

Results of the First Phase I Clinical Trial of the Novel Ii-Key Hybrid Preventive HER-2/*neu* Peptide (AE37) Vaccine

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A B S T R A C T

Purpose

HER-2/*neu* is overexpressed in breast cancer and is the source of immunogenic peptides. CD4⁺ T-helper peptides for HER-2/*neu* are being evaluated in vaccine trials. The addition of Ii-Key, a four-amino-acid LRMK modification, increases vaccine potency when compared with unmodified class II epitopes. We present the results of the first human phase I trial of the Ii-Key hybrid HER-2/*neu* peptide (AE37) vaccine in disease-free, node-negative breast cancer patients.

Patients and Methods

The dose escalation trial included five dose groups, to determine safety and optimal dose of the hybrid peptide (100 μ g, 500 μ g, 1,000 μ g) and granulocyte-macrophage colony-stimulating factor (GM-CSF; range, 0 to 250 μ g). In the event of significant local toxicity, GM-CSF (or peptide in absence of GM-CSF) was reduced by 50%. Immunologic response was monitored by delayed-type hypersensitivity and [³H]thymidine proliferative assays for both the hybrid AE37 (LRMK-positive HER-2/*neu*:776-790) and AE36 (unmodified HER-2/*neu*:776-790).

Results

All 15 patients completed the trial with no grade 3 to 5 toxicities. Dose reductions occurred in 47% of patients. In the second group (peptide, 500 μ g; GM-CSF, 250 μ g), all patients required dose reductions, prompting peptide-only inoculations in the third group. The vaccine induced dose-dependent immunologic responses in vitro and in vivo to AE37, as well as AE36.

Conclusion

The hybrid AE37 vaccine seems safe and well tolerated with minimal toxicity if properly dosed. AE37 is capable of eliciting HER-2/*neu*-specific immune responses, even without the use of an adjuvant. This trial represents the first human experience with the Ii-Key modification, and to our knowledge, AE37 is the first peptide vaccine to show potency in the absence of an immunoadjuvant.

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INTRODUCTION

Breast cancer is the most common cancer in women. Treatment is multimodal, including surgery, chemotherapy, radiation, and immunotherapy, as indicated.^{1,2} Despite this intensive therapy, many women with high-risk features, such as overexpression of HER-2/*neu* protein, will develop recurrent disease.³ HER-2/*neu* is a member of the epidermal growth factor receptor family, normally expressed during fetal development and overexpressed in 30% of breast cancer.⁴ This protein is also a source of immunogenic peptides.^{5,6}

Immunogenic peptides of HER-2/*neu* stimulate cytotoxic T lymphocytes (CTLs) to recognize and kill HER-2/*neu*-expressing cancer cells in vitro.^{5,6} Some of the peptides (E75 and GP2) are being used as clinical vaccines in patients with HER-

2/*neu*-positive breast cancer.^{7,8} Thus far, they have been shown to be safe and effective in stimulating antigen-specific immunity; more importantly, we have shown that the immunity conferred by E75 seems to have clinical benefit in decreasing breast cancer recurrence.⁹ Unfortunately, vaccine-induced immunity is not sustained without booster vaccinations.^{9,10} CD4⁺ T-helper peptides may be required to increase efficiency of induction and establishment of long-term immunity.^{11,12}

CD4⁺ T-helper peptides for HER-2/*neu* have been described; the first was G89 (HER-2/*neu*: 777-789) by Tuttle et al.¹³ A similar peptide (HER-2/*neu*: 776-790) was used in combination with two other peptides by Disis et al,¹⁴ producing encouraging immunologic responses. A novel method to increase antigen-specific stimulation of T-helper cells has been the use of the invariant protein (Ii-Key). Specifically, the addition of a four-amino-acid sequence

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(LRMK) added to T-helper peptides facilitates direct antigenic epitope charging of major histocompatibility complex (MHC) class II molecules at the cell surface.^{15,16} This enhanced epitope charging and concomitant increase in antigen presentation can increase potency 250 times or more compared with the unmodified class II epitope in vitro.^{17,18} Animal models have shown Ii-Key hybrid methodology to be highly efficient using melanoma peptides, but no human data exist to date applying this hybrid peptide.¹⁹ AE37 is the Ii-Key hybrid of HER-2/neu peptide 776 to 790 (AE36). We have performed a phase Ib trial of AE37 peptide vaccine in HER-2/neu-positive breast cancer patients to document safety and measure immunologic responses to escalating vaccine doses. The results of the first human trial of the Ii-Key hybrid technology are reported here.

PATIENTS AND METHODS

Patient Characteristics and Clinical Protocol

The trial was institutional review board approved and conducted at Walter Reed Army Medical Center (Washington, DC) under investigational new drug application #12,229. All patients had histologically confirmed, node-negative breast cancer and completed standard course of surgery, chemotherapy, and radiation (as required) before enrollment. Patients receiving hormonal therapy continued their regimens. After proper counseling/consent, breast cancer patients were enrolled and HLA typed (DNA genomic typing). Before vaccination, patients were skin tested with a panel of recall antigens (Mantoux test). Patients were considered immunocompetent if they reacted (> 5 mm) to two or more antigens.

Vaccine

The Ii-Key/HER-2/neu MHC class II peptide, AE37 (Ac-LRMKGVGSPYVSRLGICL-NH₂) was commercially produced in accordance with federal guideline current good manufacturing practices by NeoMPS Inc (San Diego, CA). Peptide purity (> 95%) was verified by high-performance liquid chromatography and mass spectrometry. Sterility and general safety testing was carried out by the manufacturer. Lyophilized peptide was reconstituted in 0.5 mL of sterile saline at the following concentrations: 100 µg, 500 µg, and 1,000 µg. The peptide was mixed with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Berlex, Seattle, WA) at varying concentrations in 0.5 mL (Table 1). The 1.0-mL inoculation was split and administered intradermally at two sites 5 cm apart in the same extremity.

Vaccination Series

The study was performed as a dose escalation trial to define vaccine and GM-CSF optimal dosing. Each dosing group consisted of three patients. The first three dose groups (patients A1-A9) received escalating amounts of AE37 peptide and fixed initial GM-CSF dose (Table 1). GM-CSF dose was chosen on the basis of our previous E75 trials. GM-CSF was reduced 50% in subsequent inoculations for local reactions of 100 mm or more or grade 2 systemic toxicities. The cutoff of 100 mm was determined from previous experience; at

100 mm and greater, the sites become confluent and local toxicity increases, and our goal is to minimize toxicity and prevent skin disruptions. All patients received six monthly inoculations. Because each patient in the 500:250:6 group (patients A4-A6) required multiple GM-CSF reductions, the 1,000:0:6 group (patients A7-A9) was inoculated without GM-CSF. One patient in this group (1,000:0:6) failed to respond with a local reaction during inoculations 1 to 3, so on the fourth inoculation, GM-CSF was reinstated to produce a local reaction. The two subsequent dose groups (patients A10-A15) received a fixed dose of AE37 (500 µg) with varying GM-CSF (Table 1).

Toxicity

Patients were observed 1 hour postvaccination for immediate hypersensitivity and returned 48 to 72 hours later to have injection sites measured and questioned in regard to local/systemic toxicities. Toxicities were graded using National Cancer Institute Common Terminology Criteria for Adverse Events v3.0 (reported on a 0-5 scale). Progression from one dose group to the next occurred only if no significant toxicity occurred in the lower dose group.

Control Peptides and Proteins

The AE37 peptide (Ac-LRMKGVGSPYVSRLGICL-NH₂) used in this study is a fusion of the Ii-Key peptide (LRMK) with the native HER-2/neu peptide (aa776-790:GVGSPYVSRLGICL). To assess whether measured immune responses are representative of reactivity against native HER-2/neu peptide, we measured immune responses against the HER-2/neu MHC class II peptide, AE36 (aa776-790:Ac-GVGSPYVSRLGICL-NH₂). In addition, a peptide (AEN) containing the Ii-Key peptide fused to a non-HER-2/neu sequence (HIV/gag 164-181:YVDRFYKTLRAEQASQEV) was used as a negative control, and tetanus toxoid (TT; List Biologicals Inc, Campbell, CA) was used as a positive control antigen for the immune assays.

Peripheral-Blood Mononuclear Cell Isolation and Cultures

Blood was drawn before each inoculation and at 1 (postvaccine) and 6 months (long term) after vaccine series completion. Forty milliliters of blood was drawn and peripheral-blood mononuclear cells (PBMCs) were isolated. PBMCs were washed and resuspended in culture medium (RPMI-1640-positive/10% FCS-positive/penicillin- and streptomycin-positive/L-glutamine) and used as a source of lymphocytes as previously described.^{7,20,21}

Proliferation Assay

PBMCs were used for monitoring of vaccine-specific proliferative activity of T lymphocytes using a standard radioactive [³H]thymidine incorporation assay. PBMCs were stimulated in absence or presence of peptide or antigen. Each of the peptides (AE36/AE37/AEN) or TT was added as triplicates to a 96-round bottom well plate, and one set of wells had no stimulant added and served as control wells. The peptides were tested at two concentrations (1 and 10 µg/mL), whereas TT was used at 1 µg/mL. PBMCs were resuspended in culture medium and added at 3 × 10⁵ cells/200µ/well. The plate was then incubated in a humidified CO₂ incubator for 4 days. On day 3 of incubation, wells were pulsed with 1 uCi/well of radioactive [³H]thymidine, and plates returned to the incubator. On day 4, cells were collected using a cell harvester (Harvester96-MachIII; Tomtec, Orange, CT) on to a filter mat and counted using a scintillation counter (MicroBeta Trilux, Perkin Elmer, Norwalk, CT). Proliferation was measured by amount of thymidine incorporation, determined as counts per minute. Average counts per minute were calculated for the triplicate cultures.

Delayed-Type Hypersensitivity

Delayed-type hypersensitivity (DTH) reaction was performed before vaccination and at 1 month after completion of vaccine series (long term). Intradermal injections, on the back or extremity (opposite side from vaccination), of 100 µg of AE37 (without GM-CSF) in 0.5 mL of saline were compared with an equal volume control inoculum of saline. DTH reactions were measured in two dimensions at 48 to 72 hours using the sensitive ballpoint pen method, and results were reported as an orthogonal mean.²²

Table 1. Dosing of Vaccine and Adjuvant

Dose Group*	Patient No.	AE37 Dose (µg)	GM-CSF Initial Dose (µg)	Schedule
100:250:6	A1, A2, A3	100	250	Monthly ×6
500:250:6	A4, A5, A6	500	250	Monthly ×6
1000:0:6	A7, A8, A9	1,000	0	Monthly ×6
500:125:6	A10, A11, A12	500	125	Monthly ×6
500:30:6	A13, A14, A15	500	30	Monthly ×6

Abbreviation: GM-CSF, granulocyte-macrophage colony-stimulating factor.
*Designated as peptide (µg):GM-CSF (µg): number of inoculations.

Statistical Analysis

P values for clinicopathologic factors were calculated using Wilcoxon test, Fisher's exact test, or χ^2 test as appropriate. *P* values for comparing pre- and postvaccine proliferative assays and DTH were calculated using the *t* test, paired or unpaired, as appropriate.

RESULTS

Patients

We enrolled and vaccinated 15 disease-free, node-negative breast cancer patients all expressing varying levels of HER-2/*neu* (ranging from IHC 1+ to 3+). No patient withdrew from this study. Patient demographics, prognostic factors, and treatment profiles are presented in Table 2.

Vaccine and Vaccination Series

Table 1 provides the dose escalation strategy utilized, and Figure 1 illustrates the robust local reactions to the hybrid vaccine. GM-CSF dose reductions (or peptide if no GM-CSF), as dictated by local or systemic reactions, are depicted. Dose groups are designated as peptide(μ g):GM-CSF(μ g):number of inoculations. The first group (100:250:6) required no dose reductions. However, all three patients in the second group (500:250:6) required GM-CSF dose reduction by the third vaccination. Given the significant local reactions, the third group (1,000:0:6) was initiated without GM-CSF; two of three patients in this group required reduction of peptide from 1,000 μ g. Another patient in this group did not show a strong local response, and GM-CSF was added back to the patients' vaccine schedule on the fourth inoculation in an escalating fashion. For the latter dose groups, a fixed amount of peptide (500 μ g) was administered with varying GM-CSF doses. Dose reductions occurred in all patients in the 500:125:6 dose group, but later in the vaccination series compared with the 500:250:6 group. No reductions occurred in the patients in the 500:30:6 dose group.

Combined Dosing Group Results

There were no grade 3 to 5 toxicities among the 15 patients receiving a total of 90 doses of AE37 \pm GM-CSF. Among all patients, maximum local toxicities were grade 1 (40%) or grade 2 (60%). Maximum systemic toxicity was mild; grade 0 (13%), grade 1 (73%), and grade 2 (13%). Grade 1 toxicities

included fatigue, nausea, myalgias, rhinitis, diarrhea, headache, and cough. Grade 2 toxicities included joint pain and stiffness. Local reaction measurement of 100 mm or greater in duration or systemic grade 2 toxicity caused 47% of patients to undergo dose reductions. Taken as a whole, the vaccine is safe with minimal local and systemic toxicity, as depicted in Figure 2A.

The AE37 peptide vaccine was capable of eliciting an immune response both in vitro and in vivo. To assess in vitro immune response, we analyzed peptide-induced proliferation of T lymphocytes by [3 H]thymidine incorporation. The average proliferative response to AE36 and AE37 increased significantly pre- to postvaccine (1 month postcompletion), and prevaccine to long term (6 months postcompletion; Fig 2B). The vaccine's in vivo effectiveness was demonstrated by significantly increased DTH comparing pre- with postvaccination (1 month postcompletion) reactions (Fig 2C). Overall, all doses of AE37 were highly immunogenic.

Toxicity per Dosing Group

Local and systemic toxicities in each dosing group are depicted respectively in Figure 3A and 3B. Local toxicity was highest in the 500:250:6 dosing group, with 100% of the patients experiencing grade 2 toxicity. Systemic symptoms were predominately grade 1, with only two patients (one per dosing group: 1,000:0:6 and 500:125:6) experiencing grade 2 toxicity.

Immunologic Response per Dosing Group

Proliferation assays. Mean proliferative responses to AE37 by dose group increased significantly pre- to postvaccine in all dose groups (Fig 4A). There was a significant increase in proliferation response at long-term follow-up compared with prevaccine in all of the 500- μ g peptide dosing groups. The patients also responded to AE36, the wild-type peptide (Fig 4B). The 500:125:6 dosing group demonstrated the most consistent proliferative responses to both AE37 and AE36 in both short- and long-term postvaccine time points. Immunologic responses were decreased in the 500:30:6 dose group.

DTH. All of the dosing groups had statistically significant increases in their pre- to postvaccine DTH responses, and reaction measurements were enhanced as peptide dose increased (Fig 4C). There was negligible decrease in DTH reactions noted with GM-CSF reduction from 250 to 125 μ g, but significant DTH decrease with GM-CSF of 30 μ g.

DISCUSSION

In our phase I clinical trial, we have shown the AE37 peptide vaccine to be both safe and effective in raising HER-2/*neu*-specific immunity both in vitro and in vivo as long as the vaccine is dosed correctly. In preclinical models, the Ii-Key hybrid peptide AE37 is more immunogenic than its wild-type counterpart²³ and is more sensitive in detecting pre-existing HER-2/*neu* immunity.^{18,24} In our trial, dose reductions were required to the point of eliminating adjuvant GM-CSF altogether, demonstrating potency of the hybrid peptide vaccine. The goal of our phase I testing was to determine safety and optimal biologic dose (OBD) with an emphasis on the community; that is, striking a balance between stimulating as strong an immune response as possible without the need for dose reductions so the vaccine can be administered safely and effectively to a large population.

The OBD of the novel AE37 hybrid vaccine seems to be 500 μ g of peptide with GM-CSF more than 30 μ g and less than 125 μ g. The OBD was established in two parts: the peptide dose and adjuvant dose. The optimal AE37 peptide dose (500 μ g) is based on the fact that two of three patients receiving 1,000 μ g of peptide required reduction in peptide to less than 250 μ g by the end of their vaccine series, as well as

Table 2. Patient Demographics, Prognostic Factors, and Treatment Profiles

Characteristic	AE37-Vaccinated Patients (n = 15)	
	No.	%
Age, years		
Median	57	
Range	44-70	
Race		
White	9	60
African American	5	33
Asian	1	7
Tumor size		
> T2 (> 2 cm)	1	7
Histologic grade		
Grade III	3	20
HER-2/ <i>neu</i> IHC 3+ or FISH+	5	33
Hormone receptor negative	4	27
No chemotherapy	12	80
No RT	4	27
Hormonal therapy	11	73

Abbreviations: IHC, immunohistochemistry; FISH, fluorescent in situ hybridization; RT, radiotherapy.

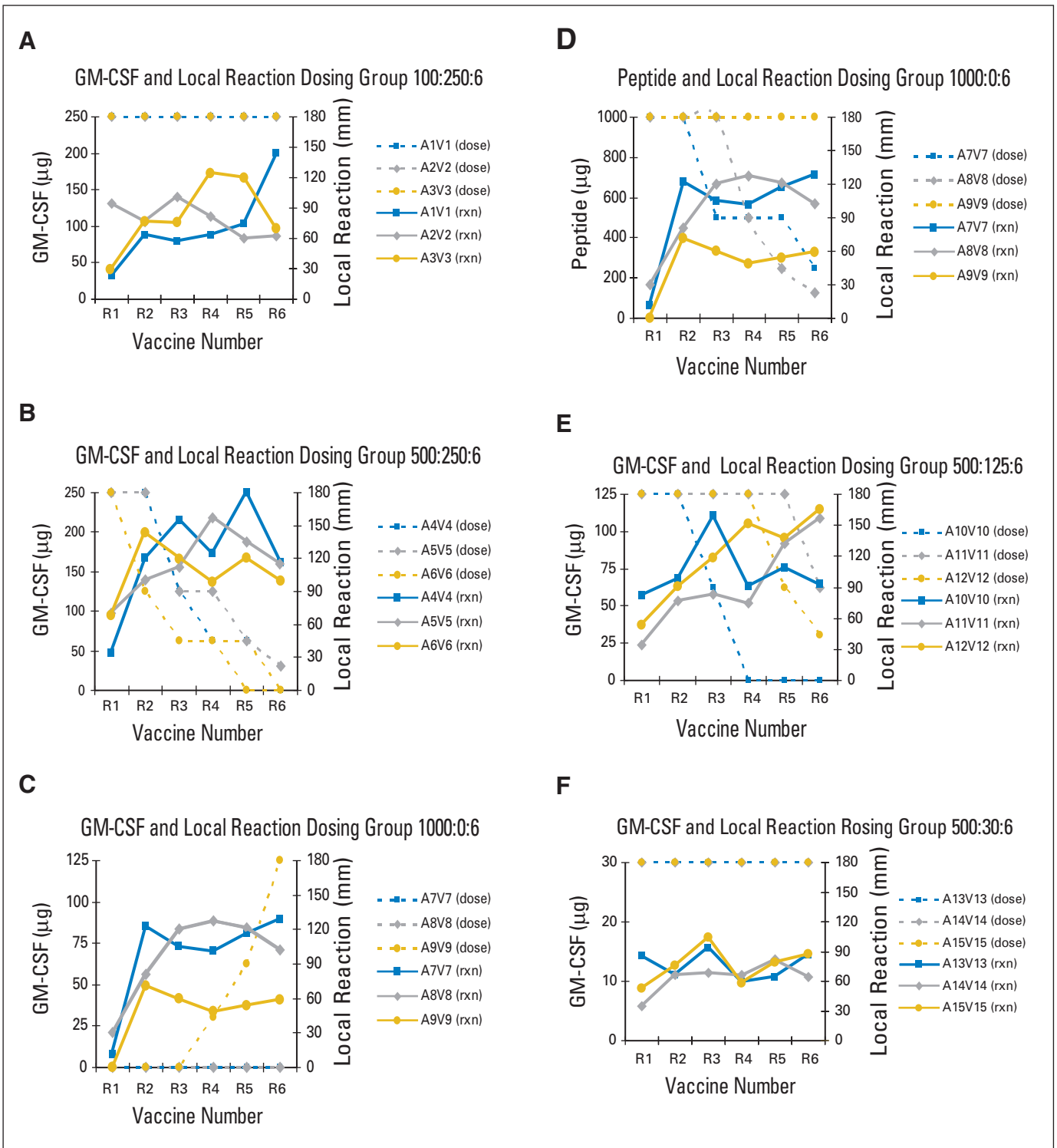


Fig 1. Local reactions by dose groups. Dose groups are designated as peptide (μg):granulocyte-macrophage colony-stimulating factor (GM-CSF; μg):number of inoculations. A local reaction (rxn; solid lines) ≥ 100 mm in duration necessitated 50% dose reduction in GM-CSF (dashed lines). GM-CSF and local reaction dosing groups: (A) 100:250:6, (B) 500:250:6, (C) 1,000:0:6, (E) 500:125:6, and (F) 500:30:6. (D) Peptide and local reaction dosing group 1,000:0:6. The 1,000- μg peptide dose group did not receive GM-CSF initially. (C) One patient required addition of GM-CSF due to low reactivity, but (D) two patients required peptide dose reductions due to robust reactions.

the fact that all patients receiving 500 μg AE37 showed significant immune responses that were sustained long term (Fig 4). We propose a GM-CSF dose of 62.5 μg because 250 and 125 μg of immunoadju-

vant were equivalent immunologically, but had significant local and systemic reactions requiring dose reductions, whereas 30 μg was well tolerated but had decreased immunologic response.

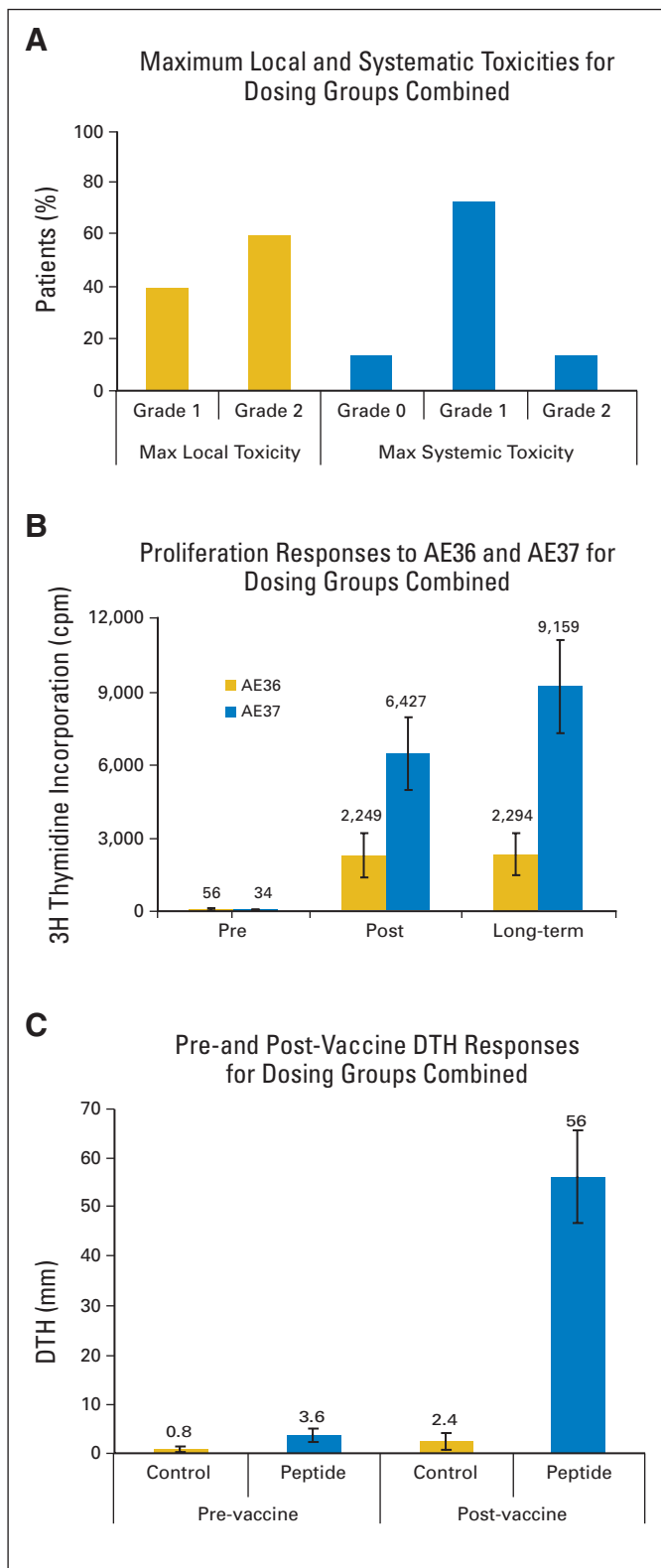


Fig 2. Combined toxicity and immunologic response. (A) Maximum (Max) local and systemic toxicities. (B) Proliferative responses (mean \pm SE) to AE36 and AE37, respectively increased significantly prevaccine (pre) to postvaccine (post; P for AE36 = .03; P for AE37 = .0002) and long-term (P for AE36 = .02; P for AE37 = .00001). (C) Delayed-type hypersensitivity (DTH; mean \pm SE) pre- and postvaccine for all dose groups combined (pre- to postvaccine DTH P = .0001).

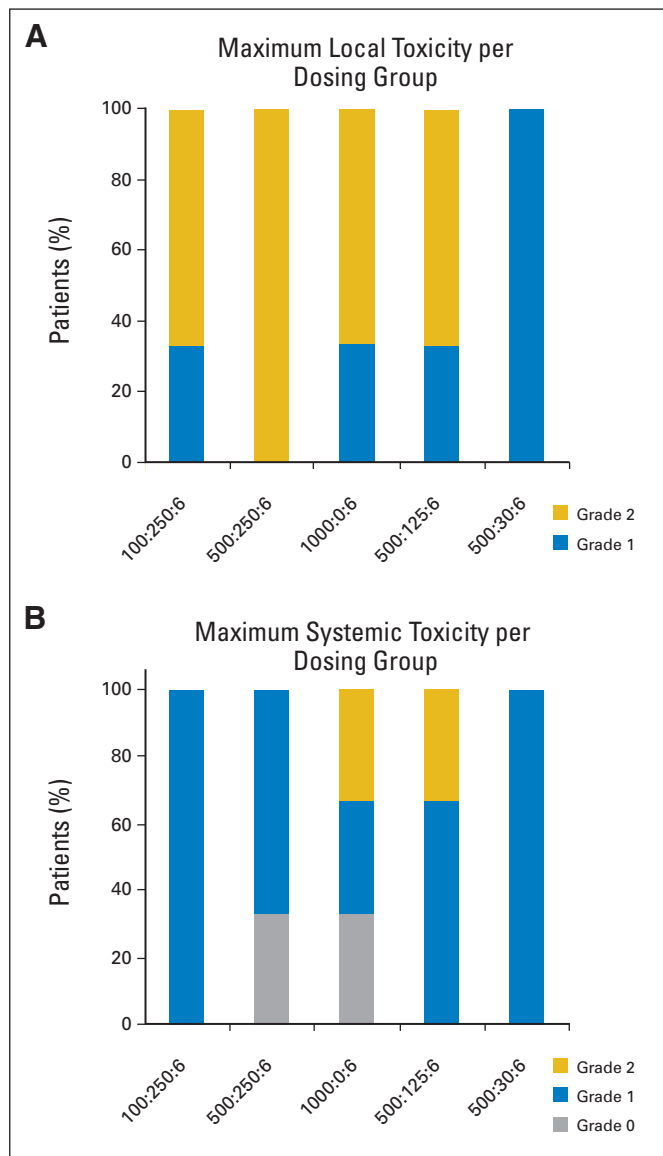


Fig 3. Maximum (A) local and (B) systemic toxicities by dose group. Toxicity was assessed in each patient after every inoculation and graded per Common Toxicity Criteria standards with each patient's maximum local and systemic toxicities depicted as a percentage of the dosing group.

GM-CSF has been used in our laboratory as an immunoadjuvant on the basis of previous data from animal and human trials in breast²⁵ and melanoma.²⁶ A randomized clinical trial in melanoma revealed that GM-CSF and Q2-21 were superior to incomplete Freund's adjuvant.²⁶ Even at low doses (< 80 μ g), immune response was induced with increasing vaccine doses.^{26,27} The 62.5- μ g GM-CSF dose is consistent with these findings. We are proceeding with this dose in our phase II trial further investigating the AE37 hybrid vaccine. As stated, our goals are to provide a safe vaccine at a consistent dose that stimulates a reproducible immune response in the general public.

Peptide vaccine trials have been ongoing for many years and have focused predominately on stimulating CTLs, which can directly kill tumor cells but lack long-lasting immunity. This AE37 peptide vaccine focuses on stimulating CD4⁺ T-helper cells with two goals: immunologic memory and persistent stimulation of CTLs.²⁸ This concept is

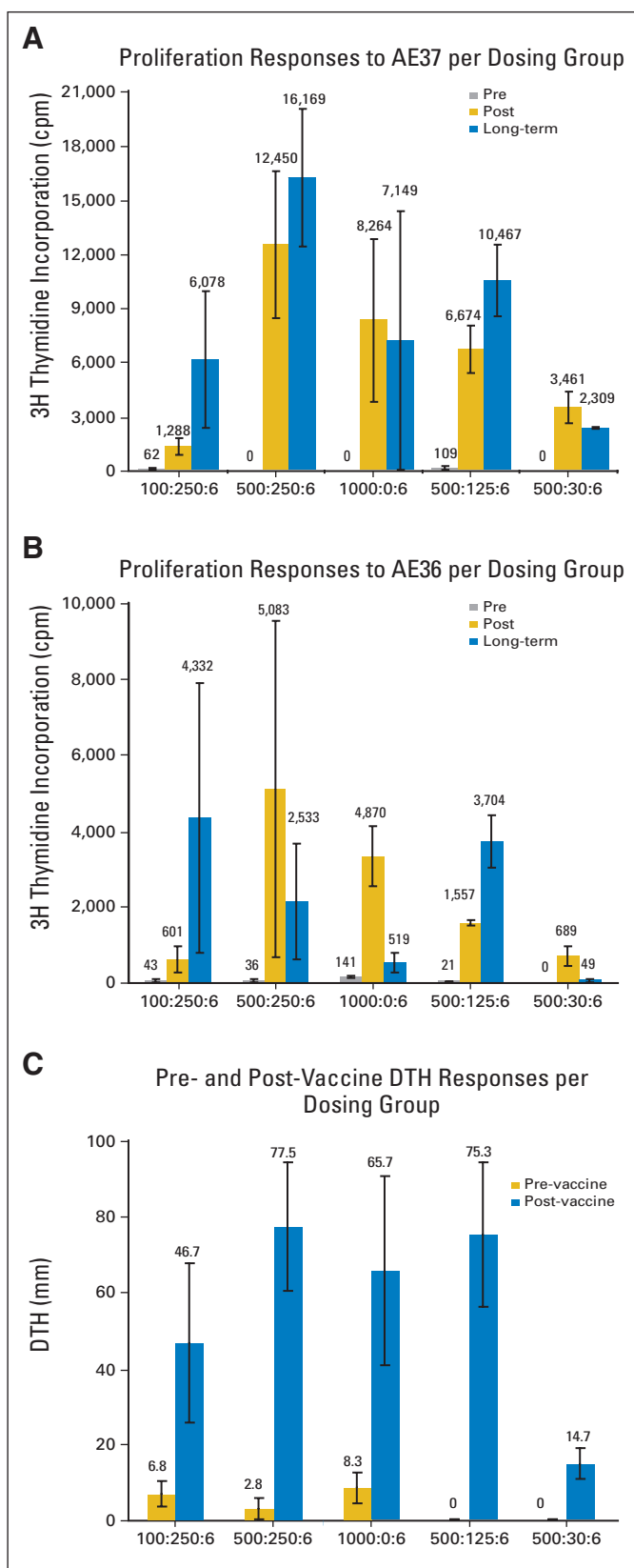


Fig 4. Immunologic responses to varying doses of AE37 vaccine. Proliferative responses (mean \pm SE) to (A) AE37 and (B) AE36 increased prevaccine (pre) to postvaccine (post; 1 month after series completion), and long-term (6 months postcompletion) in every dosing group. (C) Delayed-type hypersensitivity (DTH; mean \pm SE) pre- and postvaccine per dosing groups. cpm, counts per minute.

being studied by others, including Disis et al,²⁹ who are performing ongoing breast cancer clinical trials with class II peptide vaccines. In melanoma there are other class II peptide vaccines in clinical trials, but this is the first clinical trial using the Ii-Key modification.³⁰⁻³³

The hybrid AE37 vaccine has been designed to overcome some of the problems with peptide vaccines. By taking advantage of the Ii-Key protein interaction with class II MHC molecules, AE37 is able to directly charge MHC class II molecules with the antigenic epitope of HER-2/*neu*, bypassing the normal antigen processing pathway. The antigen can then be presented to the immune system, stimulating a specific CD4-positive T lymphocyte response. When compared with an unmodified epitope, these Ii-Key/antigenic epitope hybrids can display 250 times the potency or greater *in vitro*.^{17,18} Because of the increased potency of these hybrids in stimulating immune responses, it has been suggested that less efficiency in the interaction between epitope and MHC class II molecule can be tolerated, such that these hybrids may be active in individuals having HLA alleles with which the wild-type epitope interacts only weakly.

In our hands, the hybrid vaccine AE37 is sufficiently potent, that at times peptide did not require the use of immunoadjuvant GM-CSF. Historically, peptides alone are minimally immunogenic and require coadministration with immunologic adjuvants to induce detectable T-cell responses.^{34,35} Local and systemic reactions in the second dose group (500:250:6) necessitated dose reductions in all three patients, so the third dose group (1,000:0:6) was initiated without GM-CSF. As noted earlier, two of three patients in this group developed robust local reactions without GM-CSF and required reductions in peptide dose. Additionally, three patients in other dose groups required reductions of GM-CSF to the point of receiving peptide-only vaccinations. Further evidence of vaccine potency is seen in DTH reactions; the DTH reaction for AE37 is double the size of optimally dosed E75-peptide vaccine patients.⁹ To our knowledge, this is the first peptide vaccine tested in humans not requiring an immunoadjuvant.

Stimulating CD4⁺ T-helper cells is thought to be the key to long-term immunity, but there have been concerns implicating a subset of these cells with tissue destruction, tumorigenesis, and autoimmune diseases.¹¹ The cells associated with these pathologies are classified as CD4⁺CD25⁺FoxP3-positive T-regulatory cells (Tregs).^{36,37} Because of these theoretical concerns, and because Tregs are elevated in breast cancer patients,³⁸ we have measured Tregs in our AE37 trial. Although there was no change in the total CD4⁺ population (prevaccination, 52.4%; postvaccination, 51.0%; $P = .5$), Tregs as measured by FoxP3 (1+) in nine patients statistically decreased (prevaccination, 2.1%; postvaccination, 1.0%; $P = .0008$). Further analysis on all 15 AE37 trial patients is currently underway (manuscript in preparation).^{39,40} At present, it is unclear whether the decrease in Tregs is a result of the peptide vaccine or immunoadjuvant; this is being addressed in our phase II trial with a control arm, consisting of GM-CSF alone.

One limitation of peptide vaccines has been the preferential binding of peptides to certain MHC class I and II molecules. E75 (HER-2/*neu*:369-377) binds to HLA-A2-positive/A3-positive patients, and Tuttle^{13,41,42} has suggested that G89 peptide is preferentially recognized by HLA-DR4-positive patients; however, Salazar et al⁴³ have presented findings that the parent epitope in AE37, HER-2/*neu*:776-790 (which contains G89) interacts with a variety of MHC class II alleles. Whether the Ii-Key moiety in AE37 confers additional MHC class II promiscuity to the HE2/*neu* epitope it contains is unclear.

Variability was seen within dose groups suggesting binding preferences. Additional studies are being performed to determine optimal HLA-DR types for this specific vaccine.

There are numerous potential and theoretical advantages of HER-2/*neu*-directed vaccination therapies compared with other directed therapies like trastuzumab. These advantages include ease of administration, decreased systemic toxicity, broad applicability to all levels of HER-2/*neu* expression (1+ to 3+), and, most importantly, stimulation of broad immunologic response to include memory response with continued benefit after therapy is completed.

In conclusion, the hybrid AE37 vaccine seems safe and elicits a HER-2/*neu*-specific immune response, even without use of an adjuvant. A multicenter phase II trial of the AE37 vaccine is presently underway to further assess these intriguing findings. Eventually, our goal is to develop a combination multipeptide vaccine to stimulate antigen-specific CTL responses both directly and indirectly.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked

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