Phase I Clinical Trial of a HER-2/neu Peptide (E75) Vaccine for the Prevention of Prostate-Specific Antigen Recurrence in High-Risk Prostate Cancer Patients

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Abstract

Purpose: The E75 peptide is an immunogenic peptide from the HER-2/neu protein that is substantially expressed in prostate cancer. We are conducting a clinical trial of an E75/granulocyte macrophage colony-stimulating factor vaccine to prevent post-prostatectomy prostate-specific antigen (PSA) recurrences in high-risk prostate cancer (HRPC) patients.

Experimental Design: Prostate cancer patients at high risk for recurrence were prospectively evaluated and identified by the validated Center for Prostate Disease Research (CPDR)/CAPSURE high-risk equation. From these high-risk equation patients, 27 HER-2/neu-expressing prostate cancer patients were enrolled. HLA-A2⁺ patients (n = 17) were vaccinated, whereas HLA-A2⁻ patients (n = 10) were followed as clinical controls. Local/systemic toxicities, immunologic responses, and time to recurrence were measured.

Results: This vaccine is safe with only minor toxicities observed. Additionally, the vaccine is immunogenic with all patients showing both in vivo and in vitro phenotypic and functional immune responses, although variable. HLA-A2⁺ patients were found to have larger tumors, higher postoperative Gleason scores, and more high-risk CPDR scores than HLA-A2⁻ patients. Despite these differences, disease-free survival was not different between the vaccinated HLA-A2⁺ patients and the HLA-A2⁻ controls at a median follow up of 23 months. Three of the four vaccinated patients that recurred had rising PSAs at the initiation of the trial. Ex vivo phenotypic assays were predictive of recurrences and correlated in general with functional assays.

Conclusions: The E75 vaccine strategy is safe and effective in eliciting an immune response against the HER-2/neu protein in HRPC patients and may be useful as a preventive strategy against disease recurrence. Vaccination in response to a rising PSA may be too late.

Among men in the United States, prostate cancer is the most common cancer and the second leading cause of cancer-specific death (1). Widely accepted primary therapies are available to treat early-stage prostate cancer but suffer substantial failure rates in patients with more advanced local disease. Approximately 50% to 60% of patients who undergo radical prostatectomy for presumptively clinically localized prostate cancer will have extracapsular disease microscopically (2). Approximately 30% to 40% of these patients with extracapsular disease will have disease progression (3, 4). In those patients with disease progression, pharmacologic and/or surgical androgen ablation will almost universally fail, despite initial success, as hormone-sensitive prostate cancer evolves to become androgen-independent prostate cancer (AIPC) after antiandrogen therapy (5). Once patients develop AIPC, no effective cure exists; median survival is 9 to 12 months (6). Given the poor prognosis of this subset of patients, many investigators have attempted to identify such a group before disease progression. One clinically useful biostatistical model based on pretreatment prostate-specific antigen (PSA), prostatectomy highest Gleason sum, pathologic stage, and ethnic group has been developed to identify individuals at high risk for recurrence (2, 7). However, once identified, no standard adjuvant therapy exists to offer these high-risk individuals. Currently, these patients at high risk for recurrence are either closely followed, referred for clinical trials, offered hormonal therapy, or receive external radiation to the prostate bed.

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The paucity of effective therapies for AIPC as well as the dilemma over preventive strategies for high-risk prostate cancer (HRPC) patients have lead to intense investigations and resultant vaccine trials in the field of immunotherapy. PSA and prostate-specific membrane antigen (PSMA) are the most extensively studied prostate tumor-associated antigens (TAA) and therefore have been primarily targeted in prostate cancer vaccination trials. The majority of the results, however, have been disappointing, because PSA is either a weak immunogen or the host has been tolerized to it. To augment the immune response to PSA and PSMA, these vaccine trials have employed a variety of sophisticated methods, such as the use of recombinantly engineered vaccinia-expressing PSA (8, 9), autologous and allogenic dendritic cells pulsed with PSMA (10) or a recombinant fusion protein (11, 12), and recombinant PSA with Lipid-A formulated in liposomes in conjunction with either granulocyte macrophage-colony stimulating factor (GM-CSF) or mineral oil (13). Despite the allure of the widely expressed PSA and PSMA as vaccine targets, their lack of immunogenicity and the potentially complex procedures required to augment them requires the search for better target antigens.

HER-2/neu, a proto-oncogene in the epidermal growth factor family of tyrosine kinases, encodes for a transmembrane glycoprotein that is highly expressed in many epithelial-derived cancers (14). Several investigators have shown that the HER-2/neu protein is an immune-recognized TAA (15–17). The immunodominant epitope of HER-2/neu, E75 (KIFG-SLAFL, HER-2/neu, and 369-377), has become the most studied HER-2/neu-derived peptide both in vitro and in vivo (16, 18–21). Significant controversies in the literature exist regarding both the expression and the significance of HER-2/neu expression in prostate cancer. HER-2/neu protein overexpression in prostate cancer has been reported from 0% to 87%, with the vast majority of investigators using immunohistochemistry (22). HER-2/neu gene amplification in prostate cancers has been reported from 0% to 53% (23). The majority consensus, however, is that HER-2/neu gene amplification exists in a small percentage of the prostate cancer population (24).

Despite the difficulties of establishing a consensus of the level of HER-2/neu expression in prostate cancer, mounting evidence supports the significance of HER-2/neu in this disease (25). Craft et al. showed that androgen-dependent LNCaP cells, when induced in vitro to express HER-2/neu, can be converted into androgen-independent cells (26). In castrated mice, HER-2/neu can serve as a substitute for androgen and stimulate prostate cancer cell growth in vitro despite the absence of androgen receptor ligand. Clinical studies comparing HER-2/neu expression show an increasing percentage of patients with HER-2/neu protein overexpression with progression towards AIPC (25, 27, 28). Thus, as a patient’s clinical course evolves to the end point of AIPC, HER-2/neu may confer an increased malignant potential to prostate cancer cells through its ability to stimulate the androgen receptor pathway in the absence of androgen. When taken collectively, these studies increasingly suggest that HER-2/neu plays a role in the biological progression towards a more aggressive disease. Therefore, HER-2/neu is likely to be a promising therapeutic target in prostate cancer patients.

Our clinical trial is investigating the use of the E75 peptide mixed with GM-CSF as a simple vaccine strategy that can be easily exported to the community. We have sought to determine the safety and optimal dosing of this vaccine to produce the desired peptide-specific immunologic response. Most importantly, we are studying this vaccine in immunocompetent patients with prostate cancer who are disease free after prostatectomy but at high risk for PSA recurrence based on the Center for Prostate Disease Research (CPDR)/CaPSURE risk equation. By studying these patients, we have added the advantage of monitoring if inducing E75-specific immunity conveys a clinical benefit by preventing PSA recurrence.

Materials and Methods

Patient characteristics and clinical protocols. The Institutional Review Board of the Department of Clinical Investigation, Walter Reed Army Medical Center, approved the clinical protocol. This clinical trial is being conducted under an Investigational New Drug application (IND9187) approved by the Food and Drug Administration. All patients have prostate cancers that express HER-2/neu by immunohistochemistry. Patients have undergone a prostatectomy and have been determined to be at increased risk for recurrence according to the CPDR/CaPSURE risk equation (2, 7). After screening for eligibility criteria and proper counseling and consenting, HRPC patients are enrolled into the study and HLA typed to determine their HLA-A2 status, because E75 binds this specific HLA allele (16). HLA-A2 is found in ~40% to 50% of the general population (29). HLA-A2+ patients are vaccinated, and HLA-A2– patients are followed as matched controls for clinical recurrence. Before vaccination, patients are skin tested with a panel of recall antigens (Mantoux test = mumps, tetanus, and candida). Patients must have two of three positive (>5 mm) to be considered immunologically intact.

HLA-A2 typing. The patients’ HLA-A2 status is confirmed by indirect staining with 10 μL of anti-HLA-A2 monoclonal antibodies, BB7.2 and MA2.1 (American Type Culture Collection, Rockville, MD; 1:10 dilution of culture supernatant) at 4°C for 30 minutes followed by a 30-minute incubation with goat anti-mouse monoclonal antibodies conjugated with FITC (Becton Dickinson, Mountain View, CA) and analyzed on a BD FACScan Analyzer (Becton Dickinson).

Vaccine. The E75 peptide (KIFGSLAFL, HER-2/neu, and 369-377) was produced commercially in good manufacturing practices grade by Multiple Peptide Systems (San Diego, CA), a Food and Drug Administration–approved manufacturer. The purity of the peptide was verified by high-performance liquid chromatography and mass spectrometry, and the amino acid content determined by amino acid analysis. The peptide was purified to >95%. Sterility and general safety testing was carried out by the manufacturer. Lyophilized peptide was reconstituted in sterile 0.9% NaCl solution at the following concentrations: 100 μg in 0.5 mL, 500 μg in 0.5 mL, and 1 mg in 0.5 mL. The peptide was mixed with GM-CSF (Immunex Corp., Seattle, WA) at 250 μg in 0.5 mL, and the 1.0 mL inoculation was split and given intradermally at two sites within 5 cm of each other.

Vaccination series. Patients in the treatment group received intradermal inoculations in the same extremity to the same draining lymph node basin and were assigned to one of four dose/schedule groups. Three patients each received 100, 500, or 1,000 μg of E75 monthly for 6 months (100.6, 500.6, and 1,000.6, respectively). The variable dose helped determine the maximum tolerated as well as the optimal biological dose. A fourth group consisting of eight patients received 500 μg of peptide and 250 μg of GM-CSF but with an alternate schedule omitting the fourth and fifth inoculations (500.4).
Toxicity. The NIH Common Terminology Criteria for Adverse Events, v3.0 (March 31, 2003) definitions of adverse events were applied. Local/systemic toxicities at the injection sites were evaluated in all patients for all inoculations.

Immunologic response monitoring. Blood was drawn from patients before receiving each inoculation and at 1 and 6 months after completing the vaccination regimen. Phenotypic (HLA-A2-Ig dimer) and functional [enzyme-linked immunospot (ELISPOT) and cytotoxicity] assays were done at each time point. Patients were assessed for evidence of in vivo immunologic response by evaluation of the injection site 48 to 72 hours after each inoculation. Although a desired effect, this response was graded and reported as local toxicity. Additionally, at 1 month after completion of the vaccination regimen, a delayed type hypersensitivity (DTH) reaction was assessed with 100 µg of E75 (without GM-CSF) injected intradermally with a parallel control (0.9% NaCl solution, same volume) at a site on the back or extremity (opposite side from the vaccination site). The DTH reaction was measured in two dimensions at 48 to 72 hours using the sensitive ballpoint pen method (30) and compared with the sterile 0.9% NaCl solution control.

Peripheral blood mononuclear cell isolation and cultures. Forty milliliters of peripheral blood were drawn into CPT Vacutainer tubes (Becton Dickinson) and centrifuged for the isolation of peripheral blood mononuclear cells (PBMC) populations. The cells were washed in HBSS and resuspended in culture medium consisting of Iscove's modified Dulbecco's medium containing 5% human serum AB (Gemini Bioproducts, Woodland, CA). The PBMC were used as a source of lymphocytes and for the preparation of dendritic cells. Dendritic cells were prepared by incubating the PBMC in six-well plates for 2 hours followed by removal of the nonadherent cell population. The adherent cells remaining in the wells were cultured in the presence of AIM-V and 5% human serum AB with 100 µg/mL of GM-CSF (R&D Systems, Minneapolis, MN) and 50 µg/mL of interleukin-4 (IL-4)/mL (R&D Systems) to obtain monocyte-derived dendritic cell populations. Recombinant human necrosis factor-α (30 ng/mL) was added to the wells on day 3 to induce cell maturation. Dendritic cells were then harvested at 6 days, incubated with or without E75 for 2 hours, and then used to restimulate the PBMC cultures that had previously been stimulated with and without E75 peptide. All cultures were maintained in a humidified incubator at 37°C in 5% CO2/10 IU IL-2/mL (AMGEN, Thousand Oaks, CA). The nonadherent PBMC were grown in Iscove's modified Dulbecco's medium containing 5% human serum AB (Gemini Bioproducts) in a 48-well plate at a concentration of 2 to 3 × 106 cells/mL. Parallel cultures were stimulated with 25 µg/mL E75 or without peptide.

HLA-A2-Ig dimer assay. This assay used fresh PBMC. The HLA-A2-Ig dimer reagent (BD Pharmingen, San Diego, CA) was loaded with the peptide of interest by incubating 1 µg of dimer with an excess (5 µg) of the HER-2/neu peptide E75 (369-377) KIFGSLAFL or influenza peptide-FluM (58-66) GILGFVFTL or FBP (folate-binding protein) peptide-E37 (25-33) RIAWARTEL and 0.5 µg of β2-microglobulin (Sigma Chemical Co., St. Louis, MO) at 37°C overnight. The E37 peptide served as a negative control peptide and the influenza peptide (FluM) served as a positive control peptide. During the initial optimization phase/period of the dimer assay, a few samples were stained with “empty/mock-loaded” dimer for the negative control (this was generated by incubating the dimer in the absence of any peptide with an equivalent volume of PBS and 0.5 µg of β2-microglobulin). The incubated dimer preparations were then stored at 4°C until used. The dimer preparation was added to wells 2 × 105 cells. Human γ-globulin (Sigma G chemicals) was added and the samples were allowed to incubate for 5 minutes before adding the dimer preparations. The cells were incubated with the HLA-A2-Ig dimers for 90 minutes and then washed in PBS. HLA-A2-Ig bound to the cell surface was detected using goat anti-mouse IgG1-PE (BD Pharmingen). Murine monoclonal anti-human CD8-FITC (Sigma Chemical) was used to detect surface expression of CD8. Two-color fluorometric analysis was carried out on a BD FACSAnalyze (BD BD PharMingen). The lymphocyte population was gated on forward and side scatter, and gated events were analyzed using CellQuest software.

Enzyme-linked immunospot assay. IFN-γ-producing cells were detected using the BD ELISPOT kit. Fresh PBMC were plated into a 96-well round-bottomed plate at a concentration of 2 × 105 per well in medium containing IL-7. Cells were stimulated for 16 hours in the absence or presence of various peptides (E37 or E75 or FluM) in parallel at a concentration of 25 µg/mL. At the end of the incubation, the plates were centrifuged for 10 minutes at 1,200 rpm to pellet the cells. Following centrifugation, the supernatant was removed. The cells were then resuspended in 100 µL of medium and transferred to a 96-well ELISPOT plate precoated with IFN-γ capture antibody. The plate was incubated for 6 hours at 37°C and then washed. Biotinylated detection antibody was added and the plates were incubated overnight at 4°C. Following incubation, the plates were washed, Avidin-HRP solution was added for 1 hour, and spots were developed using AEC substrate solution. Spots were counted using the Immunospot Series 2 Analyzer and Immunospot software.

Cytotoxicity assay. Cytotoxicity was determined by a standard 4-hour chromium release assay. Briefly, a panel of HER-2/neu-expressing tumor targets previously described (22) was labeled with 100 to 150 µCi of sodium chromate (Amersham, Arlington Heights, IL) for 1.5 hours at 37°C, then washed twice and plated at 2,000 to 2,500 cells per well in 100 µL in 96-well U-bottomed plates (Costar, Cambridge, MA). Effectors were added at an effector/target (E/T) ratio of 10:1 in 100 µL/well. After 5 to 20 hours of incubation, 100 µL of culture supernatant were collected, and radioimmunoassay release was measured on a Microbeta TriLux counter (Perkin-Elmer, Gaithersburg, MD). All determinants were done in triplicate. Results are expressed as percent -- specific lysis as determined by (experimental mean cpm – spontaneous mean cpm) / (maximum mean cpm – spontaneous mean cpm) × 100, where cpm is counts per minute. The target cells used in the cytotoxicity assays consisted of HER-2/neu+, HLA-A2+ cell lines (LnsCap, SKOV3-A2, or MCF-7) or HER-2/neu-, HLA-A2- cell lines (SKOV3, AU565, or BT474). Not all tumors were used in every assay, but a minimum of two HLA-A2+ and two HLA-A2- controls were used in each experiment. In some assays, the HER-2/neu-transfected HLA-A2+ lymphoblastoid cell line 2JR/H2N was used with its corresponding negative control, 2JR (kindly provided by Dr. Keith Knutson, University of Washington, Seattle, WA). The HER-2/neu results were subtracted from the HLA-A2+ results as nonspecific. We have previously screened 12 different prostate cell lines and thus far have found no HER-2/neu- cell lines (22).

Clinical and chemical recurrences. Both vaccinated and control patients were followed for clinical and/or chemical recurrence through standard cancer screening to include physical exam, laboratory, and radiographic studies. In general, patients were followed every 3 to 4 months for the first year, every 6 months for another 2 years, and then annually thereafter. A serum PSA level of ≥0.2 ng/mL, confirmed by repeat serial laboratory examination, defined PSA recurrence.

Statistical analysis. Recurrence rates were compared between the vaccinated and control groups using survival analysis by the Kaplan-Meier method and the proportion of subjects who recurred compared using log-rank analysis. DTH versus normal 0.9% NaCl solution control responses were compared with a two-tailed, Mann-Whitney rank sum test. χ2, with or without Yates’ correction as appropriate, was used to compare frequencies of prognostic factors between vaccinated and control patients.

Results

Patients. To investigate the E75 vaccine in a preventive setting, we have enrolled 27 CPDR risk score-defined HRPC
patients who were disease free after standard therapies but at high risk for recurrence. After enrollment, patients were HLA typed, and 17 HLA-A2+ patients were vaccinated (E75 binds A2), whereas 10 HLA-A2– patients were followed prospectively as controls. Because A2 status was not known upon enrollment, we anticipated that these groups would be clinically similar. As shown in Table 1, median age, race distribution, margin status, we anticipated that these groups would be clinically similar. As shown in Table 1, median age, race distribution, margin status, and HER-2/neu expression were in fact similar between the two groups. However, a substantial proportion of the HLA-A2+ vaccinated patients had large tumors (T3b-T3c) compared with HLA-A2– controls (35% versus 10%, respectively; \( P < 0.15 \)). In addition, more than twice the percentage of vaccinated patients had high postoperative Gleason sums (8, 9) compared with the controls (47% versus 20%; \( P < 0.16 \)). A similar comparison was found using the CPDR risk equation for the highest risk patients: 59% of the vaccinated group compared with 30% of the control group were at high risk for recurrence (\( P < 0.15 \)).

Overall, HLA-A2+ patients had consistently worse prognostic factors than their corresponding control group. Although these factors did not reach statistical significance individually, taken collectively, they would suggest a higher expected recurrence rate among the HLA-A2+ patients compared with the HLA-A2– patients.

**Vaccine and vaccination series.** Good manufacturing practices grade E75 peptide (KIFGSLAFL, HER-2/neu, 369-377) was mixed with 250 \( \mu \)g of GM-CSF and injected intradermally in the same extremity on a monthly basis. Dose escalation consisted of three groups (100, 500, and 1,000 \( \mu \)g) with three patients in each inoculated monthly for 6 months (100.6, 500.6, 1,000.6, respectively). A fourth group was vaccinated with 500 \( \mu \)g but omitted the fourth and fifth vaccinations for a total of four inoculations (500.4).

**Toxicity.** Patients were observed post-vaccination for 1 hour for signs of an immediate hypersensitivity reaction and then returned 48 to 72 hours later to have their injection sites checked for local reactions and to be questioned concerning systemic toxicities. Local/systemic toxicities were graded per the Common Terminology Criteria for Adverse Events and reported on a scale of 0 to 5 (Table 2). No grade 3 to 5 systemic or local toxicities were observed or reported in the vaccinated group. Only two (12.6%) grade 2 and six (37%) grade 1 systemic toxicities were documented, whereas all patients had either a grade 1 or 2 local reaction, a desired effect. Because most toxicities were felt to be due to the direct effects of GM-CSF, the dose of this adjuvant was reduced to 125 \( \mu \)g in six patients. There seemed to be no correlation between the dose of the peptide and systemic toxicity; however, two of three patients receiving the highest peptide dose (1,000 \( \mu \)g) were found to have significant local reactions at the injection site. Therefore, we used 500 \( \mu \)g of peptide in the (alternate) schedule reduction group. Overall, 93% of the patients completed the vaccination regimen.

**In vivo immunologic response.** As a direct measure of the vaccine’s in vivo effectiveness, a DTH reaction was assessed at 1 month following completion of the vaccination series with 100 \( \mu \)g of E75 without GM-CSF injected intradermally with a 0.9% NaCl solution control at a remote site. All 16 patients that completed the vaccination series had significant DTH reactions with a median (range) induration diameter of 28 mm (11-55 mm) compared with 0.9% NaCl solution controls of 7 mm (0-20 mm; \( P < 0.01 \)). When the DTH reaction was further analyzed by dose, each dose group had significant DTH reactions compared with 0.9% NaCl solution controls (Fig. 1). The 100.6 group is not pictured, because two of the three patients had a PSA recurrence, and the effect of the disease recurrence on the DTH reactions could not be differentiated. With the exception of the 100 \( \mu \)g dosage group, the E75 inoculations showed a dose-dependent escalation with a trend towards increased median induration seen in the 1000.6 group compared with the 500.6 group of patients. However, given the local injection site reactions previously mentioned and the relatively small increase observed in the dose response analysis of the DTH between the 500 and 1,000 \( \mu \)g doses, we chose to pursue the lowest dosage, the 100 \( \mu \)g but with the same escalation schedule reduction (Fig. 1). The 100.6 group is not pictured, because two of the three patients had a PSA recurrence, and the effect of the disease recurrence on the DTH reactions could not be differentiated. With the exception of the 100 \( \mu \)g dosage group, the E75 inoculations showed a dose-dependent escalation with a trend towards increased median induration seen in the 1000.6 group compared with the 500.6 group of patients. However, given the local injection site reactions previously mentioned and the relatively small increase observed in the dose response analysis of the DTH between the 500 and 1,000 \( \mu \)g doses, we chose to pursue the lowest dose necessary to produce E75 immunity. Next, we lowered the number of inoculations to determine if a reduced schedule would also be effective. Although the median induration remained similar, the disparity of response within this group seemed idiosyncratic. Therefore, we further characterized the 500.4 group’s immunologic response within additional assays, assessing the level of vaccine-specific immunity throughout the course of the vaccination. Pre-vaccine DTH levels were not included in the initial trial design and therefore not available for comparison; however, midway through the trial, pre-vaccine DTH tests were initiated. Only one patient to date has shown a significant pre-vaccine DTH reaction.

**Dimer assay.** To better assess the vaccine-specific immunologic response of the 500.4 vaccination group, the phenotypic

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### Table 1. Prognostic factors in vaccinated HRPC patients versus control group

<table>
<thead>
<tr>
<th></th>
<th>Vaccinated HLA-A2+ (( n = 17 ))</th>
<th>Observed HLA-A2– (( n = 10 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range, y)</td>
<td>67.0 (51-78)</td>
<td>67.2 (59-73)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>76%</td>
<td>70%</td>
</tr>
<tr>
<td>African American</td>
<td>24%</td>
<td>30%</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small (T1c-T3a)</td>
<td>65%</td>
<td>90%</td>
</tr>
<tr>
<td>Large (T3b-T3c)</td>
<td>35%*</td>
<td>10%</td>
</tr>
<tr>
<td>Postoperative Gleason score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (6-7)</td>
<td>53%</td>
<td>80%</td>
</tr>
<tr>
<td>High (8-9)</td>
<td>47%†</td>
<td>20%</td>
</tr>
<tr>
<td>Positive margins</td>
<td>71%</td>
<td>70%</td>
</tr>
<tr>
<td>Old CPDR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High risk (-30)</td>
<td>59%†</td>
<td>30%</td>
</tr>
<tr>
<td>Intermediate risk (10-30)</td>
<td></td>
<td>41%</td>
</tr>
<tr>
<td>Updated CPDR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very high risk (16.7-56.3)</td>
<td></td>
<td>53%</td>
</tr>
<tr>
<td>High risk (7.1-16.7)</td>
<td>47%</td>
<td>60%</td>
</tr>
<tr>
<td>HER-2/neu mean</td>
<td>1.88</td>
<td>1.89</td>
</tr>
</tbody>
</table>

*\( P = 0.15 \).
†\( P = 0.16 \).
‡\( P = 0.15 \).
HLA-A2:Ig dimer assay was employed. Of the eight patients, one patient recurred early and was initiated on hormonal therapy; therefore, we studied the remaining seven. Figure 2 shows an example of three patients’ levels of vaccine-specific immunity as measured by the dimer assay throughout the vaccination series. All three patients initiated the vaccination series without preexisting E75-specific immunity, and vaccination caused the successful induction of E75-specific CD8+ T cells. As is typical of patients on the four-dose vaccination schedule, this immunity was most clearly evident by the RB0 time point (the level of E75-specific immunity before administration of the booster dose) and RB1 (the level of post-vaccination E75-specific immunity 1 month following the completion of the series). However, patients showed their maximal E75-specific response to vaccination at various time points during the vaccination series. This finding has prompted us to use the individualized maximal response in addition to the pre-determined time points of pre-vaccination (R0), post-vaccination (RB1), and memory (RC6) time points as measures of immunologic reaction to the vaccination.

As depicted in Fig. 3A, two of seven vaccinated HRPC patients had significant levels of preexisting E75-specific immunity. Interestingly, this did not correlate to the HER-2/neu expression of patients’ tumors. In fact, of the patients with 3+ HER-2/neu expression, none had evidence of preexisting E75 immunity, whereas the two patients with preexisting immunity had only 1+ HER-2/neu-expressing tumors. All seven vaccinated patients developed an E75-specific response to vaccination (0.43-3.64%), and six of seven showed a significant induction and/or clonal expansion of E75-specific immunity (maximal response) of >1% (range, 1.25-3.64%). Four of seven patients had significant post-vaccination E75-specific immunity of >1% (range, 1.00-2.27%), and four of seven patients had significant long-term immunity of >0.5%. The only two patients who did not reach either of these post-vaccination levels recurred.

Moreover, we observed a concordance between the in vivo DTH response and in vitro E75-specific dimer response at 1 month after vaccination (Fig. 3B). Of the three patients who showed post-vaccination E75-specific immunity of >1%, two

| Table 2. Local and systemic toxicity associated with the E75/GM-CSF vaccine |
|-----------------------------|-----------------|-------------------------------|-------------------|----------------|----------------|
| Patient | Local grade | Systemic grade | Completed series | Dose reduction* (cycle no.) |
|---------|-------------|----------------|-----------------|----------------|----------------|
| 100.6.1 | 1           | 0              | Yes             | No              |
| 100.6.2 | 1           | 0              | Yes             | No              |
| 100.6.3 | 1           | 2              | No              | No              |
| 500.6.1 | 1           | 1              | Yes             | No              |
| 500.6.2 | 1           | 0              | Yes             | No              |
| 500.6.3 | 1           | 1              | Yes             | No              |
| 1000.6.1| 2\textsuperscript{i} | 0            | Yes             | Yes (3)         |
| 1000.6.2| 1           | 1              | Yes             | No              |
| 1000.6.3| 2\textsuperscript{i} | 0            | Yes             | Yes (6)         |
| 500.4.1 | 1           | 1              | Yes             | No              |
| 500.4.2 | 2\textsuperscript{i} | 0            | Yes             | Yes (4)         |
| 500.4.3 | 1           | 0              | Yes             | No              |
| 500.4.4 | 2\textsuperscript{i} | 1            | Yes             | Yes (3)         |
| 500.4.5 | 1           | 1              | Yes             | No              |
| 500.4.6 | 2\textsuperscript{i} | 0            | Yes             | Yes (4)         |
| 500.4.7 | 1           | 0              | Yes             | No              |
| 500.4.8 | 1           | 2\textsuperscript{i} | Yes         | Yes (2)         |

\*GM-CSF decreased from 250 to 125 μg, while the peptide dose remained the same.
\textsuperscript{i}Vaccination number at which point the dose was reduced.
\textsuperscript{j}Syncope.
\textsuperscript{k}Forearm erythema/swelling.

\(\text{Fig. 1.} \) DTH response in vaccinated patients. After completion of the vaccination series, 100 μg of the E75 peptide without GM-CSF and a 0.9% NaCl solution control were placed intradermally at a site remote from the vaccination site. Induration was measured in two dimensions at 48 hours using the sensitive ballpoint pen method and compared with the 0.9% NaCl solution control. Bold bars, median DTH response per dose. NS, normal 0.9% NaCl solution controls. 500.6 patients received six inoculations of 500 μg E75, whereas 1000.6 patients received six inoculations of 1,000 μg of the E75 peptide. 500.4 patients received four inoculations, omitting the fourth and fifth monthly vaccination of 500 μg E75.
had DTH responses of >50 mm. Conversely, of the two patients with a post-vaccination E75-specific response of <0.5%, one had the smallest DTH response. In general, there was good agreement between the dimer and DTH assays with the exception of only one patient.

**Enzyme-linked immunospot assay.** To further investigate the seven patients whose PBMC had undergone flow cytometry analysis with the phenotypic dimer assay, we did the functional ELISPOT assay on four of these patients. Figure 4A shows representative results of replicated experiments. The ELISPOT assay measures the number of peptide-specific IFN-γ-producing PBMC. Similar to the dimer results, all four showed an increase in the number of E75-specific IFN-γ-producing cells after vaccination. Interestingly, the patient with the greatest maximal response of E75-specific IFN-γ-producing cells after vaccination was one of two patients with significant preexisting E75-specific immunity as measured by the dimer assay. The patient with the greatest number of IFN-γ-producing cells before vaccination had the greatest 1-month post-vaccination levels of E75-specific immunity as measured by the dimer assay. Finally, the patient with the lowest maximal number of E75-specific IFN-γ-producing cells after vaccination was the only patient who did not mount a significant maximal E75-specific immune response of >1% in the dimer assay. In fact, this patient never had a level of E75-specific CD8+ T cells of >0.43% during the course of vaccination and clinically recurred later in the study.

**Cytotoxicity assay.** To more thoroughly assess the *in vitro* functional response in this study, we did cytotoxicity assays on all patients in each dose/schedule group in the study. Figure 4B shows the pre-vaccination and maximal post-vaccination cytotoxic response against HER-2/neu-expressing tumors in these patients. In general, patients showed increased E75-specific tumor cell lysis following vaccination. As observed in the dimer assay, patients showed their maximal response at various time points throughout the vaccination series. Given this observation in both the dimer and cytotoxicity assays, we chose to use the maximal response time point as a measure of immunologic response to vaccination. The patient who showed no E75-specific cytotoxic response after vaccination was one of two patients who had E75-specific memory response of < 0.5%, and one of three patients who had post-vaccination response of < 1% on the dimer assay. This patient clinically recurred later in the study.

**Clinical response.** Despite the greater preponderance of poor prognostic factors in the HRPC HLA-A2+ group compared with their HLA-A2− controls, the PSA recurrence-free survival was similar and statistically not different in vaccinated HRPC patients compared with controls. At a median of 23 months, 72% of vaccinated patients compared with 80% of control patients remained PSA recurrence free (log-rank significance = 0.35).

Of the four vaccinated HRPC patients with PSA recurrences, all had DTH reactions that were close to the median value. However, of the four patients with recurrences, two patients’ PBMC underwent *ex vivo* phenotypic analysis and both showed weak *in vitro* immunity. More importantly, as shown in Fig. 5A, three of the four patients who recurred had rising PSA levels at the initiation of the inoculation series and therefore may not have been disease free at the time of enrollment. The fourth patient recurred over a year after the vaccination series ended (Fig. 5B).

**Discussion**

In this study, we have shown that an E75 vaccine is safe and effective at raising HER-2/neu-specific immunity both *in vivo* and *in vitro*. Our findings suggest that this immunity may have the potential to affect chemical recurrence rates if the patients are vaccinated before showing an increasing PSA. As mentioned earlier, our patients were enrolled into the study before knowing their HLA-A2 status but were subsequently placed in the appropriate category after determination of this variable. HLA-A2+ patients were vaccinated, whereas those who were negative were placed in the control arm. An interesting finding of our study was the variability of prognostic factors in the patient populations based on HLA-A2 status alone. Tumor size, postoperative Gleason score, and CPDR risk score were all worse in the HLA-A2+ group, suggesting a more aggressive disease. A correlation between certain HLA class II molecules and prognosis has been established in melanoma and suggested in some other forms of cancer (31, 32). We have also done an analysis in a larger
cohort of breast cancer patients, which has exhibited a link between HLA-A2 positivity and prognosis (33). However, larger studies are required to determine if HLA-A2 status is an independent prognostic marker in prostate cancer. For our study, the HLA-A2+ populations in HRPC patients should have had a higher recurrence rate based on all proven prognostic variables. However, after 2 years, the vaccinated HLA-A2+ patients had a similar disease-free survival as the HLA-A2- group.

Our study is the first to target prostate cancer with a preventive HER-2/neu vaccine; however, other studies have used other forms of immunotherapy to target HER-2/neu in this disease. Well-established data confirm that HER-2/neu overexpression in breast cancer represents an aggressive form of the disease that confers a much poorer prognosis with substantially shortened disease-free and overall survival (34). Therefore, HER-2/neu has been targeted using several means. Of these varying targeting strategies, a monoclonal antibody directed against HER-2/neu (trastuzumab) is by far the furthest developed and studied. In vitro effectiveness of trastuzumab has been translated into the clinical arena with encouraging success in the treatment of metastatic breast cancer patients receiving trastuzumab in combination with chemotherapy (35, 36). These findings have been recently extended to both node positive and high-risk node negative as reported at the 2005 American Society of Clinical Oncology meeting (37).

Having established a connection between HER-2/neu expression and progression to androgen insensitivity in prostate cancer, many investigators have studied the effects of trastuzumab in the context of prostate cancer. Morris et al. recently published a negative phase II trial in androgen-dependent and androgen-independent prostate cancer patients using trastuzumab alone or in combination with paclitaxel (28). However, only 25% of the patients evaluated in this study were HER-2/neu+. Moreover, the authors treated the patients with trastuzumab as a single agent (until disease progression when they added paclitaxel), in contrast to the breast cancer studies that have shown a synergistic response to treatment with trastuzumab in combination with chemotherapy. Furthermore, Ziada et al. published a phase II trial in HRPC patients receiving trastuzumab alone. They also concluded that trastuzumab alone does not show sufficient efficiency in treating HRPC patients; however, they conclude that its use in combination with cytotoxic agents should be investigated (38).

In other immunotherapeutic approaches, various prostate TAA have been targeted with vaccine immunotherapy, although most studies have focused on PSA or PSMA. Because these antigens have been shown to be weakly immunogenic, a variety of vaccination methods have been employed, but all of these trials and methods have shown disappointing results, as mentioned previously. Vaccinia, recombinantly engineered to express PSA, has been tested...
in a phase I trial (8, 9). Murphy et al. infused autologous dendritic cells pulsed with PSMA into prostate cancer patients (10). In two additional dendritic cell immunotherapy trials, autologous dendritic cells were pulsed with a recombinant fusion protein consisting of GM-CSF and prosthetic acid phosphatase (Provenge) and infused into prostate cancer patients (11, 12). Overall, the use of dendritic cells immunotherapy has shown some provocative results; however, the use of this modality as a vaccination strategy is limited in a preventive setting due to its complexity. Other investigators have looked at methods of actively immunizing prostate cancer patients with peptide- or carbohydrate-based vaccines. One such study looked at vaccination with JBT 1001, which consists of recombinant PSA with Lipid-A formulated in liposomes in conjunction with either GM-CSF or mineral oil as adjuvant (13). This study failed, as well, to show PSA-reactive T cells or any tumor-specific lysis in subsequent cytotoxicity experiments. One major disadvantage to PSA-based vaccines is that PSA exists in normal prostate tissue. Thus, tolerance has already developed to PSA before the development of cancer and may therefore be more difficult to overcome. If tolerance is broken, the immune response may not be as robust as to that of an antigen that is not normally expressed. Therefore, despite the allure of PSA and PSMA as vaccine targets, their lack of immunogenicity requires the search for better TAA.

Although PSA and PSMA vaccination attempts have been largely disappointing, HER-2/neu vaccines have been shown by many investigators to evoke a robust immune response (18–22, 39). We have previously shown the effectiveness of our E75 vaccine in high-risk breast cancer patients (40) and present evidence of a peptide-specific immune response in HRPC patients using the E75 vaccine. Although HER-2/neu expression in prostate cancer has been widely debated, we suggest that targeting HER-2/neu in this patient group is a viable option for the following reasons. First, high-density membrane HER-2/neu expression is not required for an effective cytotoxic response against the tumor. In fact, cytoplasmic HER-2/neu protein expression may be part of the key to a successful peptide vaccine strategy, because in every self-antigen, there are sequestered determinants that are unable to induce tolerance and are possibly immunogenic (39). Thus, cytoplasmic HER-2/neu protein expression may allow the necessary subdominant epitopes to be presented, thereby triggering the threshold for immune recognition and subsequent T-cell activation. In contrast to nonmalignant cells with low basal levels of HER-2/neu protein, high levels of cytoplasmic and membranous HER-2/neu protein in malignant cells may result in a necessarily different repertoire of processed peptides presented in the MHC to allow for immune recognition (39). We have shown in a previous study that PBMC from prostate cancer patients can be sensitized to a HER-2/neu peptide in vitro and subsequently kill HER-2/neu-expressing prostate cancer tumor line cells (22).

Second, it is not necessary for all the cells in a tumor to produce HER-2/neu in order for the E75 peptide vaccine to be effective. Numerous authors have shown the phenomenon of epitope spreading (18, 41). Epitope spreading is a well-documented immunologic reaction that occurs when the immune system is presented with one antigen that leads to immunity against other related antigens. Once the immune-mediated killing of cancer cells is initiated, more antigens are presented to the immune system generating secondary immune responses against the tumor. In prostate cancer, a HER-2/neu vaccination strategy would take advantage of initiating an expanding antitumor response by targeting a known immunogenic antigen and possibly expanding this antitumor response to less immunogenic but possibly more prostate-specific TAA. In this context, we have recently expanded our dimer assay to include the detection for CD8 T cells that are specific for two PSA-related epitopes and have preliminary evidence indicating the occurrence of inter-antigenic epitope spreading to PSA in some initial patients tested.

In conclusion, our study shows that our simple HER-2/neu (E75) vaccine is safe and effective in eliciting a peptide-specific immune response in HER-2/neu-expressing HRPC patients. This vaccine is also being administered to high-risk breast cancer patients who are without evidence of disease at the time of enrollment and the preliminary findings suggest a reduced recurrence rate in properly vaccinated patients (40). The current trial has exhibited an increased immune response in HRPC patients that might suggest the possibility of having an effect on their rate of PSA recurrence-free survival if patients are vaccinated before showing an increasing PSA. We suggest that an E75 vaccination strategy is a viable
option in HRPC patients due to expression, although variable, of the HER-2/neu protein as well as the phenomenon of epitope spreading in this patient cohort. Therefore, further studies are warranted to investigate the role of HER-2/neu peptide vaccination as a preventive strategy in HRPC patients.

References


Fig. 5. Recurrences as measured by rise in PSA levels in HRPC patients. A, patients enrolled with increasing PSA levels. All three patients chemically recurred during the vaccination series. Patients were started on Casodex with good hormonal response. PSA recurrence is defined as consecutively measured levels of >0.2 ng/mL. B, patients enrolled with stable PSA levels. Of 13 patients in this group, only one patient recurred 13 months after vaccination series completion.

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