

A Phase I Trial of a Human Papillomavirus DNA Vaccine for HPV16+ Cervical Intraepithelial Neoplasia 2/3

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Abstract Purpose: To evaluate the safety and immunogenicity of a therapeutic human papillomavirus (HPV)16 DNA vaccine administered to women with HPV16+cervical intraepithelial neoplasia (CIN)2/3.

Experimental Design: This phase I trial incorporated the standard '3+3' dose-escalation design with an additional 6 patients allocated to the maximally tolerated dose. Healthy adult women with colposcopically directed, biopsy-proven HPV16+ CIN2/3 received 3 i.m. vaccinations (0.5, 1, or 3 mg) of a plasmid expressing a Sig-E7(detox)-heat shock protein 70 fusion protein on days 0, 28, and 56, and underwent standard therapeutic resection of the cervical squamocolumnar junction at day 105 (week 15). The safety and immunogenicity of the vaccine and histologic outcome based on resection at week 15 were assessed.

Results: Fifteen patients were evaluable (3 each at 0.5 and 1mg, 9 at 3 mg). The vaccine was well tolerated: most adverse events were mild, transient injection-site discomfort; no dose-limiting toxicities were observed. Although HPV E7-specific T-cell responses to E7 detected by enzyme-linked immunospot assays (IFN- γ) were of low frequency and magnitude, detectable increases in response subsequent to vaccination were identified in subjects in the second and third cohorts. Complete histologic regression occurred in 3 of 9 (33%; 7-70% confidence interval) individuals in the highest-dose cohort. Although the difference is not significant, it is slightly higher than would be expected in an unvaccinated cohort (25%).

Conclusions: This HPV16 DNA vaccine was safe and well tolerated. Whereas it seems possible to elicit HPV-specific T-cell responses in patients with established dysplastic lesions, other factors are likely to play a role in lesion regression.

Cervical cancer remains the second leading cause of cancer death in women worldwide despite the fact that, for more than 5 decades, it has been possible to screen for and treat early-stage disease. Even though a highly efficacious prophylactic vaccine against the causative agent, human papillomavirus (HPV), has

been approved by the Food and Drug Administration, lack of access to healthcare in resource-poor settings is likely to limit the public health impact of the vaccine, as it has that of screening and treatment of early-stage disease in the same environments. In the absence of a broadly based preventative program, there will continue to be a need for effective therapeutic interventions for early-stage and late-stage cervical cancer.

HPV-associated neoplasia of the cervix presents a compelling opportunity to test immunotherapies after disease has been detected because expression of two non-self viral antigens, E6 and E7, is functionally required to initiate and maintain neoplastic lesions. If left undetected and/or untreated, a subset of cervical intraepithelial neoplasia (CIN)2/3 lesions will progress over a time frame of years to invasive squamous cell carcinomas. Both high-grade dysplasia and squamous cell carcinomas are associated with integration of the HPV genome into the host genome, with subsequent constitutive and functionally obligator expression of E6 and E7. However, we and others have found that between 20% and 25% of HPV16-associated CIN2/3 lesions undergo complete spontaneous regression within 15 weeks of diagnostic biopsy (1). Because conventional histopathologic assessment of tissue at time of diagnosis does not predict either spontaneous regression or lesion persistence, all CIN2/3 lesions are treated by either surgical resection or ablation. However, because a fraction of

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Received 7/7/08; revised 9/18/08; accepted 9/21/08.

Grant support: National Cancer Institute (NCI) Cancer Center Core Grant 3 P30 CA006973-45; NCI K23CA85437, NCI QuicKTrials R21 CA105696, Maryland State Cigarette Restitution Funding, and Dana Foundation (C.L. Trimble); NCI Cervical Cancer Specialized Programs of Research Excellence Project 5 P50CA098252 (T.C. Wu); and NCI Rapid Access to Interventional Development Project 048 (D.M. Pardoll).

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Note: Phase I/II Study of pNGVL4a-Sig/E7(detox)/HSP70 DNA Vaccine in Preventing Cervical Cancer in Patients with Human Papillomavirus-16-Positive Grade 2 or 3 Cervical Intraepithelial Neoplasia (<http://www.cancer.gov/clinicaltrials/NCT00121173>).

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doi:10.1158/1078-0432.CCR-08-1725

Translational Relevance

Human papillomavirus (HPV) causes 10% of malignancies in women. Despite the availability of prophylactic vaccines, because barriers to obtaining prophylaxis are significant, the need to pursue therapeutic strategies remains real. Because the viral oncoproteins (E6 and E7) are functionally required for disease initiation and persistence, they present compelling immunotherapeutic targets. In the United States, high-grade cervical dysplasia, the immediate precursor to cervical cancer, is common. We developed an HPV16 E7-targeted vaccine that had considerable therapeutic effect in the preclinical TC-1 model. Here we report the clinical, safety, and immunologic outcomes of a single-institution, investigator-initiated, phase I dose-escalation trial in women with HPV16+ high-grade dysplasia.

established high-grade dysplasias regress and lesions are accessible in a relatively noninvasive fashion, this patient population is a potentially informative cohort in which to test proof of principle for immune therapies.

Globally, HPV16 is the genotype most commonly associated with disease. Analyses of peripheral blood from patients with preinvasive dysplastic lesions have detected weak T-cell responses to E6 and E7, suggesting that these antigens are indeed presented to and recognized by the immune system (2). However, the consistently marginal or undetectable antibody responses to HPV16 proteins also suggest that genital/mucosal HPV infection does not commonly have a substantial viremic phase.

To determine if providing E7 in a potentially more immunogenic context might be beneficial for generating responses that could contribute to elimination of lesions, we developed a DNA vaccine targeting the HPV16 E7 oncoprotein, pNGVL4a-Sig/E7(detox)-heat shock protein (HSP70), which was designed to elicit a CD8 T-cell response to a mutated form of E7. This construct encodes a mutated nonfunctional E7 incapable of binding retinoblastoma protein, denoted E7(detox), thereby abrogating the transforming activity of the protein. DNA vaccines have generally exhibited low immunogenicity in humans, and we therefore linked HSP70, a chaperonin, to the E7(detox) sequence based on the notion that linkage of antigen to a heat shock protein might enhance uptake by antigen-presenting cells and MHC class I processing and presentation. We also attached a signal sequence to the hybrid antigen (E7 detox HSP70), which results in secretion of the linked E7 antigen, based on the reasoning that a secreted antigen would be more likely to gain access to professional antigen-presenting cells than one that was intracellular. In preclinical experiments, homologous prime-boost vaccination with this construct in C57BL/6 mice elicited significantly more vigorous E7-specific CD8+ T-cell responses than wild-type E7-containing DNA vaccines. These responses had therapeutic activity against established tumors expressing WTE7 (TC-1; ref. 3). Here we report the clinical and immunologic characteristics of a phase I clinical trial testing safety, immunogenicity, and histologic outcomes in

women with colposcopically directed, biopsy-proven CIN2/3 associated with HPV16.

Materials and Methods

Vaccine

The study vaccine, pNGVL4a-Sig/E7(detox)/HSP70, was manufactured by the National Cancer Institute Rapid Access to Interventional Development program. It is composed of a closed circular DNA plasmid expressing HPV16E7 mutated at aa 24 and 26, linked to sequences coding for Sig and for HSP70 (Fig. 1). Both bulk and formulated vaccine showed expression of E7(detox) in transfected human embryonic kidney cells, confirming that clinical material had the capacity to support translation of the vaccine antigen.

Study design

This phase I dose-escalation study, J0323, was conducted as an open-label, single-site study at the Johns Hopkins Medical Institutions. This trial incorporated the standard '3+3' dose-escalation design with an additional 6 patients allocated to the maximally tolerated dose. Human experimental guidelines of the Department of Health and Human Services were followed in the conduct of clinical research, and the protocol was reviewed and approved by the Johns Hopkins Institutional Review Board. The primary objective was safety and tolerability of the study vaccine. Secondary end points included histology at week 15 (7 wk post-vaccination) and systemic T-cell response measured by γ -IFN enzyme-linked immunospot assay (ELISpot).

Patients referred for colposcopic evaluation of high-grade squamous intraepithelial lesion pap smears were approached for participation. Eligibility criteria included ability to give informed consent, immune competence, HIV seronegativity, nonpregnant status, and presence of residual, visible lesion after a colposcopically directed, biopsy-confirmed diagnosis of CIN2/3. Patients included in this analysis had lesions associated with HPV16, the genotype most commonly associated with cervical cancer and its precursor lesions. Patients underwent standard colposcopic evaluation and diagnosis at study entry ($t = 0$), an interval colposcopic exam at week 8, standard cone or loop electrosurgical excision procedure resection of the squamocolumnar junction at week 15, and had a postoperative exam at week 19. At each study visit, colpographs, cervical swabs, and peripheral blood were obtained.

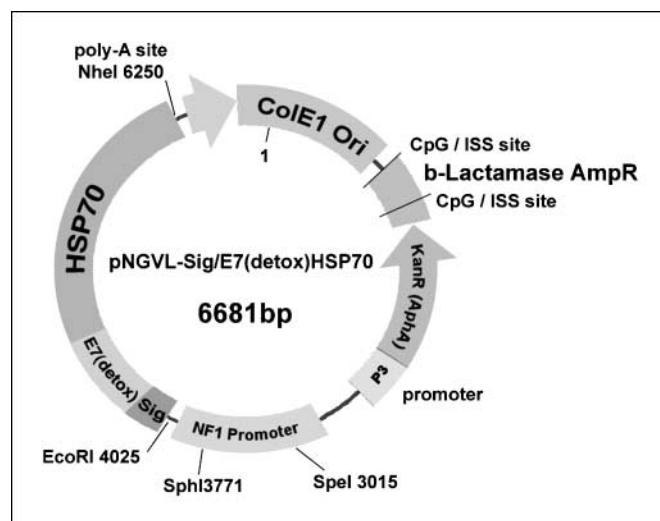


Fig. 1. Schematic of pNGVL4a-Sig/E7(detox)/HSP70.

Table 1. Demographics of study cohort

Age (y)	
18-20	3 (20%)
21-30	7 (47%)
31-40	3 (20%)
41-50	2 (13%)
Race	
American Indian/Alaskan	0
Asian	1 (7%)
African American	3 (20%)
Caucasian	9 (60%)
Hispanic	2 (13%)

The study protocol included 3 vaccinations with 1 of 3 doses of study vaccine, 0.5, 1.0, or 3.0 mg, by i.m. injection in alternating deltoid muscles. Immunizations were administered on day of enrollment (day 0), and weeks 4 and 8. At the highest dose, 3 mg, the vaccination was split equally between the left and right deltoids. Subjects were monitored throughout the study with physical assessments, including serial colposcopic exams and subject self-assessment for local and systemic symptoms by diary cards for 7 d after each vaccination. Adverse events were assessed for severity by the criteria in the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. HIV antibody testing was assessed by the standard institutional protocol. Complete human leukocyte antigen typing was carried out by the Johns Hopkins Immunogenetics Core Laboratory. HPV testing was carried out real-time by the Johns Hopkins Molecular Pathology Core Laboratory with the use of the HPV16-specific TaqMan real-time PCR method developed by Gravitt et al. (4). Histology at diagnosis and at therapeutic resection at week 15 was read first as part of routine care and subsequently reviewed by the study pathologists.

End point evaluations

Colposcopic evaluation. Using DenVu digital imaging software, we obtained serial colposcopic images over the 15-wk interval.

Cell preparation. Peripheral blood mononuclear cells (PBMC) were obtained at study entry (screening/week 0), and at weeks 8, 15, and 19. Cells were prepared by density centrifugation with Lymphoprep (Axis-Shield PoC AS), resuspended in freezing medium (90% heat-inactivated fetal bovine serum+10% DMSO), and cryopreserved. ELISpot assays were done on thawed specimens. Average viability was 68.77%.

ELISpot assays. MultiScreen-IP plates (polyvinylidene difluoride; Millipore) were coated overnight at 4°C with 1 µg per well of antihuman IFN-γ antibody 1-D1K (Mabtech). The plates were washed 3 times and blocked with culture medium (Iscove's Modified Dulbecco's Medium with 10% human AB serum; Invitrogen and Gemini Bio-Products, respectively) for 2 hr at 37°C. Cryopreserved PBMCs were thawed, washed, and resuspended ($5-10 \times 10^6$ cells) in 5 mL culture medium in 50-mL tubes. Cells were incubated for 2 hr at 37°C in a CO₂ incubator, filtered through a nylon cell strainer to remove debris, and resuspended at a final concentration of 4×10^6 cells/mL in culture medium. PBMCs were plated in triplicates at 2×10^5 cells/well/0.1 mL with peptide antigens at 2 µg/mL or medium alone. The following antigens were used: HPV16 E6-overlapping or E7-overlapping 15-mer peptides (synthesized at 60% purity, confirmed by high-performance liquid chromatography; Sigma-Genesis) combined in pools and a CEF peptide pool (Mabtech). After 20-hr incubation at 37°C, cells were removed by 6 washes with PBS/0.05% Tween 20 followed by 2 washes with PBS alone. Captured IFN-γ was detected by the addition of 200 ng/well of biotinylated antihuman IFN-γ antibody 7-B6-1 (Mabtech) followed by 2-hr incubation at 37°C. After washing, as above, avidin-peroxidase complex (Vectastain Elite ABC kit;

Vector Laboratories) was added for 1 hr at room temperature. Plates were washed again 8 times as above, and peroxidase staining was done with 3-amino-9-ethylcarbazole (Sigma) substrate in acetate buffer for about 5 min. The reaction was stopped by rinsing the plates under running distilled water. After drying overnight, spot numbers were evaluated on the KS ELISpot Automated Reader System with KS ELISpot software (Zeiss). The mean numbers of spot-forming cells from triplicate wells were calculated and expressed as spots per 1×10^6 PBMC. The mean spot numbers from wells with PBMCs incubated with medium alone (background) were subtracted from the means of PBMCs stimulated with peptides. Values ≥ 20 spots/ 1×10^6 PBMC after background subtraction and ≥ 2 SD above background were considered as positive.

A second protocol involving a single cycle of *in vitro* stimulation was used to carry out a separate set of analyses of peripheral blood responses. This protocol differed from the above in the following ways: on day 3, 1 ml of R10-AB medium containing 50 IU/mL recombinant human interleukin 2 (PeproTech) was added into each well. On days 5 and 7, half of the culture medium was replaced with fresh R10-AB containing 50 IU/mL recombinant human interleukin 2. On day 9, the cells were harvested, washed, and resuspended with R10-AB medium and plated into 24-well plates overnight. ELISpot assays were done on day 10 with the use of a commercially available ELISpot kit (Mabtech Inc.). A response for this set of assays was defined as positive if the number of spots minus the background was 2-fold above background, at least 50 spots/ 10^5 PBMC. For individual subjects for whom the background was <10 spots, the background was defined as 25.

Measurement of antibody responses. Antibodies to HPV16 E6 and E7 were measured by ELISA with the use of the glutathione *S*-transferase (GST) capture method of Sehr et al.(5) with minor modifications.

Statistical methods

The sample size was based upon the standard 3+3 dose-finding design with 3 dosing levels and an additional 6 individuals at the maximally tolerated dose. Given the low toxicity levels observed in prior DNA vaccine studies, the expected sample size was 15 patients, 9 for dose escalation and 6 additional for the maximally tolerated dose. The analyses were primarily descriptive in nature. Summary statistics (means, SDs, proportions, and confidence intervals) were used to descriptively characterize the cohorts numerically. Plots of the outcome variables, including T-cell response to the vaccine antigen, E7, over time, were used to describe the change in markers pre-vaccination and post-vaccination. Comparisons between groups were made with the use of *t* test or Wilcoxon rank sum tests.

Results

Study population

A total of 70 patients were screened and 16 participants enrolled for vaccination between December 10, 2003 and December 4, 2006. The mean age of study participants was 29.3 years (SD, 8.97; Table 1). Reasons for screening failure included no visible ectocervical high-grade lesion and not HPV16. The first patient enrolled was taken off study for

Table 2. Adverse events reported by study cohort

Toxicity	No. reporting by grade			
	Grade 1	Grade 2	Grade 3	Grade 4
Vaccine site tenderness	4	0	0	0
Malaise/flu-like symptoms	3	0	0	0
Fatigue	5	0	0	0
Vaginal discomfort/discharge	2	0	0	0

noncompliance after she had undergone vaccination twice. All of the subsequently enrolled subjects received all 3 injections, and completed 41 weeks of clinical observation. No patient had progression of disease during the study window. A total of 3 subjects (3 of 9; 33%) had complete histologic regression of disease at week 15. All of the regressors were in the highest-dose cohort (3 mg). Colposcopic regression did correlate with histology at week 15.

Vaccine safety and tolerability

No dose-limiting toxicities were observed (Table 2). Transient local reactivity was reported in 5 of 15 (33%), with the worst severity being mild. Systemic symptoms (malaise, myalgia, headache) after vaccination were also reported by 5 of 15 subjects. However, the worst severity in each case was mild. Two patients in the highest-dose cohort reported transient, mild systemic symptoms after one or more vaccinations, suggestive of a dose effect.

Vaccine-induced antibodies

Vaccination did not elicit antibody responses. We identified measurable titers at study entry of anti-E6 IgG antibody in 3 of 15 (20%) and of anti-E7 IgG antibody in 2 of 15 (13.3%) patients. The antibody titers to E7 were not boosted after vaccination with the E7 DNA construct in any dose cohort (data not shown). Measurable antibody titers to E7 at study entry did not correlate with γ -IFN immune response measured in PBMC.

HPV-specific T-cell responses in PBMC

Direct ex vivo ELISpot. The frequency of T-cell responses to pooled E7 overlapping peptides was measured in longitudinally obtained peripheral blood with the use of γ -IFN ELISpot assays. With the use of the overnight assay, HPV16 E7-specific T-cell responses were identified at baseline in 3 of 15 subjects. E7-specific response was increased in one of these patients subsequent to vaccination at week 15 and was stable in one other subject with a preexisting response. In the 3rd patient, response to E7 declined. Four patients in whom no detectable E7 response was found pre-vaccination had measurable responses post-vaccination.

Five patients had measurable responses to E6 at study entry. None of those subjects showed an increase in either E6 or E7 responses subsequent to vaccination. Two of the individuals in the highest-dose cohort, both regressors, had measurable

responses to E6 (Fig. 2). Overall, responses to E6 were not of greater absolute magnitude in regressors compared with non-regressors at either of the two time points. ($P = 0.4228$ and $P = 0.4964$, respectively). However, it should be noted that one of the regressors was the only individual to maintain a high E6 response before and after the vaccine.

We measured responses to E7 at 6 months and, unexpectedly, detected responses to E7 in 5 of 9 (55.6%) of subjects in the highest-dose cohort. No new responses to E7 were detected in subjects in the lower-dose cohorts.

In vitro stimulation followed by ELISpot. Because the detectable immune responses to E7 were low in frequency, in a second round of testing, we did a single cycle of *in vitro* stimulation and did ELISpot assays subsequently (Fig. 3A). With the use of this method, overall 5 of 15 patients had detectable responses pre-vaccination, and 8 patients (8 of 12; 53%) showed an increase in frequency of responses post-vaccination in the second and third dose cohorts (Fig. 3B). Peripheral blood immune responses to the vaccine antigen measured with the use of either method, although detectable and suggestive of a dose response, were not sustained.

Discussion

In the United States, CIN2/3 is common, with an estimated age-adjusted incidence ranging from 30 to 60 per 100,000 women (6). If left undetected and/or untreated, a subset of CIN2/3 lesions will progress to invasive squamous cell carcinomas. Conversely, complete histologic regression does occur spontaneously in up to 25% of HPV16-associated high-grade dysplasia (1, 7). Because conventional histopathologic assessment of biopsy tissue at the time of diagnosis does not predict regression, all CIN2/3 lesions are treated by either surgical resection or ablation. Surgical resection of cervical dysplasias has been associated with subsequent risk of preterm delivery in several large studies (8–10). The development of immunotherapeutic strategies would prevent morbidity even in high-resource settings. Moreover, because a subset of CIN2/3 lesions does indeed regress, this patient population is a potentially informative cohort in which to identify immune correlates of lesion regression versus persistence.

This single-plasmid HPV16 DNA vaccine construct was well tolerated in healthy patients with biopsy-confirmed CIN2/3

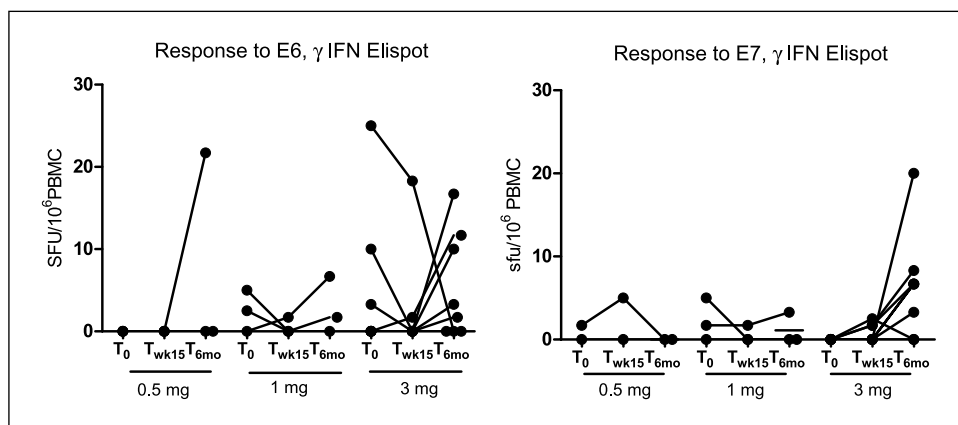
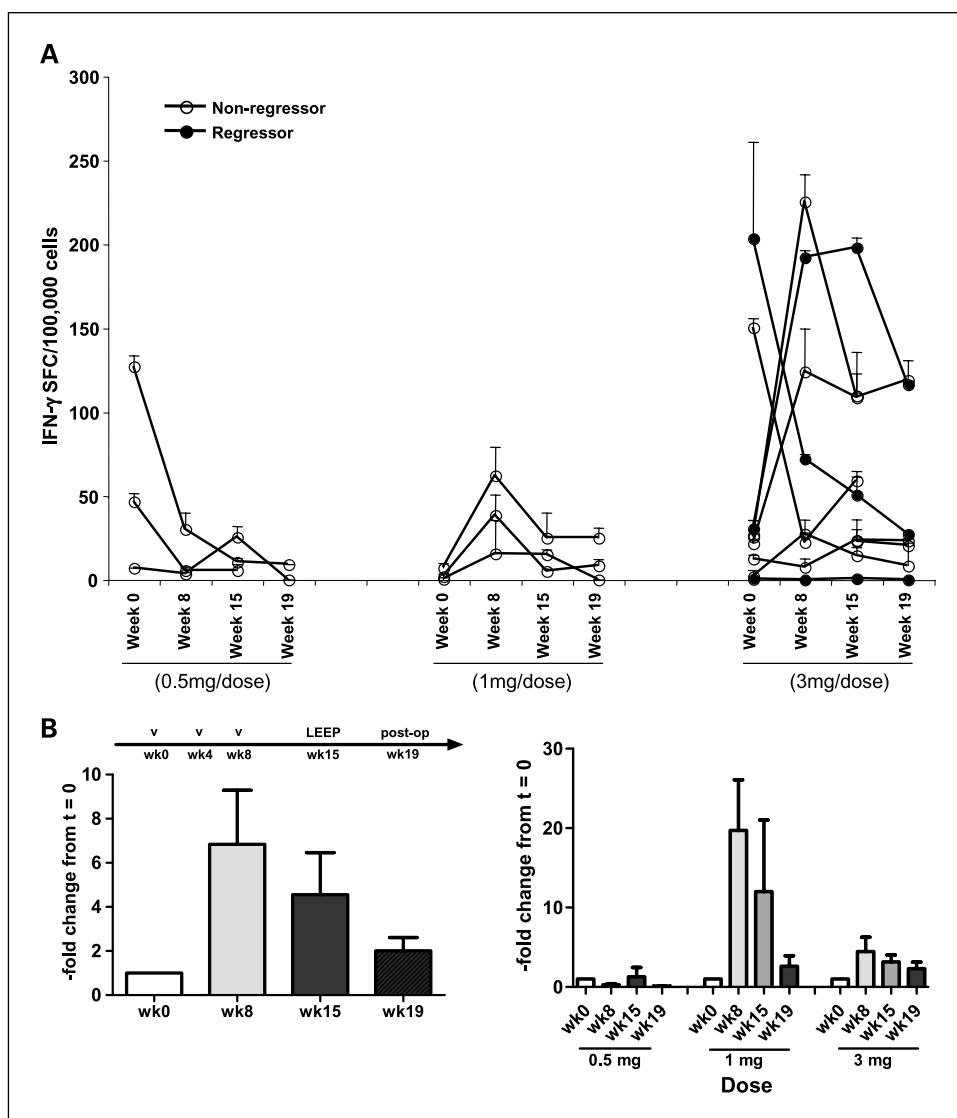


Fig. 2. T-cell responses to HPV16 E6-overlapping and E7-overlapping peptides pre-vaccination and post-vaccination using direct *ex vivo* ELISpot on unfractionated PBMCs.

Fig. 3. A, T-cell responses to HPV16 E7-overlapping peptides after a single cycle of *in vitro* stimulation using ELISpot on unfractionated PBMCs. B, fold change in T-cell response to E7 post-vaccination using ELISpot on unfractionated PBMCs.



associated with HPV16. In addition, 3 of 9 patients in the highest-dose cohort had complete histologic regression of established CIN2/3 in the study window. With the use of an overnight ELISpot assay, no patients had significant changes in recognition of E7 in the initial 19 weeks. However, we did detect new responses in 5 of 9 (55.6%) subjects in the highest-dose cohort. With the use of an assay that included a cycle of *in vitro* stimulation, T-cell responses to E7 were increased subsequent to vaccination in 8 of 15 patients. However, overall HPV-specific T-cell responses were not of greater magnitude than those we have identified in unvaccinated subjects with HPV16+ CIN2/3. Vaccination was not associated with changes in serum IgG antibody titers to the vaccine antigen within the study window.

The long interval before detection of responses was unexpected. However, maturing clinical data from other investigators have also identified new responses to DNA vaccine antigens after a protracted interval (11). Other investigators assessing IFN- γ responses to heterologous DNA prime-viral vector-based boost vaccination regimens have also uncovered different

kinetics of response to vaccine antigens in direct *ex vivo* assays compared with cultured assays of patient-matched specimens (12). Responses identified with the use of cultured assays correlated with long-lasting T-cell responses. The identification of the kinetics of response to vaccination will have obvious implication in the design of boosting strategies in humans.

Many investigators have shown the safety, tolerability, and feasibility of DNA vaccination in cohorts encompassing a spectrum of health, from healthy naive cohorts to patients with end-stage disease. Whereas DNA vaccine constructs are relatively simple to engineer and to produce, in humans the potency of DNA vaccination alone is limited. In fact, the immunogenicity of the DNA vaccines in humans becomes obvious only after heterologous boosting. Clinical experience with DNA vaccination with the use of other antigenic targets, notably malaria and HIV, has uncovered greater efficacy of priming when DNA vaccination is used as a priming vaccination in a heterologous prime-boost regimen compared with homologous vaccination strategies (13–17). In our preclinical model, heterologous E7-targeted vaccination with

the use of DNA priming and vaccinia-based boosting is considerably more immunogenic than homologous vaccination with either DNA-DNA or vaccinia-vaccinia, or vaccinia-DNA regimens (18). Because our target patient population is essentially healthy, our first trial needed to show the safety of the vaccine construct used alone. It does seem possible to elicit measurable changes in HPV-specific T-cell responses in patients with established preinvasive HPV disease.

Preexisting adaptive immune responses to E6, presumably reflecting previous exposure and immune recognition, did not seem to be associated with ability to respond to vaccination with this E7-targeted construct. In this cohort, we identified endogenous responses to E6 more frequently than responses to E7, and these were, overall, of greater magnitude. Several lines of evidence suggest that the E6 oncoprotein may well be more immunogenic than E7. Whereas functional T-cell recognition of both antigens have been measured in peripheral blood in unvaccinated clinical cohorts, memory CD4+ T-cell responses against HPV16 E6 are found frequently in healthy subjects without disease (19, 20), and, conversely, the absence of CD4 recognition of E6 correlates with higher incidence of disease (21). In a recently published phase I trial assessing vaccination with E6 and E7, long peptides in subjects with late-stage cervical cancer, subjects who underwent vaccination with both E6 and E7 in the same limb displayed dominant responses to E6; vaccination with E6 peptides and E7 peptides targeting separate sets of draining lymph nodes, in contrast, increased the magnitude of response to E7 and did not affect the magnitude of responses to E6 (22). Therefore, in our next clinical trial, we will assess the effect of heterologous, viral-based boost vaccination on immunogenicity and will also include E6 as an immunotherapeutic target.

Finally, another plausible reason that HPV-specific immune responses measured in the peripheral blood do not correlate completely with regression of CIN2/3 is that relevant immune variables are likely to be compartmentalized at the site of the lesion. Because high-grade dysplastic lesions develop only in

the clinical setting of a chronic, mucosally sequestered viral infection in the context of many coexisting commensal organisms, the immunologic 'default' set point is likely one of localized immune 'privilege' (i.e., one in which immunogenicity is suppressed; ref. 23). Although localized immune suppression is widely observed in the clinical setting of many frankly invasive diseases, it is likely to play a role well before transition to malignancy in the case of HPV-associated dysplasia. Indeed, Frazer et al. indicate a critical role for the generation of local lesional inflammation for effective tumor immunotherapy (24). In an animal model that used HPV16 E6 and E7 as nominal antigens, even in the face of circulating antigen-specific effector T cells, local acute inflammation was necessary to allow antigen-specific effector T cells to eliminate epithelium expressing these antigens, even in primed hosts. Early clinical data suggest an increased likelihood of response to local immune modulation in the presence of preexisting measurable HPV-specific T-cell responses (25). Based on these data, our next clinical trial of sequential heterologous vaccination will also assess the effect of topical imiquimod on access to lesions.

Disclosure of Potential Conflicts of Interest

C.L. Trimble has served a consultant for Merck and for Takeda.

Acknowledgments

We thank the study volunteers, and the Johns Hopkins Hospital Outpatient Center, Johns Hopkins Bayview Medical Center, and Johns Hopkins at Green Spring Station staff.

GMP reagent was manufactured, and underwent toxicology and release testing by NCI Rapid Access to Interventional Development (Barry Kobrin, Jason Yovandich, Stephen Creekmore, Sheryl Ruppel, Karen Schweikart, Joseph Tomasciewski, Douglas Gaum, Steven Giardina).

The following reagents were generously provided by Michael Pawlita, German Cancer Research Center (DKFZ), Heidelberg, Germany: glutathione casein, cleared lysate from *Escherichia coli* overexpressing GST-HPV16 E6, GST-HPV16 E7, and GSTalone.

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Clin Cancer Res 2009;15:361-367.

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