Combined Clinical Trial Results of a HER2/*neu* (E75) Vaccine for the Prevention of Recurrence in High-Risk Breast Cancer Patients: U.S. Military Cancer Institute Clinical Trials Group Study I-01 and I-02

George E. Peoples,[1,2] Jarrod P. Holmes,[2,3] Matthew T. Hueman,[2,4] Elizabeth A. Mittendorf,[5] Asna Amin,[2,4] Steven Khoo,[2,4] Zia A. Dehqanzada,[2,4] Jennifer M. Gurney,[2] Michael M. Woll,[2] Gayle B. Ryan,[2] Catherine E. Storrer,[2] Dianna Craig,[6] Constantin G. Ioannides,[5] and Sathibalan Ponniah[2]

Abstract

Purpose: E75 is an immunogenic peptide from the HER2/*neu* protein, which is overexpressed in many breast cancer patients. We have conducted two overlapping E75 vaccine trials to prevent recurrence in node-positive (NP) and node-negative (NN) breast cancer patients.

Experimental Design: E75 (HER2/*neu* 369-377) + granulocyte macrophage colony-stimulating factor was given intradermally to previously treated, disease-free NP breast cancer patients in a dose escalation safety trial and to NN breast cancer patients in a dose optimization study. Local and systemic toxicity was monitored. Immunologic responses were assessed using *in vitro* assays and *in vivo* delayed-type hypersensitivity responses. Clinical recurrences were documented.

Results: One hundred and eighty-six patients were enrolled in the two studies (NP, 95; NN, 91). Human leucocyte antigen A2 (HLA-A2) and HLA-A3 patients were vaccinated ($n = 101$), whereas all others ($n = 85$) were followed prospectively as controls. Toxicities were minimal, and a dose-dependent immunologic response to the vaccine was shown. Planned primary analysis revealed a recurrence rate of 5.6% in vaccinated patients compared with 14.2% in the controls ($P = 0.04$) at a median of 20 months follow-up. As vaccine-specific immunity waned over time, the difference in recurrence lost significance at 26 months median follow-up (8.3% versus 14.8%); however, a significant difference in the pattern of recurrence persisted.

Conclusions: E75 is safe and effective in raising a dose-dependent HER2/*neu* immunity in HLA-A2 and HLA-A3 NP and NN breast cancer patients. More importantly, E75 may reduce recurrences in disease-free, conventionally treated, high-risk breast cancer patients. These findings warrant a prospective, randomized phase III trial of the E75 vaccine with periodic booster to prevent breast cancer recurrences.

Authors' Affiliations: [1]Department of Surgery, General Surgery Service, Brooke Army Medical Center, Fort Sam Houston, San Antonio, Texas; [2]Cancer Vaccine Development Program, U.S. Military Cancer Institute, Uniformed Services University of the Health Sciences; [3]Department of Medicine, Division of Hematology and Medical Oncology, National Naval Medical Center, Bethesda, Maryland; [4]Department of Surgery, General Surgery Service, Walter Reed Army Medical Center, Washington, District of Columbia; [5]U.T.M.D. Anderson Cancer Center, Houston, Texas; and [6]Joyce Murtha Breast Care Center, Windber Medical Center, Windber, Pennsylvania

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Requests for reprints: George E. Peoples, M.D., FACS, Department of Surgery, General Surgery Service, Brooke Army Medical Center, 3851 Roger Brooke Drive, Fort Sam Houston, San Antonio, TX 78234. Phone: 210-916-1117; Fax: 210-916-6658; E-mail: george.peoples@amedd.army.mil.

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Breast cancer is the most common cancer diagnosis in women and the second leading cause of cancer-related death among women.[7] Despite advances in standard treatment, a significant proportion of breast cancer patients will ultimately die from recurrent disease, especially aggressive subsets, such as those overexpressing HER2/*neu*. HER2/*neu* is a protooncogene expressed in many epithelial malignancies (1). Overexpression of HER2/*neu* is found in 20% to 25% of breast cancer and confers a poor prognosis (2).

Novel approaches are needed to further improve outcomes among breast cancer patients, and one such approach is immunotherapy. Trastuzumab is a monoclonal antibody that targets the HER2/*neu* protein and is an effective treatment of metastatic breast cancer (3). Several recent large trials have shown that adjuvant trastuzumab decreases recurrence rates compared with chemotherapy alone (4–6).

Another mode of immunotherapy in treating cancer is vaccines. Tumor-associated antigens are proteins expressed by

[7] Ries LAG, Harkins D, Krapcho M, et al. (eds.). *SEER Cancer Statistics Review, 1975-2003*, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2003/, based on November 2005 SEER data submission, posted to the SEER Web site, 2006.

Table 1. NP and NN trial designs

Patient group	No. patients HLA-A2$^+$(HLA-A3$^+$)	Peptide dose*(μg)	GM-CSF dose*(μg)	Months vaccinated †
NP				
100.6	2 ‡	100	250	0, 1, 2, 3, 4, 5
500.4	6	500	250	0, 1, 2, 5
500.6	6	500	250	0, 1, 2, 3, 4, 5
1,000.4	9 (+2)	1,000	250	0, 1, 2, 5
1,000.6	16 (+4)	1,000	250	0, 1, 2, 3, 4, 5
NN				
500.125.3	10	500	125	0, 1, 5
500.125.4	10	500	125	0, 1, 2, 5
500.250.4	10 (+3)	500	250	0, 1, 2, 5
500.250.6	10 (+2)	1,000	250	0, 1, 2, 3, 4, 5
1000.250.6	6	1,000	250	0, 1, 2, 3, 4, 5
Total	85 (+11)§			

*Peptide was suspended in 0.5 mL sterile saline and combined with GM-CSF and sterile saline to a final volume of 1.0 mL per inoculation.
† Vaccines were administered every 3-4 wk.
‡ One patient assigned to 100.6 group withdrew, and no replacement at that dose group was designated.
§ One hundred one patients enrolled to vaccine arm, and five withdrew.

tumors capable of eliciting a specific immune response. Two sources of tumor-associated antigens that have been extensively investigated in breast cancer are mucin-1 and HER2/*neu* (7). Several immunogenic peptides capable of inducing CTLs have been described from the HER2/*neu* protein (8, 9). E75 (KIFGSLAFL, HER2/*neu*, 369-377) is the most studied HER2/*neu*-derived peptide in laboratory and clinical studies (10–16).

E75 has been used in several clinical trials as an anticancer vaccine, either as a single-peptide vaccine combined with different immunoadjuvants (10–12) loaded on to autologous dendritic cells and reinfused (13, 14) or embedded in longer peptides capable of binding human leucocyte antigen (HLA) class II molecules to recruit CD4 helper T cells (15, 16). Each approach is safe and effective at stimulating E75-specific immunity, but combining E75 with an immunoadjuvant is the simplest approach and is at least as effective as the others at inducing peptide-specific immunity. Both granulocyte macrophage-colony stimulating factor (GM-CSF) and incomplete Freund's adjuvant have been used as immunoadjuvants with E75 (10–12, 17). GM-CSF seems to be a better vaccine immunoadjuvant (18).

Little is known about the clinical efficacy of E75, possibly due to the advanced nature of disease in most patients in the initial clinical trials. Our approach has been to administer E75 as a preventive vaccine in disease-free patients at high risk for recurrence. We have previously reported clinical trial results using E75 in prostate cancer (19), as well as preliminary results from our node-positive (NP) breast cancer (20) trial. In both trials, we vaccinated disease-free patients after completion of standard therapies. Additionally, we are conducting a similar trial in node-negative (NN) breast cancer patients. The goals of these trials are to document safety, immunogenicity, and clinical efficacy of the E75 vaccine. Here, we report the combined clinical results of our NN and NP breast cancer vaccine trials.

Materials and Methods

Patient characteristics and clinical protocols. The NP and NN trials were approved by the local institutional review boards and conducted at Walter Reed Army Medical Center and Joyce Murtha Breast Care Center under an investigational new drug application (BB-IND#9187). All patients had histologically confirmed breast cancer and completed a standard course of surgery, chemotherapy, and radiation therapy (as required) before enrollment. Patients on hormonal therapy were continued on their specific regimen. After proper counseling and consenting, breast cancer patients were enrolled to the appropriate trial (NP or NN) and then HLA typed because E75 binds primarily HLA-A2 found in ~40% to 50% of the general population (21). HLA-A2$^+$ patients were vaccinated, and HLA-A2$^-$ patients were observed prospectively for clinical recurrence. Before vaccination, patients were skin tested with a panel of recall antigens (mumps, tetanus, and *Candida*). Patients were considered immunocompetent if they reacted (>5 mm) to two or more antigens.

HLA-A3$^+$ patients. During the trials, it was determined that E75 could be used in HLA-A3$^+$ patients based on binding affinity data obtained from two commonly used HLA-peptide binding algorithms: BIMAS[8] (22) and SYFPEITHI[9] (23). Additionally, preclinical evaluation showed that E75-stimulated HLA-A3$^+$ CTL could lyse HLA-A3$^+$ HER2/*neu*-expressing cancer cells.[10]

Vaccine. The E75 peptide was commercially produced in good manufacturing practices grade by NeoMPS, Inc. Peptide purity (>95%) was verified by high-performance liquid chromatography and mass spectrometry, and the amino acid content was determined by amino acid analysis. Sterility and general safety testing was carried out by the manufacturer. Lyophilized peptide was reconstituted in sterile saline at 100, 500, or 1,000 μg in 0.5 mL. The peptide was mixed with GM-CSF (Berlex) in 0.5 mL, and the 1.0-mL inoculation was split and given intradermally at two sites 5 cm apart. All inoculations were given in the same extremity.

Vaccination series. The NP trial was designed as a two-stage safety trial with escalating doses of peptide in the initial stage and alterations of schedule in the latter stage. Details of the vaccine series have been previously published (20). Briefly, three to six patients were each assigned to receive four or six monthly injections of 100, 500, or 1,000 μg of E75 (100.6, 500.4, 500.6, 1,000.4 and 1,000.6, respectively; Table 1). Groups were ultimately expanded to determine and confirm

[8] http://bimas.dcrt.nih.gov/molbio/hla_bind/
[9] http://www.syfpeithi.de/
[10] Unpublished data.

optimal dosing in NP patients, accounting for the larger number of patients in the latter dose groups.

The NN trial was designed to further delineate optimal biological dosing by varying the dose of GM-CSF and altering the inoculation schedule. Patients with non–HER2/*neu*-expressing tumors were allowed in this trial to determine the feasibility of vaccinating a presumably antigen-naive host. Ten patients were assigned to each dose group to receive three, four, or six monthly injections over 5 months (Table 1).

Toxicity. Patients were observed 1-h postvaccination for immediate hypersensitivity and returned 48 to 72 h later to have their injection sites measured and questioned about toxicities. Toxicities were graded by National Cancer Institute Common Terminology Criteria for Adverse Events, v3.0 and reported on a scale from 0 to 5. Progression from one dose group to the next occurred only if no significant toxicity occurred in the lower dose group. Patient-specific results are reported based on maximal local and systemic toxicity occurring during the series.

Peripheral blood mononuclear cell isolation and cultures. Blood was drawn before each vaccination and at 1 month (postvaccine) and 6 months (long term) after vaccine series completion. Blood (50 mL) was drawn, and peripheral blood mononuclear cells were isolated. Peripheral blood mononuclear cells were washed and resuspended in culture medium and used as a source of lymphocytes as previously described (19, 20, 24).

HLA-A2:immunoglobulin dimer assay. The presence of $CD8^+$ E75-specific cells in freshly isolated peripheral blood mononuclear cells from patients was directly assessed by using the dimer assay as previously described (19, 20, 24). Briefly, the HLA-A2:immunoglobulin dimer (PharMingen) was loaded with the E75 or control peptide (E37, folate-binding protein; 25–33; RIAWARTEL) by incubating 1 μg of dimer with an excess (5 μg) of peptide and 0.5 μg of β_2-microglobulin (Sigma) at 37°C overnight then stored at 4°C until used. Peripheral blood mononuclear cells were washed and resuspended in PharMingen stain buffer (PharMingen), added at 5×10^5 cells/100 μL per tube in 5-mL round-bottomed polystyrene tubes (Becton Dickinson), and stained with the loaded dimers and antibodies. In each patient, the level of $CD8^+$ E75-specific cells was determined in response to each successive vaccination, and all postinoculation measurements were averaged for each patient and compared with their preinoculation levels.

Delayed type hypersensitivity. In both trials, a delayed type hypersensitivity (DTH) reaction was assessed with 100 μg of E75 in 0.5 mL of normal saline (without GM-CSF) and 0.5 mL normal saline as a volume control 1 month after completion of the vaccine series as described previously (20). The DTH reaction was measured in two dimensions at 48 to 72 h by using the sensitive ballpoint-pen method and reported as the orthogonal mean and compared with control (25). In the NN trial, a DTH was done prevaccination, also.

Clinical recurrences. All patients were observed for clinical recurrence per standard of care cancer screening as dictated by the patient's primary oncologist. A patient was considered recurrent if biopsy proved or if treated for recurrence by the primary oncology team.

Statistical analysis. Recurrence rates were compared between groups using survival analysis by the Kaplan-Meier method and the proportion of subjects using log-rank analysis. *P* values for clinicopathologic factors were calculated using Wilcoxon, Fisher's exact test, or χ^2 as appropriate. *P* values for comparing prevaccination and postvaccination dimer levels were calculated using Wilcoxon and for DTH using Student's *t* test.

Results

Patients

We have enrolled 186 patients in both E75 vaccine trials (NP, 95; NN, 91) who were disease-free after standard therapy but at high risk for recurrence. After enrollment, patients were HLA typed. $HLA\text{-}A2^+$, and later $HLA\text{-}A3^+$, patients (n = 101) were vaccinated (49 NP and 52 NN; 90 $HLA\text{-}A2^+$ and 11 $HLA\text{-}A3^+$). All other patients (n = 85) were assigned to observation. Five vaccine patients (NP, 4; NN, 1) and four observation patients (NP, 2; NN, 2) withdrew, though none due to toxicity.

Table 2. Demographic and prognostic factors for vaccinated and observation patients

	Vaccinated, $HLA\text{-}A2^+$, $HLA\text{-}A3^+$ (n = 96)*	Observed, $HLA\text{-}A2^-$, $HLA\text{-}A3^-$ (n = 81)†	*P*
Median age, y	58.9	55.1	0.33
Range, y	32-80	34-87	
Race			
White, %	89.6	81.5	
Other, %	10.4	18.5	0.12
Tumor size			
T1, %	69.8	60.5	0.20
T2-T4, %	30.2	39.5	0.20
Histologic grade			
I-II, %	64.5	59.5	0.50
III, %	35.5	40.5	0.50
NP, %	46.9	56.8	0.19
Median + nodes (NP only)	2.0	2.5	0.17
Range	1-25	1-15	
HER2/*neu* IHC 3+ or FISH+, %	25.8	28.4	0.32
Hormone receptor negative, %	31.6	17.3	0.03
X-ray therapy, %	71.9	80.2	0.20
Chemoprevention, %	65.6	78.8	0.05
Adjuvant Herceptin, %	5.2	3.7	0.60

*One hundred one patients enrolled to vaccine arm: two switched to observation, one withdrew for adjuvant trastuzumab, one withdrew due to an extended unrelated illness, and one patient withdrew for personal reasons.
† Eighty-five patients enrolled to observation arm: two were lost to follow-up, and four withdrew to another vaccine trial. Two patients were gained from the vaccine arm.

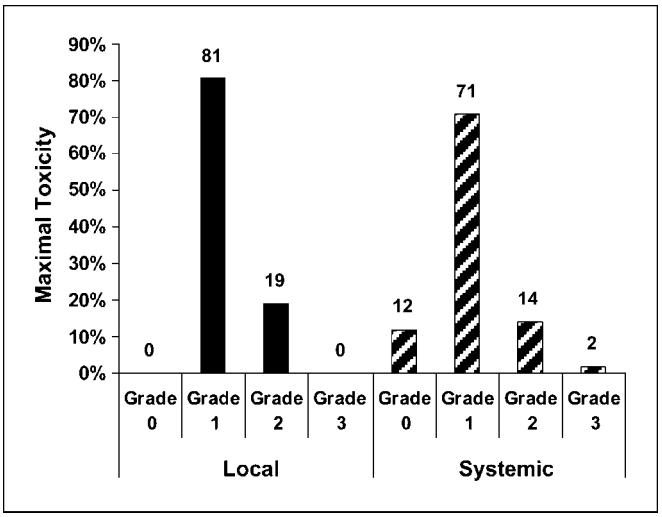

Fig. 1. Maximum local and systemic toxicity experienced by patients vaccinated with E75. Local toxicity (erythema and induration at injection site) is a desired effect showing a response to the vaccine. The most common grade 2 local toxicities were pruritis or discomfort requiring medication. Most common systemic toxicities were bone pain, flu-like symptoms, and fatigue (commonly associated with GM-CSF) and lasted <24 h. The two grade 3 systemic toxicities were angioedema of the tongue (after sixth inoculation) and bony pain.

Therefore, 96 vaccinated patients and 81 observation patients were available for analysis. Demographics and prognostic factors for both groups are presented in Table 2. Fifty-three (all NP, 24 vaccinated and 29 observation) of the 186 patients have been previously reported but are included here with 2 years of additional follow-up (20).

The two groups were equivalent in most standard prognostic categories, and there was no difference in the time from completion of primary treatment to enrollment between control (median, 8.4 months; range, 0-87.8 months) and vaccinated patients (8.9 months, 0-198 months; $P = 0.45$). However, more vaccinated patients were hormone receptor negative, and therefore, fewer patients in the vaccine group were on adjuvant hormonal therapy. In looking at the individual trials, more vaccinated patients in the NN trial compared with controls had HER2/*neu*-overexpressing tumors (25.0% versus 7.1%; $P < 0.05$), and fewer received adjuvant radiotherapy (64.7% versus 85.7%; $P < 0.05$).

Although there was no difference in the nodal status of the HLA-A3 subset compared with the HLA-A2 subset (54.5% versus 45.9%; $P = 0.59$), they tended to have smaller tumors (90.9% T1 versus 65.7%; $P = 0.08$), were less likely to have hormonally insensitive tumors (18.2% versus 29.6%; $P = 0.4$), and had less HER2/*neu*-overexpressing tumors (0% versus 31.5%; $P = 0.028$).

Toxicity

Local and systemic toxicities were mild, and all patients completed the vaccine series. Local toxicities were grade 1 (81%) and grade 2 (19%). Systemic toxicity was minimal, grade 0 (12%), grade 1 (71%), grade 2 (14%), and grade 3 (2%) (Fig. 1), with no grade 4 or grade 5 systemic toxicities observed. Because toxicities observed are consistent with GM-CSF, a 50% dose reduction in GM-CSF was instituted in the event of significant local or systemic reactions (18.7% of patients).

There was no difference in the toxicity profile among the HLA-A3$^+$ patients compared with the HLA-A2$^+$ patients, and the local reactions were just as robust. Grade 2 local toxicity was 20% compared with 18%, respectively, suggesting similar *in vivo* immunogenicity.

Immune response

Dimer. E75-specific CTL were assessed in fresh *ex vivo* peripheral blood mononuclear cells by the dimer assay (24) before each vaccination and at 1 (postvaccination) and 6 (long term) months. The dimer assay has been previously shown to correlate with functional immune assays (cytotoxicity and cytokine secretion; ref. 20). As we have described previously (19, 20), a pattern of increasing CD8$^+$ E75-specific CTL was observed during the vaccine series, peaking and then receding to a plateau by completion.

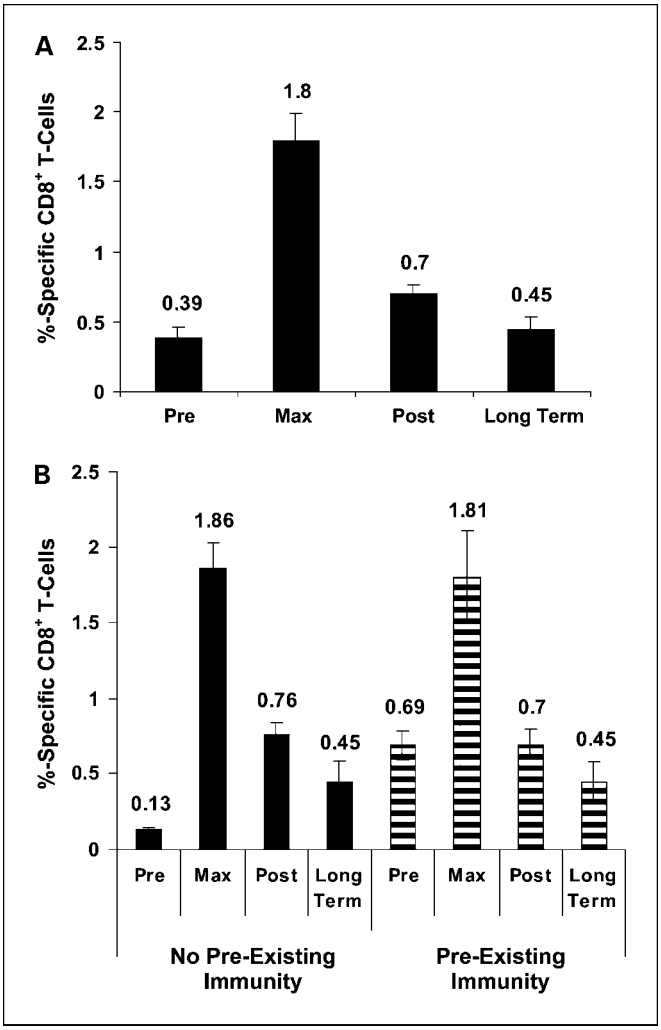

Fig. 2. *A,* vaccine-induced E75-specific CTL for all patients. The median levels of CD8$^+$ E75-specific CTL were significantly increased from prevaccination levels (0.39%; range, 0-3.28%) to a maximum level (1.8%; range, 0.4-12.2%; $P < 0.0001$) and postvaccination level (0.70%; range, 0.06-2.91%; $P = 0.002$). There was no difference between prevaccine levels and long-term (6 mo) levels of specific CD8$^+$ T cells. *B,* vaccine-induced E75-specific CTL based on preexisting immunity. Patients with and without preexisting immunity showed identical patterns in response to E75 vaccination with similar median maximum and postvaccination levels achieved for both. However, in those patients without preexisting immunity, there was a significant increase in dimer levels from prevaccine to 6 mo postvaccine; 0.13% (range, 0-0.28%) versus 0.45% (0-2.68%); $P < 0.0001$.

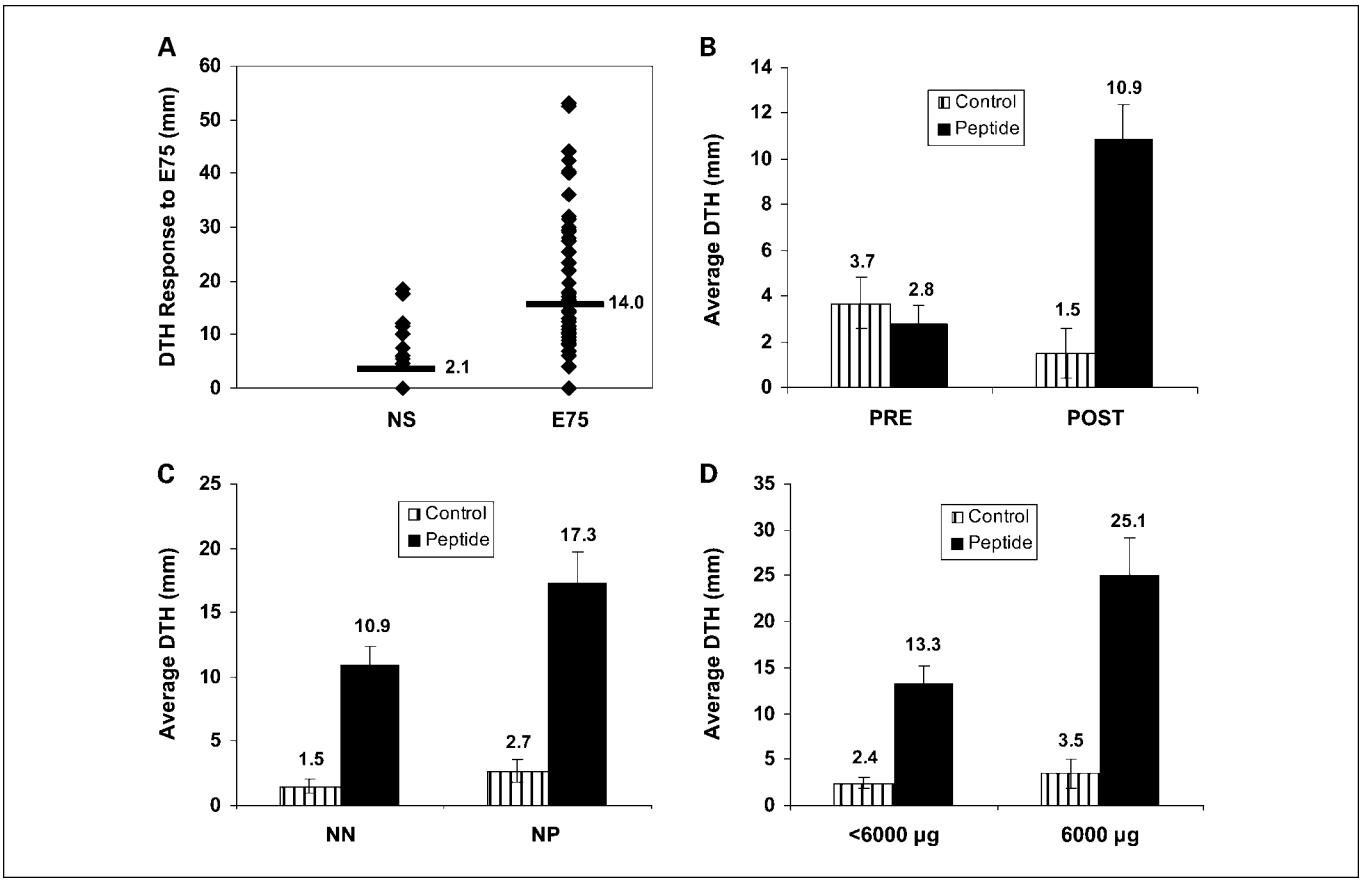

Fig. 3. *A,* DTH for all patients postvaccination. Control, 2.1 ± 0.5 mm compared with peptide, 14.0 ± 1.4 mm; $P < 0.0001$. *B,* prevaccine and postvaccine DTH for NN patients. There was no difference in saline control versus peptide prevaccination. At postvaccination, there was a significant increase in DTH response to E75 peptide compared with postvaccine control ($P < 0.001$) and compared with prevaccination E75 DTH ($P < 0.001$). *C,* postvaccination DTH by trial. NP patients had significantly larger DTH responses compared with NN patients (17.3 ± 2.4 mm versus 10.9 ± 1.5 mm; $P = 0.02$). This may be due to a difference in the median total vaccine dose in the NN group (2,000 μg versus 4,000 μg; $P < 0.0001$). *D,* postvaccination DTH by dose groups. Patients receiving <6,000 μg E75 had significantly smaller DTH responses compared with patients receiving a total of 6,000 μg (13.3 ± 1.9 mm versus 25.1 ± 4.0 mm; $P = 0.008$).

The cumulative dimer responses for all patients are shown in Fig. 2A. There was a statistically significant increase in the median $CD8^+$ E75-specific cells from prevaccine to postvaccination and to maximum levels (the maximum level of specific CD8 did not occur at the same time point for every patient, but for the majority of the patients, it was detected after the third or fourth dose of vaccine and very rarely at any of the other time points). Long-term levels were not different from the prevaccination levels. Only 48.3% of patients maintained significant residual immunity (defined as dimer >0.5) 6 months postvaccination.

Preexisting immunity to E75 (defined as dimer >0.3) was found in 42.7% of patients (Fig. 2B). The same pattern of dimer response, with an inducible maximal response, was seen regardless of initial dimer levels. Interestingly, the patients who showed preexisting immunity seemed to return to their baseline dimer levels after completion of the vaccination series, whereas those who lacked preexisting immunity had a significant increase in their long-term dimer levels. The exact relevance of the prevaccination E75-specific CD8 levels and its effect on the inducible and long-term level of immunity is yet to be elucidated but may bear on future vaccination strategies in terms of patient selection and/or dosing.

In vivo immune response

To measure the vaccine's *in vivo* effectiveness, a postvaccine DTH was measured 1 month after vaccine series completion with 100 μg of E75 injected intradermally with a saline volume control. Among all vaccinated patients, 74% had a positive postvaccine DTH with an average induration to E75 of 14.0 ± 1.4 mm compared with control of 2.1 ± 0.5 mm ($P < 0.0001$; Fig. 3A).

NN patients had a prevaccine DTH, as well as postvaccine DTH (Fig. 3B). At prevaccination, there was no difference in DTH between E75 and control. At postvaccination, the DTH response to E75 was statistically larger than control and the E75 DTH was significantly different postvaccine compared with prevaccine (10.9 ± 1.5 mm versus 2.8 ± 0.8 mm; $P < 0.0001$).

NP patients had a larger postvaccination E75 DTH response than NN patients (Fig. 3C), a difference likely due to the NN patients receiving much lower amounts of E75 overall. Assessing DTH responses as a function of dose, those patients receiving 6,000 μg of E75 had a significantly larger DTH reaction compared with those patients receiving <6,000 μg of peptide (25.1 ± 4.0 mm versus 13.3 ± 1.9 mm; $P = 0.008$; Fig. 3D).

The $HLA\text{-}A3^+$ patients had comparable postvaccination DTH to the $HLA\text{-}A2^+$ (10.5±2.7 mm versus 15.1±1.9 mm; $P = 0.38$).

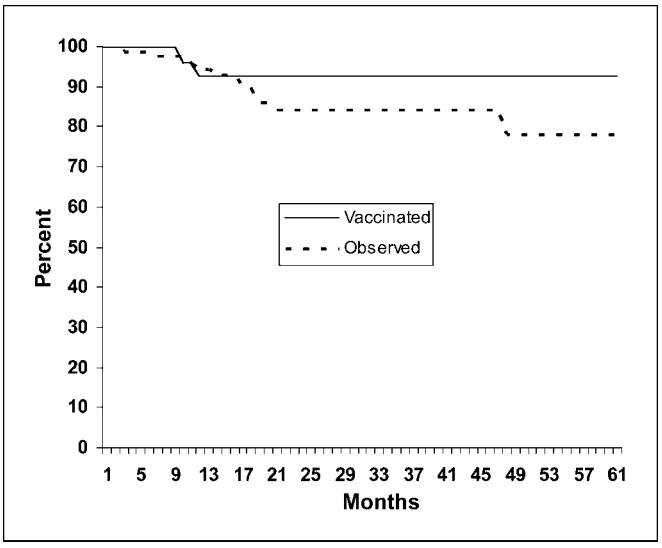

Fig. 4. Kaplan-Meier disease-free survival curves at 20 mo median follow-up. For 171 enrolled patients, the recurrence rate in the vaccinated group was 5.6% compared with 14.2% in the observation group ($P = 0.04$) at a median follow-up of 20 mo. The disease-free survival rates in the vaccinated and control groups were 92.5% and 77%, respectively.

Clinical response

Per protocol design, primary analysis was initiated at 18 months median follow-up. At completion of this analysis, 171 patients had been enrolled, and the recurrence rate in the vaccinated group was 5.6% compared with 14.2% in the observation group ($P = 0.04$) at a median follow-up of 20 months (range, 1-60 months). The disease-free survival rates in the vaccinated and control groups were 92.5% and 77%, respectively (Fig. 4). There were four deaths in the observation group (overall survival, 95.1%) compared with only one death in the vaccinated group (overall survival, 99%; $P = 0.1$).

We have extended the follow-up of both trials to 5 years despite waning immunity and lack of a booster inoculation in the protocol design. An updated analysis has documented additional recurrences in both groups including a late recurrence in the vaccine group at 58 months. Currently, at a median follow-up of 26 months, there are 186 patients enrolled, and the recurrence rate is 8.3% in the vaccine group compared with 14.8% in the observation group ($P = 0.15$). Interestingly, there is a different distribution of recurrences among these patients. Bone-only recurrence accounted for 50% of the recurrences in the control patients (6 of 12) and 0% of the vaccinated recurrent patients ($P = 0.04$). A more detailed analysis of the recurrence patients is under way.

Among the HLA $A3^+$ patients, the recurrence rate was similar to the HLA-$A2^+$ patients (9.1% versus 8.2%).

Discussion

We present here the combined results of our two clinical trials using a simple vaccine strategy consisting of the E75 HER2/*neu* peptide mixed with GM-CSF and given intradermally to disease-free NP or NN breast cancer patients. This is the largest trial to date using a HER2/*neu*-based peptide vaccine, as well as the largest trial of a preventive vaccine strategy in breast cancer. Our results show that this vaccine is safe and effective at stimulating HER2/*neu*-specific immunity and may reduce the recurrence rate among vaccinated HLA-$A2^+$ and HLA-$A3^+$ patients compared with prospectively observed control patients.

The vaccine produced minimal toxicity with all patients completing the vaccine series. Systemic toxicity primarily reflected side effects associated with GM-CSF, and 18.7% of patients required a 50% reduction in GM-CSF dose. We have used GM-CSF exclusively because it has been proved effective by multiple groups (17); however, recent concerns have been raised that GM-CSF may be immunosuppressive at high doses and contribute to bone metastasis in an animal model of breast cancer (26). In our study, the opposite was observed with the complete absence of bone metastasis in vaccinated patients.

We have shown a dose-dependent enhancement of DTH reactions in our vaccinated patients. DTH has previously been shown to be an important measure of immune response to peptide vaccines (27); however, we found no difference in either *in vitro* immune assays or DTH responses among vaccinated patients who ultimately recurred compared with nonrecurrent vaccinated patients (data not shown). The lack of a reliable immunologic monitoring assay showing clinical correlation for vaccine trials was recently raised by the Cancer Vaccine Consortium-Sabin Institute (28). We are investigating the use of the clinically validated circulating tumor cell assay (CellSearch System, Veridex) for monitoring our vaccine trials. In a pilot series, we have found that circulating tumor cells are readily detectable in NP clinically disease-free patients and that levels of circulating tumor cells are significantly reduced after vaccination (29).

Overall, the vaccinated patients were at higher risk for recurrence than the observed patients. More patients in the vaccine group were steroid hormone receptor negative and not on hormonal therapy. HLA-A2 has previously been implicated as a negative prognostic factor in ovarian (30) and prostate cancer (19, 31), and our results here extend this concept to breast cancer.

At our primary analysis, there was a statistically significant difference in recurrence rates between vaccinated and observed patients. However, this statistical finding did not extend out to 26 months due to additional recurrences, including a vaccine patient that recurred at 58 months. We have documented that E75 immunity wanes over time with only 48% of patients maintaining significant residual immunity at 6 months. As a result, a booster program has been initiated. Additionally, these are mixed trials with a total of seven different dose groups. Only one of the eight recurrent vaccinated patients received what is now determined to be the optimal biological dose of the vaccine. Interestingly, a difference in recurrence pattern was observed between the control and vaccine patients. Fifty percent of the observation patients had bone-only recurrences consistent with published rates (32–34), whereas no vaccine patients had bone-only recurrence. This surprising finding is being further investigated. Although there was a higher incidence of visceral metastases among vaccinated patients, the death rate was substantially lower, further suggesting a potential clinical benefit to the vaccine.

Although the E75 peptide has been exclusively tested in HLA-$A2^+$ patients (50% of the population), additional data suggest that E75 binds HLA-$A3^+$ (15% of the population; ref. 21). We

extended the E75 vaccine to HLA-A3$^+$ patients, and the toxicity profile, DTH reactions, and recurrence rates were similar to HLA-A2$^+$ patients. This suggests expanded use of the E75 vaccine in HLA-A3$^+$ patients, therefore addressing two-thirds of the general population with a single-peptide vaccine.

These data show that E75 is safe and effective at stimulating a HER2/*neu*-specific immune response and may prevent recurrence. These intriguing findings should be confirmed in a randomized, controlled phase III trial, enrolling only HLA-A2$^+$ and HLA-A3$^+$ patients.

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