Baseline count	ELISA titer ratio relative to time zero		
			Time 1	Time 2	Time 3
E7-specific $CD4^+$ count ratios relative to baseline					
05:Low	1	15	3.87	5.20	3.13
05:Low	2	105	1.16	1.51	1.36
05:Low	3	100	2.56	2.64	2.91
10:Med	4	120	2.09	3.11	1.87
10:Med	5	71	3.52	3.44	4.28
10:Med	6	17	2.35	1.59	1.24
15:High	7	91	1.29	1.99	1.92
15:High	8	69	1.93	2.04	2.38
15:High	9	112	1.42	1.78	1.61
15:High	10	91	2.10	3.05	2.75
Median ratio			2.10	2.34	2.15
Signed-rank test P value[a]			0.001	0.001	0.001
KLH-specific $CD4^+$ count ratios relative to baseline					
05:Low	1	49	6.55	9.02	11.22
05:Low	2	89	3.15	4.16	5.17
05:Low	3	60	3.35	5.05	5.37
10:Med	4	22	8.82	12.27	11.36
10:Med	5	12	30.83	34.17	40.83
10:Med	6	7	30.29	37.14	52.86
15:High	7	19	12.63	20.53	14.79
15:High	8	31	8.42	11.97	13.29
15:High	9	27	10.74	15.56	15.93
15:High	10	27	12.96	19.3	18.48
Median ratio			9.78	13.92	14.04
Signed-rank test P value[a]			0.001	0.001	0.001

[a] One-sided test of whether the median ratio is equal to (versus greater than) 1.00. Under a Bonferroni-adjusted alpha value of 0.0,167, all of the ELISA titer increases for both E7 and KLH are considered statistically significant.

TABLE 4. $CD8^+$ ELISpot counts over time for individual patients

Dose group	Patient	Baseline count	ELISA titer ratio relative to time zero		
			Time 1	Time 2	Time 3
E7-specific $CD8^+$ count ratios relative to baseline					
05:Low	1	17	1.88	1.35	4.06
05:Low	2	110	0.61	0.39	0.66
05:Low	3	47	1.30	1.60	1.70
10:Med	4	34	4.44	5.26	0.88
10:Med	5	75	2.35	2.15	2.27
10:Med	6	41	0.93	2.44	1.39
15:High	7	96	0.78	0.73	0.56
15:High	8	150	1.09	1.27	1.35
15:High	9	111	1.36	1.72	1.91
15:High	10	79	1.42	1.71	1.28
Median ratio			1.33	1.66	1.37
Signed-rank test *P* value[a]			0.053	0.066	0.080
KLH-specific $CD8^+$ count ratios relative to baseline					
05:Low	1	36	5.19	8.64	9.44
05:Low	2	41	4.17	4.88	5.12
05:Low	3	30	5.67	7.00	7.30
10:Med	4	12	16.75	20.58	24.08
10:Med	5	9	24.44	28.89	23.33
10:Med	6	4	31.00	40.00	59.00
15:High	10	41	6.12	6.80	6.49
15:High	7	41	3.73	4.17	4.20
15:High	8	72	3.47	5.14	5.69
15:High	9	39	5.41	7.08	6.15
Median ratio			5.54	7.04	6.90
Signed-rank test *P* value[a]			0.001	0.001	0.001

[a] One-sided test of whether the median ratio is equal to (versus greater than) 1.00. Under a Bonferroni-adjusted alpha value of 0.0,167, all of the KLH-specific $CD8^+$ increased, but none of the E7-specific $CD8^+$ increases is considered statistically significant.

relative to those at baseline had a median of 1.33 (ranges of 0.61 to 4.44) at time 1 (signed-rank test, $P = 0.053$), a median of 1.66 (range of 0.39 to 5.26) at time 2 (signed-rank test, $P = 0.066$), and a median of 1.37 (range of 0.56 to 4.06) at time 3 (signed-rank test, $P = 0.080$); under an adjusted alpha value of 0.017, none of the medians was considered significantly greater than 1.00.

Figure 5 shows that the frequency ratio relative to that of the baseline of $CD4^+$ T cells showing reactivity to E7 and KLH has statistically significant elevations over time zero. Repeated-measures analysis of ELISpot count log ratios yielded mixed-model estimates of total variance (intrapatient correlation) of 0.034 (81%) for E7-specific $CD4^+$ cells and 0.041 (96%) for KLH-specific $CD4^+$ cells. One-sided location tests from the repeated-measures analysis of E7-specific $CD4^+$ yielded statistically significant elevations for seven out of nine combinations of dose and time (Fig. 5). One-sided location tests of dose groups averaged over time yielded *P* values below the adjusted alpha value of 0.0066 for all three dose groups, with equivalent increases in $CD4^+$ count of 2.43-fold ($P = 0.0030$) for the low-dose group, 2.43-fold ($P = 0.0030$) for the medium-dose group, and 1.96-fold ($P = 0.0056$) for the high-dose group. The location tests establish that all dose groups, at nearly all time points, showed a statistically significant increase over baseline in E7-specific $CD4^+$ T-cell counts. There was little difference among postbaseline time points averaged across dose groups ($F_{2,14} = 1.94$, $P = 0.18$ for time main effect), little difference among dose groups averaged across time points ($F_{2,7} = 0.35$, $P = 0.73$ for dose main effect), and little evidence for a significant time-by-dose interaction ($F_{4,14} = 1.29$, $P =$

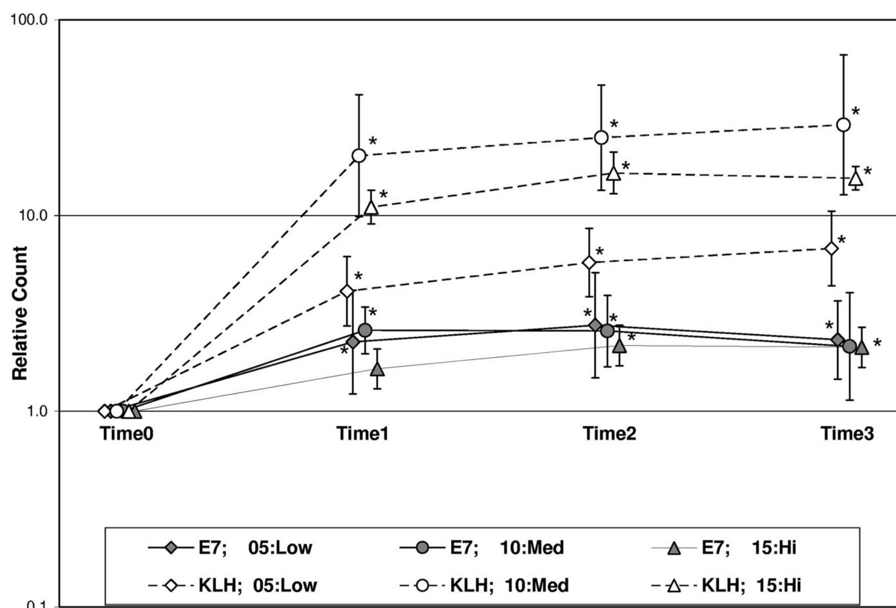

FIG. 5. $CD4^+$ ELISpot counts versus E7 and KLH, ratios relative to time zero. ELISpot analysis for enumeration of IFN-γ-secreting $CD4^+$ T cells specific for E7 (gray symbols) or KLH (open symbols) before and after multiple in vivo vaccinations of patients receiving the low, intermediate, and high doses of the vaccine. IFN-γ secretion was evaluated 24 h after stimulation of T cells with autologous unpulsed DC (negative control), HPV16/18 E7-pulsed DC, or anti-CD3 monoclonal antibody OKT3 (positive control). Positive control stimulation with the OKT3 antibody was more than 600 spots. Duplicate counts per patient against the indicated antigen at the time points shown were averaged after subtracting the background (i.e., unpulsed DC), converted to ratios (relative counts) relative to time zero to adjust for baseline differences, and analyzed as log10(ratios) in repeated-measures ANOVA. Symbols (error bars) represent means (SDs) based on log10(ratios); symbols marked with an asterisk show (at $P < 0.0033$) a statistically significant elevation above time zero.

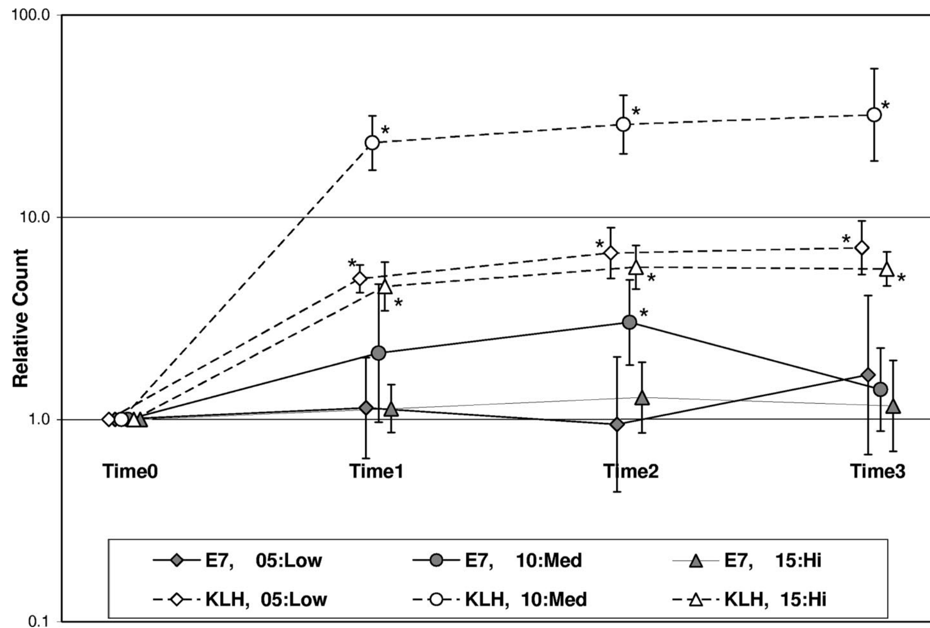

FIG. 6. CD8+ ELISpot counts versus E7 and KLH, ratios relative to time zero. ELISpot analysis for the enumeration of IFN-γ-secreting $CD8^+$ T cells specific for E7 (gray symbols) or KLH (open symbols) before and after multiple in vivo vaccinations of patients receiving the low, intermediate, and high doses of the vaccine. IFN-γ secretion was evaluated 24 h after stimulation of T cells with autologous unpulsed DC (negative control), HPV16/18 E7-pulsed DC, or anti-CD3 monoclonal antibody OKT3 (positive control). Positive control stimulation with the OKT3 antibody was more than 600 spots. Duplicate counts per patient against the indicated antigen at the time points shown were averaged after subtracting the background (i.e., unpulsed DC), converted to ratios (relative counts) relative to time zero to adjust for baseline differences, and analyzed as log10(ratios) in repeated-measures ANOVA. Symbols (error bars) represent means (SDs) based on log10(ratios); symbols marked with an asterisk show (at $P < 0.0033$) a statistically significant elevation above time zero.

0.32), collectively establishing that time and dose had little influence on the magnitude of increase in E7-specific $CD4^+$ counts that resulted from the vaccinations. Examination of KLH-specific $CD4^+$ count log ratios via one-sided location tests from the repeated-measures analysis yielded statistically significant elevations for all nine out of nine combinations of dose and time (Fig. 5), establishing that all dose groups at all time points showed KLH-specific CD4+ T-cell counts that were statistically significant compared to that at baseline. The main effect for time was statistically significant ($F_{2,14} = 49.99$, $P < 0.0001$) and consistent with an upward trend in $CD4^+$ count ratios, evident in Fig. 5. This figure also shows a roughly constant spread over time of about fivefold on average between the medium and low doses, which is consistent with a statistically significant dose main effect ($F_{2,7} = 8.33$, $P = 0.014$) and statistically significant dose simple effects at time 1 ($F_{2,14} = 9.02$, $P = 0.0030$), time 2 ($F_{2,14} = 8.06$, $P = 0.0047$), and time 3 ($F_{2,14} = 7.41$, $P = 0.0064$). The time-by-dose interaction was not significant ($F_{4,14} = 2.14$, $P = 0.13$), a finding consistent with the approximately parallel behavior displayed by the dose groups in Fig. 5 with respect to the time trends in their KLH-specific $CD4^+$ count ratios.

Figure 6 shows the frequency ratio relative to that of the baseline of $CD8^+$ T cells showing IFN-γ positivity after stimulation with E7 or KLH, with statistically significant elevations over time zero. Repeated-measures analysis of ELISpot count log ratios yielded mixed-model estimates of total variance (intrapatient correlation) of 0.064 (57%) for E7-specific $CD8^+$ cells and 0.017 (79%) for KLH-specific $CD8^+$ cells. For E7-specific $CD8^+$, one-sided location tests of count log ratios yielded statistically significant elevations for only one out of the nine combinations of dose and time (Fig. 6). For dose group ratios averaged over time, all location-test P values were above the adjusted alpha of 0.0067, with one-sided P values (equivalent increases over baseline) of 0.260 (1.21-fold) for the low-dose group, 0.018 (2.08-fold) for the medium-dose group, and 0.240 (1.19-fold) for the high-dose group. This shows that DC vaccination generally failed to raise E7-specific $CD8^+$ T-cell counts by amounts that could be considered statistically significant. The repeated-measures ANOVA of log ratios for E7-specific $CD8^+$ yielded statistically insignificant effects for time ($F_{2,14} = 0.21$, $P = 0.81$), for dose ($F_{2,7} = 1.30$, $P = 0.33$), and for the time-by-dose interaction ($F_{4,14} = 2.31$, $P = 0.11$). In contrast, for KLH-specific $CD8^+$, one-sided location tests of count log ratios yielded statistically significant elevations for all nine out of nine combinations of dose and time (Fig. 6), establishing that all dose groups at all time points showed statistically significant increases in KLH-specific $CD8^+$ T-cell counts compared to baseline values measured prior to the commencement of vaccination. The main effect for time was significant ($F_{2,14} = 12.42$, $P = 0.0008$) and consistent with a slight but steady increase in KLH-specific $CD8^+$ counts, as seen in Fig. 6 from time 1 to time 3 for all dose groups. The dose main effect was significant ($F_{2,7} = 34.80$, $P = 0.0002$) and consistent with the figure's depiction of a nearly fivefold average elevation in count ratios for medium-dose compared to the other two groups. The time-by-dose interaction was not significant ($F_{4,14} = 0.39$, $P = 0.81$), a finding consistent with the

striking parallelism shown in Fig. 6 for the dose groups with respect to the time trends in their KLH-specific $CD8^+$ count ratios.

Collectively, these results demonstrated a significant cellular immune response to vaccination with KLH-primed DC and a significant response by $CD4^+$ T cells to vaccination with E7-primed DC. In contrast, when $CD8^+$ T-cell responses to vaccination with E7-primed DC were evaluated among the 10 patients as a group, although 8 out of 10 patients demonstrated $CD8^+$ T-cell counts during or immediately after immunization that were increased in comparison to the prevaccination baseline levels, $CD8^+$ T-cell responses to vaccination with E7-primed DC were not significant.

FOXP3 and CD25 expression by $CD4^+$ T cells before and after DC vaccination. To characterize in more detail the nature of $CD4^+$ T-cell responses induced following DC immunization and evaluate the effect, if any, of the vaccination on the percentage of $CD4^+$/$FOXP3^+$ Treg cells, blood samples collected before vaccination (i.e., time zero) and after the last DC vaccination (i.e., time 3) were analyzed by flow cytometry for the expression of CD25 and CD4 surface antigens and intracellular FOXP3. Data from all 10 patients are presented. No significant differences between the percentages of $CD4^+$ $FOXP3^+$ Treg cells were identified when prevaccination Treg cell levels were compared to postvaccination levels (i.e., mean $\pm$ standard deviation [SD] Treg at time zero = 4.5 $\pm$ 2.6, range 1.5 to 10, versus 4.4 $\pm$ 2.3, range 0.8 to 8.3 [P = 0.6] at time 3). In contrast, when the number of DC-activated $CD4^+$ $CD25^+$ $FOXP3^-$ T cells were analyzed, we found that vaccine-activated $FOXP3^-$ $CD4^+$ $CD25^+$ T cells were significantly increased after DC-based vaccination (i.e., 5.6 $\pm$ 4.9, range 1.9 to 16.6, versus 7.2 $\pm$ 5.8, range 3.1 to 23.1 [$P < 0.01$, by Student's *t* test] at time 3).

For patients 2 and 7, for whom a decreased E7-specific CD8 T-cell response was observed with ELISpot assays postvaccination, we found an increase in $FOXP3^+$ $CD4^+$ T cells from time zero to time 3 (from 5.3% to 6.9% for patient 2 and from 2.9% to 6.0% for patient 7). However, both patients also showed increases in the number of $FOXP3^-$ $CD4^+$ $CD25^+$-activated T cells (from 16.5% to 23.1% for patient 2 and from 3.2% to 6.4% for patient 7), and both patients showed increased frequencies of E7-specific $CD4^+$ T cells with IFN-γ ELISpot assays (Table 3).

DISCUSSION

In this phase I dose escalation study, we investigated whether HPV16/18 full-length E7 antigen-pulsed mature monocyte-derived autologous dendritic cell vaccination can generate or boost an anti-E7 immune response in patients with stage IB or IIA cervical cancer. In addition, the effect of the dose of E7-pulsed DC on the immune response elicited in cervical cancer patients was also evaluated. Safety, toxicity, skin-test DTH reactions, and the induction of serological and cellular immunity against HPV16/18 E7 and KLH were monitored. In preclinical studies using E7-loaded DC, we observed an efficient induction of antigen-specific T cells from cervical cancer patients as well as from healthy individuals (20, 23). Autologous DC pulsed with full-length E7 oncoprotein of the HPV genotype involved in the disease offers the significant advantage of potentially presenting multiple immunogenic CTL epitopes, as well as allowing DC to tailor those peptides fitting self-HLA molecules without the necessity for previous knowledge of the individual HLA type. This approach, unlike that of HPV-peptide-based vaccinations, may be more practical for large-scale vaccination protocols. Furthermore, because the selective loss of one HLA restriction element from cervical tumor cells is a much more frequent finding than the total loss of HLA expression (5, 27), it is likely that the use of full-length E7 oncoprotein-based vaccination, presenting peptides potentially capable of binding multiple HLA class I restriction elements (10, 18, 20), might be a superior form of vaccination. In accord with this view, recombinant full-length E7-pulsed autologous DC consistently elicit specific T-cell responses against E7-infected autologous tumor cells in HLA-unselected patients harboring HPV16/18-positive cervical cancer in vitro as well as in vivo (10, 18, 20, 23).

From our previous studies (18, 20, 23) as well as those of others (10), DC vaccination is a safe and potentially effective therapeutic approach for patients with recurrent/metastatic cervical cancer refractory to standard treatment modalities. Unfortunately, however, the majority of patients with advanced cervical cancer have been heavily pretreated with radiation and/or multiple regimens of palliative chemotherapy and are often profoundly immunocompromised at the time of vaccination treatment (11, 19, 21). Here, we describe the results of the first clinical trial using recombinant full-length HPV16/18 E7-pulsed autologous DC vaccination as an adjuvant therapy for patients harboring early-stage cervical cancer. Our observations for all patients treated with autologous HPV E7 antigen-loaded DC confirm the tolerability and safety of the cellular vaccine. We investigated serologic responses of the vaccinated patients by a highly sensitive and specific antigen-capture ELISA. Remarkably, all immunized patients responded with increased titers of E7 antibody at all postvaccinations time points relative to those at baseline. We identified de novo E7-specific antibody responses from 5 of the 10 patients, and 5 other patients showed elevations in preexisting E7-specific antibody titers after vaccination. Similarly, progressively higher levels of KLH-specific antibody titers were detectable in 10 out of 10 patients after vaccination with KLH-loaded DC. Interestingly, the mean increases in antibody titer against E7 and KLH were not significantly dependent upon DC dose. These findings support the high in vivo immunogenicity of DC vaccines previously reported for healthy volunteers, where a single injection of less than 3×10^6 mature DC was able to rapidly expand T-cell immunity against a variety of antigens (7).

Cellular immune responses toward tumor-associated antigens are considered crucial for antitumor effects in vivo. In order to distinguish the cellular immune response induced by the E7-pulsed DC vaccination, we separated the PBL collected before and during the vaccination treatment into $CD4^+$ and $CD8^+$ T-cell components. $CD4^+$ Th1 cells are considered key players in the generation of potent cytotoxic T lymphocyte immune responses against tumor cells, and the inability to mount and/or maintain effective antitumor immune responses in vivo has often been attributed to the lack of generation of sufficient tumor-specific T-cell help (3, 14, 15, 28). Although we found considerable variability in T-cell responses among

patients, we observed a significant increase in the number of IFN-γ-secreting $CD4^+$ T cells in all 10 of our immunized patients, in whom 6 out of 10 also demonstrated a persistent elevation in the number of circulating IFN-γ-secreting $CD8^+$ T cells at all time points tested by ELISpot. The lack of a collectively significant $CD8^+$ response in DC vaccinees reflected the findings that (i) two patients showed postvaccination decreases in E7-specific $CD8^+$ T cells at all three time points following treatment onset, and (ii) two other patients showed cell count increases relative to their baseline counts at some time points but decreases at others.

The reason $CD8^+$ T-cell responses were not detected consistently in all 10 vaccinated patients is not completely understood. The use of cryopreserved blood samples, necessary for simultaneous evaluation in the same assay of samples collected at different time points during the vaccination protocol, may be a potential explanation for the variability in the $CD8^+$ T-cell results. However, this hypothesis seems unlikely because of the high viability of all tested blood samples at the time of thawing as well as the presence in each assays of both negative and positive control samples. It is interesting that in a recent clinical trial using similar in vitro techniques to evaluate immunologic responses in a smaller group of patients harboring recurrent cervical cancer, we found a consistent increase in the number of E7-specific $CD8^+$ T cells in all vaccinated patients when they were immunized with HPV16/18 E7-DOTAP-loaded autologous DC (24). On the basis of these findings, we are tempted to speculate that the use of DOTAP (a liposome transfection reagent previously shown to significantly facilitate the cytoplasmic incorporation of exogenous antigens for major histocompatibility complex class I (MHC-I)-restricted presentation to $CD8^+$ T cells), in addition to the different patient population enrolled (i.e., late-stage versus early-early-stage cervical cancer patients), may at least partially explain why $CD8^+$ T-cell counts were not consistently increased in all 10 patients. Consistent with this view, MHC-II-restricted $CD4^+$ T-cell activation by E7-pulsed DC, which is less likely to be significantly affected by DOTAP pulsing (24; also Santin et al., unpublished data), was consistently detected in 100% of the vaccinated patients. In this regard, phenotypic characterization of vaccine-responsive $CD4^+$ T cells by flow cytometry demonstrated a significant increase in $CD4^+$ $CD25^+$ $FOXP3^-$ T cells compared to that at prevaccination levels. More importantly, no significant differences in the percentages of $CD4^+$ $FOXP3^+$ Treg cells were identified when prevaccination Treg cell levels were compared to postvaccination levels for all 10 immunized patients. Taken together, these data suggest that in early-stage HPV-infected cervical cancer patients, unlike myeloma patients vaccinated with unpulsed DC (2), fully mature DC do not significantly increase the number of tolerogenic $CD4^+$ $FOXP3^+$ T cells but do significantly increase the number of antigen-specific DC-activated $CD4^+$ $CD25^+$ $FOXP3^-$ T cells. Finally, these immunologic results further support the notion that the activation of $CD4^+$ T cells, particularly IFN-γ-producing Th1 cells, may be critical for the simultaneous activation in vivo of the humoral and cellular arms of the immune system against HPV-infected tumors. Similarly, significant increases in the numbers of IFN-γ-secreting KLH-specific $CD4^+$ and $CD8^+$ T cells in 10 out of 10 of our immunized patients were demonstrated.

To rule out the presence of immunosuppression before vaccination as well as to confirm the successful induction of systemic immunity against E7 and KLH in vivo, all cancer patients were tested with a panel of recall DTH antigens in addition to E7 and KLH. Before they were immunized, all patients were able to respond to at least one recall antigen, whereas none furnished a positive DTH response against E7 or KLH. In contrast, positive DTH reactions against HPV16 and HPV18 E7 and KLH antigens were consistently identified after vaccination for all patients, although with considerable variability in magnitude. Collectively, these results indicate a systemic cellular immune reaction to E7 and KLH antigens and suggest that this therapeutic vaccination strategy may stimulate strong immunity against HPV-infected tumor cells in patients who had previously failed to clear HPV16/18-infected cervical lesions.

In conclusion, we have shown the safety and the immunologic potential of an autologous DC vaccine against an HPV16/18 E7 antigen. Antibody and $CD4^+$ T-cell responses were elicited from all patients enrolled in the study. The safety of the vaccination approach described here combined with the potential to increase host immunity against HPV-infected tumor cells may enable the immune system to clear a limited tumor burden and/or prevent disease recurrence in immunocompetent cervical cancer patients. Phase II trials of HPV E7 antigen-pulsed DC vaccination for the treatment of patients with stage IB or IIA HPV16/18-infected cervical cancer at high risk of recurrence are warranted.

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