Combining a Recombinant Cancer Vaccine with Standard Definitive Radiotherapy in Patients with Localized Prostate Cancer

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Abstract

Purpose: Many patients with clinically localized prostate cancer develop biochemical failure despite excellent local therapy perhaps due to occult metastatic disease. One potential solution is the utilization of a well-tolerated systemic therapy (e.g., vaccine) in concert with local therapy.

Experimental Design: We present a randomized phase II clinical trial designed to determine if a poxviral vaccine encoding prostate-specific antigen (PSA) can induce a PSA-specific T-cell response when combined with radiotherapy in patients with clinically localized prostate cancer. Thirty patients were randomized in a 2:1 ratio into vaccine plus radiotherapy or radiotherapy-only arms. Those patients in the combination arm received a "priming" vaccine with recombinant vaccinia (r V) PSA plus r V containing the T-cell costimulatory molecule B7.1 (r V-B7.1) followed by monthly booster vaccines with recombinant fowlpox PSA. The vaccines were given with local granulocyte-macrophage colony-stimulating factor and low-dose systemic interleukin-2. Standard external beam radiation therapy was given between the fourth and the sixth vaccinations.

Results: Seventeen of 19 patients in the combination arm completed all eight vaccinations and 13 of these 17 patients had increases in PSA-specific T cells of at least 3-fold versus no detectable increases in the radiotherapy-only arm ($P < 0.0005$). There was also evidence of *de novo* generation of T cells to well-described prostate-associated antigens not found in the vaccine, providing indirect evidence of immune-mediated tumor killing. The vaccine was well tolerated.

Conclusion: This vaccine regimen can be safely given in patients undergoing radiation therapy for localized prostate cancer, with the majority of patients generating a PSA-specific cellular immune response to vaccine.

Prostate cancer is the most common noncutaneous malignancy among men in the United States. An estimated 230,100 men will be diagnosed with prostate cancer and 29,900 will die from the disease during 2004 (1). Although the majority of patients currently are diagnosed with clinically localized disease, 30% to 40% of patients will fail local definitive therapy (radiation or surgery) within 10 years as evidenced by an increase in prostate-specific antigen (PSA; refs. 2–4). This is often due to occult metastatic disease at the time of local therapy. Patients who are at high risk for recurrence, based on well-described risk factors,

have improved overall survival if they are given systemic androgen deprivation therapy (ADT) in addition to radiation therapy (5–7). This approach, however, does not benefit all patients and is associated with significant side effects. Thus, given the number of patients who develop prostate cancer, undergo definitive therapy, and then develop biochemical failure, it is reasonable to evaluate whether other systemic approaches may be combined with definitive local therapy to elicit a clinical effect.

One potential systemic treatment is the addition of an immunotherapeutic method to standard definitive radiotherapy. This approach may be able to not only assist in targeting the cancer within the prostate gland but also target the occult metastatic disease. The initial step in creating a vaccine for tumor immunotherapy is to choose the target antigen. Because PSA is expressed essentially only in prostatic epithelial cells (normal and malignant), and the prostate gland is nonessential, this antigen is an enticing choice. The fact that PSA is secreted and not membrane bound limits its use as a target for humoral immunity but not its use as a target of specific cellular immune system attack. Cells, including tumor cells, present endogenously expressed proteins on their surface in the form of peptide MHCs. CTLs recognize and are activated by specific peptides in the context of the appropriate MHC class I molecule on antigen-presenting cells (APC). This activation can in turn lead to killing of tumor targets by the peptide-specific CTLs.

Naive T cells require two signals to proliferate and stimulate a CTL response. The first is peptide antigen presented via MHC binding to the T-cell receptor. The second signal is modulated through a costimulatory molecule on the APC, such as B7.1, which binds to CD28 on T cells. The net result of these signals is the production of multiple cytokines, including interleukin (IL)-2 and IFN-γ, in both CD4 and CD8 T cells. In the absence of costimulation, weak antigens, such as tumor-associated antigens (TAA), presented to T cells via APC may result in anergy of those TAA-specific T cells. The concept of combining an admixture of recombinant vaccinia (rV) vaccine containing a specific antigen (e.g., PSA) with rV containing the costimulatory molecule B7.1 derives from the work of Hodge et al. (8). They examined T-cell response to vaccinated tumor-bearing mice with varying ratios of vaccinia vectors containing TAAs and B7.1. They found that in mice vaccinated with an admixture of rV-TAA and rV-B7.1 there was a synergistic T-cell lymphoproliferative response over either vector alone. This response was optimal at a 3:1 ratio of rV-TAA to rV-B7.1.

It has been shown previously that recombinant poxvirus vectors can be employed successfully to induce immune responses to the inserted "self" TAA transgenes in both preclinical models and clinical trials (8–14). Furthermore, it has been shown that T cells stimulated to recognize PSA can specifically kill PSA-expressing tumor cells (9, 10) and that these PSA-based vaccines are well tolerated with no dose-limiting toxicities noted (12–14). It has also been shown in both preclinical studies (15) and clinical studies (16, 17) that a primary vaccination with rV followed by multiple boosting vaccinations with recombinant avipox vaccine is superior to the reciprocal regimen or the continued use of one vaccine.

We and others have shown that radiation can cause increased expression of Fas, MHC class I molecules, and intracellular adhesion molecule-1 among other cell surface proteins (18–20). Each of these radiation-induced biological effects has the potential to make the tumor more susceptible to immune-mediated killing. Furthermore, it has recently been shown that vaccines can have a synergistic antitumor benefit with local tumor radiotherapy in a preclinical model (21). On the other hand, there are reports of decreases in nonspecific measures of the immune system following radiation therapy (22, 23).

This clinical trial is the first to combine a vaccine with definitive external beam radiation therapy (EBRT) for prostate cancer and the first to use an admixture of viral vectors, one encoding a TAA (PSA) and another encoding a costimulatory molecule. In addition, to our knowledge, this is the first published clinical trial looking at the effect of radiation therapy on specific immune responses.

Materials and Methods

Patient selection and trial design. Thirty patients with prostatic adenocarcinoma who were considered candidates for definitive EBRT (low, intermediate, or high risk for biochemical failure) were enrolled onto a randomized phase II trial approved by the National Cancer Institute institutional review board and conducted at the National Cancer Institute (Bethesda, MD). Patients were randomized in a 2:1 ratio to EBRT with vaccine or EBRT alone (see Fig. 1). The EBRT-only arm was used to control for radiation-induced changes, such as the induction of local inflammation and initiation of apoptosis, either of which could potentially stimulate PSA-specific T-cell responses. Patients were stratified by ADT versus no ADT and EBRT alone versus EBRT with
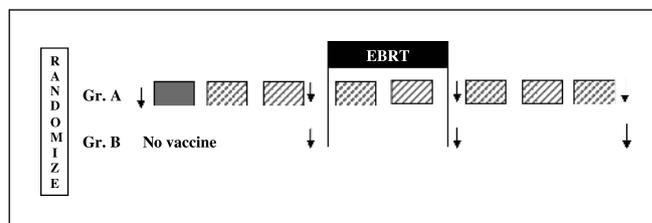

Fig. 1. Patients in group A received three monthly vaccines before EBRT, whereas those in group B were allowed to start EBRT on enrollment. The initial vaccine was given with an admixture of rV-PSA and rV-B7.1 (*solid gray box*) with follow-up monthly boosts given with recombinant fowlpox PSA (*boxes with the hatched lines*).

brachytherapy boost. The study was designed to have 20 patients receive radiation therapy with vaccine and 10 without vaccine to have 80% power to detect a 1 SD difference in the change in T-cell precursor frequencies compared with baseline, with a one-tailed 0.05 α level test. Because the primary end point of this trial was immunologic with the ELISPOT assay as the readout, all patients were required to be HLA-A2 positive. Patients needed to be Zubrod performance status 0 or 1 and have adequate hematologic, hepatic, and renal function. In addition, patients were required to have no evidence of an immunocompromised state as defined by nonreactive HIV testing, no diagnosis of altered immune function, no prior radiotherapy to >50% of nodal groups, no prior splenectomy, and no concurrent steroid use. Prior vaccinia exposure (for smallpox vaccination) was required.

Exclusion criteria were known egg allergy, active cases or history of skin disorders (such as eczema, extensive psoriasis, varicella zoster, impetigo, or burns), history of seizures, serious intercurrent illnesses, a noncutaneous malignant process, and close contact with either immunocompromised individuals, those with the above skin conditions, or children ages <5 years. All patients gave written informed consent in accordance with federal, state, and institutional guidelines and the principles embodied in the Declaration of Helsinki.

Vaccine formulation. Each of the three viral vaccine productions was manufactured by Therion Biologics Corp. (Cambridge, MA) as part of a Collaborative Research and Development Agreement between Therion Biologics and the Laboratory of Tumor Immunology and Biology, National Cancer Institute. Vaccines were then provided by the Cancer Therapy Evaluation Program, National Cancer Institute. rV-PSA (NSC 697729) and rV-B7.1 (NSC 699018) were prepared from virus derived from the Wyeth (New York City Board of Health) strain of vaccinia. This was selected based on its favorable toxicity profile. The rV-PSA was constructed by insertion of the entire human *PSA* gene into the viral genome, whereas the rV-B7.1 was constructed by insertion of the entire human *B7.1* costimulatory molecule gene into the viral genome. The priming vaccine consisted of 3.51×10^8 plaque-forming units of rV-PSA admixed with 1.17×10^8 plaque-forming units of rV-B7.1 (3:1 ratio) given s.c. A sterile, nonadherent dressing (i.e., "Telfa") was used to cover the site. The recombinant fowlpox PSA (NSC 694450) also contains the entire gene for human PSA inserted into the replication-defective avian fowlpox virus. This vector, used for each of the vaccine boosts, was injected s.c. in alternating sites at 1.5×10^9 plaque-forming units.

Treatment plan. The primary objective of this study was to determine if a PSA-specific T-cell response to the vaccine regimen could be mounted in the face of radiation therapy. Because local radiation-induced inflammation of the prostate may cause PSA-specific T cells, a control arm with no vaccine was used. Safety and biochemical failure [American Society for Therapeutic Radiology and Oncology definition (24)] were secondary and exploratory end points, respectively. Radiation therapy could be given to the patients by their local radiation oncologist and guidelines suggested total external beam dose to be ≥70 Gy, with 1.8 to 2.0 Gy per fraction. Because ADT is part of standard care for high-risk patients and is often used in intermediate-risk patients, it was given at the discretion of the treating radiation oncologist.

The rV-PSA/rV-B7.1 admixture was given as a priming vaccination and recombinant fowlpox PSA was given for each of seven subsequent monthly boosts. All vaccines were given on day 2 of each 28-day cycle with sargramostim [granulocyte-macrophage colony-stimulating factor (GM-CSF)] 100 μg/d given s.c. at the same site as the vaccination on days 1 to 4 and aldesleukin (IL-2) 4 MIU/M^2 given s.c. in the abdomen on days 8 to 12. The dose and schedule of GM-CSF and IL-2 were based on previous preclinical and clinical studies (12, 13, 25, 26). GM-CSF has been shown to increase recruitment of dendritic cells and enhance clinical responses to vaccine. IL-2 has been shown in preclinical studies to enhance the effectiveness of poxviral vector vaccines (27) and is widely used as a biological adjuvant in antitumor immunologic protocols (28, 29). If a patient experienced a grade 3 toxicity due to IL-2 or GM-CSF, that cytokine was reduced to 50% of the previous dose for subsequent administrations. Standard EBRT was given between the fourth and the sixth vaccinations.

The patients were seen monthly for 9 months with weekly laboratory and telephone follow-up for the first 4 weeks. After the first 9 months, patients were followed every 3 months until biochemical failure or 2 years, whichever came first. Complete interval histories, physical examinations, blood chemistries, hemogram, and serum PSA were obtained. All patients were evaluated for toxicity by the Common Toxicity Criteria version 2 and the vaccinia toxicity grading scale published previously (12).

Collection of peripheral blood mononuclear cells. Apheresis was obtained at four time points for patients on the vaccine arm: before vaccine, after three cycles of vaccine, after five cycles of vaccine, and after all eight cycles of vaccine. Briefly, 5×10^8 to 2×10^9 mononuclear cells were obtained by a single-access "four-pass" mononuclear cell procedure on the Haemonetics V-50 instrument, during which 2.0 liters of whole blood were processed at a flow rate of 70 to 80 mL/min. Radiation commenced after the second apheresis and concluded before the third apheresis. At the other monthly intervals, peripheral blood mononuclear cells (PBMC) from 60 mL of blood were collected in heparinized tubes. The mononuclear fraction of both apheresis packs and tubes was separated by Ficoll-Hypaque density gradient separation, washed thrice, and frozen in 90% heat-inactivated human AB serum and 10% DMSO at −80°C at a concentration of 1×10^7 cells/mL until assayed.

ELISPOT. Cells were thawed and cultured overnight in RPMI 1640 complete (Life Technologies, Inc., Gaithersburg, MD) at 37°C at 5% CO_2 before performing the ELISPOT assay. A modified ELISPOT assay that detects IFN-γ production was used to determine the T-cell precursor frequency to PSA3 peptide (VISNDVCAQV) and Flu peptide (mp 58-66 GILGFVFTL) in both prevaccination and postvaccination PBMC as described previously (30). Briefly, 96-well milliliter HA plates (Millipore Corp., Bedford, MA) were coated with 100 μL/well of capture monoclonal antibody against human IFN-γ at a concentration of 10 μg/mL for 12 hours at room temperature. Plates were blocked for 30 minutes with RPMI 1640 plus 10% human AB serum. PBMCs (2×10^5) were added to each well. PSA3-pulsed C1R-A2 cells were added into each well as APC at an effector-to-APC ratio of 1:1. Unpulsed C1R-A2 cells were used as a negative control. HLA-A2 binding Flu peptide was used as a positive peptide control. Cells were coincubated for 24 hours and lysed with PBS-Tween (0.05%). Biotinylated anti-IFN-γ antibody diluted to 2 μg/mL in PBS-Tween containing 1% bovine serum albumin was added and incubated overnight in 5% CO_2 at 37°C. Plates were then washed thrice and developed with avidin alkaline phosphatase (Life Technologies, Grand Island, NY) for 2 hours, after which each well was examined for positive dots. The number of spots in each well was counted by two separate investigators in a blinded manner, and the frequency of responding cells was determined for a total of 6×10^5 effector cells plated. The identical assay was done to look for antigen cascade. The HLA-A2-restricted peptides used were the MUC-1 agonist (ALWGQDVTSV), a PSMA-1 peptide (LLHETDSAV), a PAP peptide (ALDVYNGLL), and a PSCA peptide (AILALLPAL). HIV pol peptide (ILKEPVHGV) was used as negative control.

Serologic analysis. Serum was collected from patients before the first vaccination (prevaccination) and 1 month after the eighth vaccination. Serum was cryopreserved for analysis of antibodies to PSA, B7.1, and GM-CSF. Anti-PSA antibody (IgG) was quantified in the serum of each patient by ELISA as described previously (31). Antibodies specific for B7.1 were quantified by fluorescence-activated cell sorting capture assay as described previously (31). Detection limit was 4 ng/mL. Anti-GM-CSF antibody was quantitated by ELISA as described previously (32).

Culture of dendritic cells from peripheral blood mononuclear cells. PBMCs from patient 3 were obtained from heparinized blood. PBMCs were separated using lymphocyte separation medium gradient (Organon Teknika, Durham, NC) as described previously (33). Dendritic cells were prepared using a modification of the procedure described by Sallusto and Lanzavecchia (34). PBMCs (1.5×10^8) were resuspended in AIM-V medium containing 2 mmol/L glutamine, 50 μg/mL streptomycin, and 10 μg/mL gentamicin (Invitrogen Life Technologies, Carlsbad, CA) and allowed to adhere to a T-150 flask (Corning Costar Corp., Cambridge, MA). After 2 hours at 37°C, the nonadherent cells were removed with a gentle rinse. The adherent cells were cultured for 6 to 7 days in AIM-V medium containing 100 ng/mL recombinant human GM-CSF and 20 ng/mL recombinant human IL-4. The culture medium was replenished every 3 days.

Generation of T-cell lines. Modification of the protocol described by Tsang et al. (35) was used to generate MUC-1-specific CTL and PSA-specific CTL. To generate T-cell lines T-3-MUC-1 and T-3-PSA, autologous dendritic cells were used as APCs. Autologous non-adherent cells were then added to the peptide-pulsed APCs at an effector-to-APC ratio of 10:1. Cultures were then incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO_2. The cultures were then supplemented with recombinant human IL-2 at a concentration of 20 units/mL for 7 days; the IL-2-containing medium was replenished every 3 days. The 3-day incubation with peptide and 7-day IL-2 supplement constituted one *in vitro* stimulation cycle. Primary cultures were restimulated with peptide-pulsed autologous dendritic cells as described above on day 11 to begin the next *in vitro* stimulation cycle.

Cytotoxic assay. Tumor cells (MCF-7, LNCaP, and SK-Mel-24) were labeled with 50 μCi ^{111}In-labeled oxyquinoline (Medi-Physics, Inc., Arlington, IL) for 15 minutes at room temperature. Target cells (0.3×10^4) in 100 μL RPMI 1640 complete were added to each of 96 wells in flat-bottomed assay plates (Corning Costar). Effector cells were suspended in 100 μL RPMI 1640 complete supplemented with 10% pooled human AB serum and added to the target cells at various E:T ratios. The plates were then incubated at 37°C in 5% CO_2 for 16 hours. Supernatant was harvested for gamma counting with the use of harvester frames (Skatron, Inc., Sterling, VA). Determinations were carried out in triplicate, and SDs were calculated. Specific lysis was calculated with the use of the following formula (all values in counts per minute):

$$\% \text{ Lysis} = \frac{\text{Observed release} - \text{ Spontaneous release}}{\text{Total release } - \text{ Spontaneous release}} \times 100$$

Spontaneous release was determined from wells to which 100 μL RPMI 1640 complete was added. Total releasable radioactivity was obtained after treatment of targets with 2.5% Triton X-100.

Detection of cytokines. Supernatants of T cells exposed for 24 hours to peptide-pulsed autologous APC were screened for secretion of IFN-γ using an ELISA kit (R&D Systems, Minneapolis, MN). The results were expressed in pg/mL.

Results

The baseline characteristics of the enrolled patients are shown in Table 1. Nineteen patients were randomized to the vaccine with EBRT arm and 11 on the EBRT alone arm. Of the 19 patients on the combination, 17 completed all eight vaccinations,

Table 1. Patient characteristics and treatment

	Arm A, vaccine + radiation therapy	Arm B, radiation therapy alone
No. patients	19	11
Age, median (range)	59 (50-77)	70 (56-80)
Race/ethnicity, *n* (%)		
White	16 (84.2)	8 (72.7)
Black	2 (10.5)	2 (18.2)
Hispanic	2 (10.5)	0 (0)
Asian	0 (0)	1 (9.1)
Gleason, *n* (%)		
5	2 (10.5)	0 (0)
6	5 (26.3)	3 (27.3)
7	5 (26.3)	5 (45.5)
8	3 (15.8)	2 (18.2)
9	4 (21.1)	1 (9.1)
Median	7	7
Stage, *n* (%)		
$T_{1c}N_0M_0$	6 (31.6)	5 (45.5)
$T_{2a}N_0M_0$	4 (21.1)	0 (0)
$T_{2b}N_0M_0$	2 (10.5)	1 (9.1)
$T_{2c}N_xM_0$	1 (5.3)	0 (0)
$T_{3a}N_0M_0$	0 (0)	1 (9.1)
$T_{3b}N_0M_0$	3 (15.8)	4 (36.4)
N_1	3 (15.8)	0 (0)
PSA at diagnosis (ng/mL), median (range)	14.15 (3.84-206)	8.00 (4.5-23)
Risk of biochemical failure, *n* (%)		
Low	2	2
Intermediate	6	2
High	11	7
PSA on-study (ng/mL)		
Median	9.86	4.53
Range	0.17-122.26	0.20-9.50
ADT		
Given	15 (78.9)	9 (81.8)
Not given	4 (21.1)	2 (18.2)

1 patient decided not to wait for EBRT and dropped out after one vaccine cycle, and 1 patient was diagnosed with muscle-invasive bladder cancer after three cycles of vaccine and went off study to undergo a cystoprostatectomy. Of the 11 patients in the EBRT-only arm, 8 completed EBRT and had follow-up laboratories drawn for immunologic variables. One patient decided to get brachytherapy only (not allowed on this trial), one patient decided against definitive therapy after enrolling (and received ADT only), and one patient had severe radiation therapy–associated diarrhea and could not travel to the clinic for follow-up. No patient in the vaccine arm and two patients in the no vaccine arm elected to receive brachytherapy with EBRT, and all but three patients in the vaccine arm and one patient in the no vaccine arm elected to receive ADT with EBRT.

The vaccine was tolerated well with only grade ≤2 toxicity related to the vaccine itself; however, there were some grade 3 toxicities attributed to IL-2 and one each attributed to GM-CSF and EBRT (see Table 2). Many of these grade 3 toxicities were asymptomatic (lymphopenia or hyperglycemia

in known diabetics). All of the episodes of hyperglycemia were in patients diagnosed with non-insulin-dependent diabetes mellitus. Because of the IL-2 side effects, 89 of 138 (65%) cycles of vaccine were given with reduced IL-2 doses and only 1 of 17 patients had no reduction in the dose of IL-2. Not all dose reductions were due to grade 3 toxicities—some were due to patient choice. The observed lymphopenia seemed to be more likely due to the EBRT than IL-2, as 3 of 27 cycles with IL-2 and EBRT and 1 of 7 cycles of EBRT without IL-2 (11-14%) were associated with grade 3 lymphopenia, whereas 3 of 87 (3.4 %) cycles containing IL-2 given before or after EBRT were associated with grade 3 lymphopenia. It should be noted that the use of low-dose GM-CSF (for local immunologic effects) given with radiation was not associated with the excess toxicity seen using higher doses of GM-CSF (for systemic effect) given with combination chemotherapy and radiotherapy (36).

There was no induction of PSA-specific T-cell responses in the no vaccine arm. All PSA-specific T cells analyzed from PBMC obtained before radiotherapy, immediately following radiotherapy, and ~3 months after radiotherapy (see Fig. 1) were <1/200,000 in the ELISPOT assay with or without PSA peptide. The majority of vaccinated patients had an increase in their PSA-specific T-cell numbers; 13 of 17 had an increase of at least 3-fold at some point following the vaccination (see Table 3); $P < 0.0005$ by a two-sided Fisher's exact test in comparison with 0 of 8 with EBRT alone. There were six basic patterns of PSA-specific T-cell response following vaccination. Two patients had no evidence of an increase in PSA-specific T cells at any time point following vaccination. Four patients had

Table 2. Toxicities to vaccine

	Grade 2, *n* (%)	Grade 3, *n* (%)
Vaccine		
Injection site reaction	45 (41)*	0 (0)*
GM-CSF		
Dyspnea†	1 (1)	1 (1)
Arthralgia	1 (1)	0 (0)
IL-2		
Constitutional symptoms		
Fatigue	23 (21)	7 (6)
Fever	4 (4)	2 (2)
Arthralgias	7 (6)	0 (0)
Metabolic/laboratory		
Hyperglycemia‡	7 (6)	4 (4)
Blood/bone marrow		
Lymphopenia	17 (16)	6 (6)
Gastrointestinal		
Dehydration/anorexia	2 (2)	1 (1)
Diarrhea	7 (6)	0 (0)
Pulmonary		
Dyspnea	8 (7)	0 (0)

*% of cycles with toxicity (% patients with toxicity).
†Patient also had history of asthma.
‡All in known diabetics.

Table 3. Immune responses (ELISPOT)

Patient	Sample	Flu peptide	PSA3 peptide
1*	Pre	1/23,077	<1/200,000
	Post 3	1/21,429	<1/200,000
	Post 8	1/21,429	<1/200,000
2*,†	Pre	1/14,286	<1/200,000
	Post 3	1/8,955	1/26,087
	Post 8	1/13,363	1/50,000
3*,†	Pre	1/100,000	<1/200,000
	Post 3	1/150,000	1/50,000
	Post 8	1/150,000	1/46,154
4†,‡	Pre	1/42,857	1/50,000
	Post 3	1/46,154	1/37,500
	Post 8	1/46,154	1/15,789
5§	Pre	1/31,579	<1/200,000
	Post 3	1/20,690	1/46,154
	Post 8	1/15,385	1/30,000
6*,†	Pre	1/21,429	1/200,000
	Post 3	1/20,000	1/54,545
	Post 8	1/13,043	1/22,222
7§	Pre	1/18,182	<1/200,000
	Post 3	1/27,273	1/42,857
	Post 8	1/11,765	1/15,000
8*,†	Pre	1/17,647	<1/200,000
	Post 3	1/31,579	<1/200,000
	Post 8	1/28,571	1/66,667
9*,†,‖	Pre	1/7,692	<1/200,000
	Post 3	1/31,579	<1/200,000
	Post 8	1/9,836	<1/200,000
10*,†	Pre	1/26,087	1/85,714
	Post 3	1/11,111	1/54,545
	Post 8	1/15,385	1/100,000
11‡	Pre	1/75,000	1/100,000
	Post 3	1/60,000	1/85,714
	Post 8	1/100,000	<1/200,000
12*,†	Pre	1/46,154	1/100,000
	Post 3	1/37,500	1/150,000
	Post 8	1/35,294	1/200,000
13*,†	Pre	1/60,000	1/150,000
	Post 3	1/60,000	1/37,500
	Post 8	1/60,000	1/200,000
14*	Pre	1/15,789	<1/200,000
	Post 3	1/21,429	1/28,571
	Post 8	1/27,273	<1/200,000
15†,‡	Pre	1/30,000	<1/200,000
	Post 3	1/33,333	1/66,667
	Post 8	1/31,579	1/20,000
16§	Pre	1/66,667	1/100,000
	Post 3	1/66,667	1/15,789
	Post 8	1/54,545	1/75,000
17†,‡	Pre	1/2,913	<1/200,000
	Post 3	1/3,175	1/31,579
	Post 8	1/4,444	1/200,000

*Concomitant ADT started >1 month before vaccination.
†Whole pelvic radiation.
‡Concomitant ADT started within 1 month before vaccination.
§No ADT.
‖Patient 9 had a postvaccine 5 PSA-specific precursor frequency of 1/24,000.

increases in PSA-specific T cells before radiotherapy (1 month after the third vaccination) that became blunted after radiotherapy (1 month after the fifth vaccination) and then increased again (1 month after the eighth vaccination). Four additional patients had increases in PSA-specific T cells before radiotherapy (1 month after the third vaccination) that also became blunted and did not recover following radiotherapy. One patient had an increase in PSA-specific T cells (1 month after the third vaccination) that remained stable immediately following radiotherapy and then decreased after an additional 3 months of vaccine. Another group of four patients had either increased or stable numbers of PSA-specific T cells at each of the successive measurements with no substantial decrease following radiotherapy. Finally, two patients had no increase in PSA-specific T cells before radiotherapy but developed an increase following radiotherapy (1 month after the fifth vaccination) that was sustained in one patient. Thus, unlike the patterns of radiotherapy blunting the immune response seen in some patients, it is apparent that radiation may have actually enhanced the PSA-specific immune response in a minority of patients. Of these 17 patients, 11 had whole pelvis EBRT, whereas six did not. Three of 6 patients who did not have pelvic EBRT had decreases in immune response following EBRT, whereas 5 of 11 who had pelvic EBRT had similar decreases in the immune response. Interestingly, both patients who had measurable PSA-specific T-cell responses only after radiation therapy had whole pelvic radiation. Thus, the field of radiation did not seem to have a large effect on a patient's ability to maintain the PSA-specific immune response during radiotherapy.

The majority of the patients (18 of 30) had high-risk disease, with only 4 of 30 having low-risk disease (Table 1). Of the 10 patients with Gleason ≤7 tumor, 8 of them had a detectable increase in their PSA-specific T cells of at least 3-fold at some time point following vaccine versus 4 of 7 who had a Gleason score of 8 to 10. If one were to look at the published risk factors (37), 2 of 2 with low-risk, 3 of 4 with intermediate-risk (all with ≥50% of cores positive), and 7 of 11 with high-risk had a detectable increase in their PSA-specific T cells of at least 3-fold at some time point following vaccine. Thus, there may be a correlation with patients with lower-risk tumors being more likely to mount an immune response, but there are too few patients to justify any conclusions.

Preclinical studies and some clinical trials have revealed distinct immune responses not only to TAAs found in the vaccine but also to multiple other TAAs found in the tumor cells, a phenomenon known as epitope spreading and/or antigen cascade. In a recent preclinical study, this antigen cascade is associated with tumor cell destruction. PBMC from eight HLA-A2-positive vaccinated patients were evaluated for the presence of T cells directed against known HLA-A2 epitopes of four prostate cancer–associated antigens both before vaccination and after three vaccinations. The presence of T cells directed against a known HIV HLA-A2 epitope was also evaluated. None of the eight patients had detectable levels of T cells to HIV at either time point. Although all were negative for T cells directed against these antigens prevaccination, six of eight patients developed T-cell responses to at least one of the prostate-associated TAAs postvaccination. Theses included the generation of T cells directed against PSMA, PAP, PSCA, and/or MUC-1 (Table 4).

Table 4. Antigenic cascade (ELISPOT)

Patient	Sample	PSMA	PAP	PSCA	MUC-1	HIV
3	Pre	<1/200,000	<1/200,000	<1/200,000	<1/200,000	<1/200,000
	Post 3	<1/200,000	**1/85,714**	**1/85,714**	1/23,077	<1/200,000
6	Pre	<1/200,000	<1/200,000	<1/200,000	<1/200,000	<1/200,000
	Post 3	**1/85,714**	<1/200,000	<1/200,000	**1/60,000**	<1/200,000
7	Pre	<1/200,000	<1/200,000	<1/200,000	<1/200,000	<1/200,000
	Post 3	1/200,000	**1/85,714**	<1/200,000	<1/200,000	<1/200,000
8	Pre	<1/200,000	<1/200,000	ND	1/80,000	<1/200,000
	Post 3	**1/62,500**	<1/200,000	ND	**1/46,154**	<1/200,000
11	Pre	<1/200,000	<1/200,000	<1/200,000	<1/200,000	<1/200,000
	Post 3	<1/200,000	<1/200,000	<1/200,000	**1/40,000**	<1/200,000
12	Pre	<1/200,000	1/200,000	1/200,000	<1/200,000	<1/200,000
	Post 3	<1/200,000	<1/200,000	<1/200,000	**1/35,294**	<1/200,000

In light of the ELISPOT results in which we observed generation of a T-cell response postvaccination to both PSA and MUC-1, we conducted studies to determine if we can generate T-cell lines from the postvaccination PBMCs that were specific for either PSA or MUC-1 epitopes. The 10-mer PSA and MUC-1 peptides used have been described previously (10, 38). Autologous dendritic cells were pulsed with PSA or MUC-1 peptide to generate T cells from PBMC as described in Materials and Methods. As observed in the results in Table 5, both a PSA-specific T-cell line and a MUC-1-specific T-cell line were generated. It should be noted that each line was stimulated to produce IFN-γ only with the peptide used to generate it. As an additional negative control, a carcinoembryonic antigen peptide was also employed. Experiments were then conducted to determine if these T-cell lines could lyse human tumor cells endogenously expressing either MUC-1 or PSA (Table 6). Three HLA-2 lines were employed. The LNCaP prostate cancer line, which is PSA positive and MUC-1 negative, was lysed only by the PSA-specific T-cell line. Conversely, the MCF-7 breast cancer line, which is MUC-1 positive and PSA negative, was lysed only by the MUC-1-specific T-cell line. Neither the MUC-1-specific nor the PSA-specific T-cell line lysed the melanoma line, which is negative for both PSA and MUC-1. Previous studies (10, 38) have shown that the CD8+ T-cell lines generated by both PSA and MUC-1 peptides employed here are MHC restricted.

The proportion of regulatory CD4+CD25+ T cells was also analyzed by flow cytometry (see Table 5). The majority of patients had normal levels of regulatory T cells (10-18% of CD4+ cells); however, three patients had above normal levels (20-31% of CD4+ cells) of this T-cell subset as described in Discussion. The first 14 patients to complete treatment had serologic analysis for the production of antibodies to B7.1, GM-CSF, and PSA. The vaccine induced no production of antibodies to B7.1 or PSA and only one patient had an increase in antibodies to GM-CSF <1:50 prevaccination and 1:400 following the eighth vaccination.

The patients in the vaccine arm have a median follow-up of 20.0 months, with 2 of 17 patients having biochemical failure (see Discussion). The patients in the no vaccine arm have a median follow-up of 25.1 months, with two of nine patients evaluable who developed biochemical failure at 17 and 24 months after initiation of radiotherapy.

Two patients in the combination arm developed metastatic disease. Both of these patients had clinical evidence of lymph node–positive disease at diagnosis. One was a patient with clinical D1 disease ($T_{2c}N_1M_0$ with Gleason 4 + 4 and PSA of 63 at diagnosis) who had been treated with ADT for 9.2 months before enrolling on study. During that time, his lymphadenopathy had regressed; however, his PSA nadired and before commencing radiation had started to increase despite ADT. A bone scan performed just before initiating radiotherapy showed no abnormal uptake. About 6 months after initiating radiation therapy, the patient's PSA was again increasing and a complete restaging was done with evidence of multiple liver metastases. He went on to get chemotherapy with a docetaxel-based regimen and subsequently died of his disease 16.6 months after enrolling on trial. The second patient had a $T_{2c}N_1M_0$ Gleason 4 + 5 tumor with a PSA at diagnosis of 5.4. He met the definition of biochemical failure 10 months after completing radiation therapy and was found to have bilateral adrenal metastasis. This patient

Table 5. Establishment of T-cell lines from post-vaccination PBMC of a prostate cancer patient, using autologousdendritic cells pulsed with PSA or MUC-1 peptide, shows reactivity to both PSA and MUC-1 epitopes

T-cell line	APC only	APC + carcinoembryonic antigen peptide	APC + MUC-1 peptide	APC + PSA peptide
T-3-PSA	<15.6	<15.6	<15.6	314.5
T-3-MUC-1	<15.6	<15.6	673.4	<15.6

NOTE: T-3-PSA and T-3-MUC-1 were established by stimulating T cells isolated from patient 3 with autologous dendritic cells pulsed with PSA peptide or MUC-1 peptide for three *in vitro* stimulation. The effector-to-APC ratio was 10:1. Peptides were used at a concentration of 20 μg/mL. Carcinoembryonic antigen peptide was used as a negative control. Culture supernatants (24 hours) were collected and screened for the secretion of IFN-γ. Results are expressed in pg/mL IFN-γ.

Table 6. Ability of T-cell lines (T-3-PSA and T-3-MUC-1) established from a prostate cancer patient postvaccination to lyse human tumor cells

T-cell lines	MCF-7	LNCaP	SK-Mel-24
T-3-PSA			
40:1	9.2 (1.2)	22.8 (1.1)*	0.5 (0.4)
20:1	8.2 (0.6)	15.8 (0.8)*	0.6 (2.0)
10:1	3.4 (1.9)	3.7 (0.4)	1.0 (0.3)
T-3-MUC-1			
40:1	50.8 (4.6)†	5.7 (1.6)	0.2 (0.6)
20:1	41.9 (7.4)†	4.1 (1.2)	0.2 (1.1)
10:1	4.5 (1.5)	5.3 (2.5)	0 (1.2)

NOTE: T-3-PSA and T-3-MUC-1 cell lines were established by stimulating T cells isolated from patient 3 with autologous dendritic cells pulsed with PSA peptide or MUC-1 peptide for three *in vitro* stimulation. A 16-h ^{111}In release assay was done on MCF-7, LNCaP, and SK-Mel-24 cells. Results are expressed in % lysis (SD). MCF-7 (human breast carcinoma cell line: HLA-A2[+], MUC-1 positive and PSA negative), LNCaP (prostate cancer cell line: HLA-A2[+], PSA positive and MUC-1 negative), SK-Mel-24 (human melanoma cell line: HLA-A2[+], MUC-1 negative and PSA negative).
*$P < 0.01$, two-tailed t test, comparing lysis to SK-Mel-24 cells and MCF-7.
†$P < 0.01$, two-tailed t test, comparing lysis to SK-Mel-24 cells and LNCaP.

currently has stable disease on a docetaxel-based regimen 29 months after developing androgen-independent prostate cancer.

Two of the patients who had lymph node involvement and eventually developed biochemical failure in the vaccine arm had good initial immune responses to the vaccine (4- to 10-fold increase in the number of PSA-specific T cells). The third patient with lymph node involvement had a PSA at diagnosis of 95, a Gleason of 4 + 3, and $T_{2b}N_1M_0$ disease (patient 4). This patient was treated with ADT, vaccine, and EBRT and his PSA remains undetectable 40 months after diagnosis. He also had a good immune response to vaccine with an increase in his number of PSA-specific T cells to 1:15,000 circulating PBMC by 1 month following his final vaccination.

Discussion

There have been reports that radiotherapy can decrease nonspecific immune system responses and that these responses may remain suppressed for several months following radiation (22, 23). However, these studies involved radiation of multiple lymph node chains. Other reports have shown that there are long-term decreases in T-cell subsets following radiation therapy, largely in the naive T-cell populations (39, 40). Definitive radiation for localized prostate cancer does not involve extensive lymph node chains within standard treatment ports. Additionally, the strategy employed in this trial of vaccinating patients before radiotherapy would allow for the formation of a memory response that is less likely to be susceptible to radiation therapy. Despite this, there was evidence of decreased circulating levels of PSA-specific T cells following radiation therapy in at least 7 of the 17 patients evaluated. It is unclear if this is a direct effect of radiation on the PSA-specific T cells (possibly due to trafficking to tumor

within the radiation therapy ports) or the effect of radiation on the tumor cells causing tumor cell destruction and consequent trafficking of the T cells to the tumor site or a combination of both. Alternatively, it may be that vaccination with a weak antigen produces short-lived memory cells and that the radiation decreased the ability to induce PSA-specific T cells in some patients. Because the level of T cells to influenza matrix peptide remained constant in most patients, it is unlikely that this was an effect on the immune system in general.

There are several drawbacks to the method of immune monitoring we conducted in these patients. First, we were limited by identifying circulating antigen-specific T cells. As mentioned above, radiation, with its known ability to up-regulate MHC class I and adhesion molecules on the tumor surface, may have caused higher-affinity binding of antigen-specific T cells to the tumor and may have led to greater antigen-specific T-cell destruction directly by radiation (18–20). Thus, it is possible that this accounts for the decreased number of peripheral PSA-specific T cells specific for PSA following radiation seen in some patients. Although we were not able to obtain biopsies to ascertain if there was evidence of this as part of the study, a subsequent trial with the use of radiation to augment poxviral vector-induced immunity has recently opened and is designed to help answer this question. Another disadvantage of this immunologic readout results from the focus on only one HLA-A2 PSA epitope, PSA-3, when there may have been other epitopes within the PSA protein to which the patient responded. Both of these limitations could result in underestimating the true immune responsiveness of the vaccine. In addition, it should be noted that the blood used for analysis was taken 28 days after the previous vaccine and thus does not capture the possible increase in effector cells typically seen 1 to 2 weeks following vaccination. In addition, unlike other previously published immunologic results, we did not perform *in vitro* stimulation of the T cells that could lead to artificial overestimation of the numbers of PSA-specific T cells in the circulating blood. In addition, we did not further manipulate cells *in vitro* by looking only at the CD8^{+} cells, which will enrich for the proportion of PSA-specific T cells by ~4-fold.

There are multiple considerations for immune enhancement in this vaccine strategy, including but not limited to the effect of cytokines as well as that of hormonal therapy and radiation therapy. For example, one can attribute the effects of radiation as contributing to an immune response to be due in part to the generation of a local inflammatory response that in turn can cause recruitment of additional T cells, which can then be sensitized by the localized disease within the prostate; in addition, radiation-induced up-regulation of Fas, intracellular adhesion molecule-1, PSA, or MHC molecules could facilitate immune-mediated killing (18, 20).

In addition, there may be a synergistic effect of the radiation with ADT that can influence sensitization of the T-cell population. The majority of the literature, largely from research on autoimmune diseases, supports the immunosuppressive effects of androgens (41–44). Androgens have been reported to inhibit cellular immunity, immunoglobulin synthesis, and production of cytokines. This action is explained by the existence of androgen receptors in T cells (45). Thus, ADT may cause an increase in immune responses. It should

be noted, however, that several reports have shown a direct correlation between the level of androgens and the number of CD8^{+} cells (46–48). Although there were no substantial differences in the PSA-specific immune responses of the 3 of 17 patients who were not treated with ADT compared with those who were, there are too few patients to answer this question with any certainty.

There is another trial that compared the identical vaccine regimen with second-line hormonal therapy in patients with nonmetastatic disease who had rising PSA with castrate levels of testosterone (49). This trial yielded similar proportions of patients with PSA-specific immune responses, similar levels of those responses, and a similar side effect profile.

It is unknown what level of immune response is needed to induce immune-mediated tumor killing. However, there is indirect evidence that there may be immune-mediated tumor killing precipitated by the vaccine. Although none of the patients were vaccinated against PAP, MUC-1, PSMA, or PSCA, six of eight patients tested had roughly double or greater levels of one or more of these prostate-associated antigen-specific T cells following initiation of the vaccine and before radiotherapy. No patient was started on any other antitumor therapy during this period. Thus, it is possible that the immune responses to these other prostate-associated antigens postvaccination were induced by some level of immune targeting of the prostate gland induced by the vaccine. The PSA-specific T cells induced could have led to the killing of some prostate cells, leading to their processing by APC and in turn causing an induction of "antigen cascade" with the generation of T cells specific for these other prostate-associated antigens. Evidence of antigen cascade has been reported by others (50, 51) and has been seen in additional studies with these vaccines (52). One of six patients tested in the no vaccine arm had an increase in T cells specific for one prostate-associated antigen (PSMA) following radiation therapy. Four of the six patients with evidence of antigen cascade in the vaccine group had decreased levels of circulating T cells to these other prostate-associated antigens following radiation therapy similar to the decreases in circulating levels of PSA-specific T cells.

To further support the concept of antigen cascade, a T-cell line (T-3-MUC-1) was generated from a patient who had induction of MUC-1-specific T cells as measured by ELISPOT assay following three vaccinations. This cell line reacted specifically to a HLA-A2-restricted MUC-1 peptide and specifically killed MUC-1-expressing tumor cells. The only therapy that was initiated in this patient during the time of the induction of this response was a PSA-based vaccine, indicating that the induction of a response against MUC-1 was likely the result of immune-mediated tumor killing. Previous studies by others (53) have shown that tumor-infiltrating lymphocytes from patients with ovarian cancer can lyse ovarian cancer cells in a non-MHC-restricted manner. Specificity experiments showed that these tumor-infiltrating lymphocytes targeted an epitope within the MUC-1 core tandem repeats. However, the peptide we employed is a 9-mer peptide that is outside the tandem repeat region and the T cells generated to this peptide have been shown previously to be HLA-A2 restricted (38).

A minority of patients (2 of 17) had relatively high levels of circulating PSA-specific T cells (1/50,000 and 1/85,714) before

commencing vaccine. This is not dissimilar to previously described PSA-specific T-cell precursor frequencies of unvaccinated patients with prostate cancer (13). This may be due to an underlying immune response to PSA produced by the cells in the tumor or normal prostate gland as has been published previously (54). In addition, it is possible that ADT, which has been shown to cause apoptosis with an influx of lymphocytes into tumor, may have had an effect on this (55). This would be expected to be greatest when ADT is started in conjunction with vaccination. We did not see any obvious increase in response when the ADT was started within 1 month of vaccination (see Table 3); however, there were only four patients in this group and only one who started the androgen deprivation within 20 days before vaccine.

It is not known if adding active immunotherapy to definitive radiation therapy could affect clinical outcomes in this population of patients. Only much larger randomized controlled clinical trials will be able to adequately assess clinical outcomes. This small pilot study was designed to determine if immune responses could be generated as a consequence of vaccinations in the face of radiation therapy and if this combination was safe. Because we have shown that the combination seems to be safe and that specific immune responses can indeed be generated in the majority of patients, future trials can be designed that evaluate the question of clinical outcome.

Conclusion

This vaccine regimen can induce a PSA-specific immune response in the majority of patients undergoing local radiation therapy. In combination with radiotherapy, the vaccine regimen is well tolerated with virtually all the toxicity related to IL-2. Radiation therapy was associated with a decrease in the number of circulating PSA-specific T cells in PBMC in some patients due to mechanisms unclear at this time. However, in many of these patients, T-cell responses to PSA again increased postradiation and following subsequent vaccinations. There is indirect evidence that immune-mediated killing of prostate cells is seen before radiation therapy, with the majority of patients tested having *de novo* formation of T cells specific for antigens not found in the vaccine but found on prostate cells. In addition, patient-derived T-cell lines raised to PSA and MUC-1 epitopes were able to specifically lyse tumors containing PSA and MUC-1, respectively. Ideally, a well-tolerated vaccine such as this would augment radiotherapy-induced killing and lead to antigen cascade with the induction of a polyclonal response that could eradicate occult metastatic disease. Further studies are warranted with similar vaccines and strategies to determine whether the immune responses seen to date can translate into improved clinical outcomes.

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Combining a Recombinant Cancer Vaccine with Standard Definitive Radiotherapy in Patients with Localized Prostate Cancer

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