Timed Sequential Treatment With Cyclophosphamide, Doxorubicin, and an Allogeneic Granulocyte-Macrophage Colony-Stimulating Factor–Secreting Breast Tumor Vaccine: A Chemotherapy Dose-Ranging Factorial Study of Safety and Immune Activation

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ABSTRACT

Purpose
Granulocyte-macrophage colony-stimulating factor (GM-CSF)–secreting tumor vaccines have demonstrated bioactivity but may be limited by disease burdens and immune tolerance. We tested the hypothesis that cyclophosphamide (CY) and doxorubicin (DOX) can enhance vaccine-induced immunity in patients with breast cancer.

Patients and Methods
We conducted a 3 × 3 factorial (response surface) dose-ranging study of CY, DOX, and an HER2-positive, allogeneic, GM-CSF–secreting tumor vaccine in 28 patients with metastatic breast cancer. Patients received three monthly immunizations, with a boost 6 to 8 months from study entry. Primary objectives included safety and determination of the chemotherapy doses that maximize HER2-specific immunity.

Results
Twenty-eight patients received at least one immunization, and 16 patients received four immunizations. No dose-limiting toxicities were observed. HER2-specific delayed-type hypersensitivity development in most patients who received vaccine alone or with 200 mg/m² CY. HER2-specific antibody responses were enhanced by 200 mg/m² CY and 35 mg/m² DOX, but higher CY doses suppressed immunity. Analyses revealed that CY at 200 mg/m² and DOX at 35 mg/m² is the combination that produced the highest antibody responses.

Conclusion
First, immunotherapy with an allogeneic, HER2-positive, GM-CSF–secreting breast tumor vaccine alone or with CY and DOX is safe and induces HER2-specific immunity in patients with metastatic breast cancer. Second, the immunomodulatory activity of low-dose CY has a narrow therapeutic window, with an optimal dose not exceeding 200 mg/m². Third, factorial designs provide an opportunity to identify the most active combination of interacting drugs in patients. Further investigation of the impact of chemotherapy on vaccine-induced immunity is warranted.

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INTRODUCTION

More effective treatments have led to a clear decrease in breast cancer mortality, but up to 40% of diagnosed patients ultimately relapse.¹ The best drugs available have limited impact on the survival of patients with disseminated breast cancer. Innovative treatments that complement existing therapies are urgently needed to improve disease outcomes in advanced, treatment-resistant patients.

Active immune-based therapies, such as vaccines, have several advantages that could complement standard breast cancer treatments. First, they can engage the host antitumor response rather than targeting the tumor directly. Second, the immune system can specifically recognize an unlimited number of target antigens preferentially expressed by diseased cells relative to normal tissue. Third, immunotherapy could yield a durable treatment response due to immunologic memory. Several vaccines for metastatic breast cancer have been tested with modest success.² These studies demonstrated vaccine safety, but immune responses were frequently inconsistent, observed in small numbers
of patients, or not clearly associated with clinical benefit. The lack of clinical success is most likely due to suboptimal immunization strategies that fail to consider immune tolerance and disease burdens, inadequate targets, or both.3

Tumor cells genetically modified to express granulocyte-macrophage colony-stimulating factor (GM-CSF) can induce potent T-cell–dependent immunity capable of curing tumor-bearing mice.4 Early clinical trials of GM-CSF–secreting tumor vaccines in diverse solid tumors demonstrated their safety and bioactivity, with some suggestive evidence of clinical benefit.5-13 However, vaccination alone is unlikely to induce an immune response of sufficient magnitude and potency to cause tumor regression when immune tolerance and measurable tumor burdens are present.

Some chemotherapy drugs can augment immunotherapy when given in proper dose and sequence.14 In the immune tolerant HER2/neu (neu-N) transgenic mouse model of mammary cancer, an HER2-targeted, GM-CSF–secreting vaccine alone is ineffective against established HER2-positive tumors.15 In contrast, sequencing the vaccine with low doses of cyclophosphamide (CY) and doxorubicin (DOX) induces curative HER2-specific immune responses in up to 30% of tumor-bearing neu-N mice.16 CY can abrogate the suppressive influence of CD4+CD25+ regulatory T cells (Tregs), allowing the activation of potent, tumor-specific CD8+ T cells.17 Accumulating data implicate Tregs as a major barrier to effective T-cell immunity in advanced cancer patients.18-23

On the basis of these data, we conducted a clinical evaluation of an allogeneic, HER2-positive GM-CSF–secreting breast tumor vaccine alone or in sequence with low doses of CY and DOX. This phase I study was designed to assess the safety and immunologic activity of chemotherapy-modulated vaccination in patients with stable metastatic breast cancer. Modeling responses to HER2 as a sentinel measure of immunologic activity, the study used a factorial design24 to identify the CY and DOX dose combination that maximizes the vaccine-induced immune response.

PATIENTS AND METHODS

**Study Design**

The study protocol has been published.25 This was a dose-ranging study of CY and DOX in a 3 × 3 factorial design to determine the dose combination that maximizes vaccine-induced immunity (Fig 1A). Vaccine alone was first given to six patients; the remaining 22 patients were enrolled in the dosing schema sequencing chemotherapy with vaccination. CY and DOX were each tested at three doses encompassing those efficacious in the preclinical neu-N model, yielding a total of nine design points. Enrollment initially followed a predefined path through the nine design points for safety.
The study was conducted in accordance with the principles of good clinical practice and the ethical principles stated in the Declaration of Helsinki. It was approved by The Johns Hopkins University School of Medicine Institutional Review Board, the National Institutes of Health Recombinant DNA Advisory Committee, and the US Food and Drug Administration for Biologics, Evaluation and Research. A modification to the original design was approved by the Institutional Review Board and the US Food and Drug Administration and was implemented July 6, 2006. This modification, based on early safety and immune data, altered the range of CY doses from 250, 350, and 450 mg/m² to 200, 250, and 350 mg/m², allowed flexibility to enter eligible patients onto the design points compatible with their prior cumulative DOX dose, and revised the sample size from 30 to a range of 22 to 30.

**Patient Selection**

Twenty-eight patients with metastatic breast cancer stable for ≥ 28 days were enrolled at the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center between January 15, 2004, and January 9, 2008. Eligible patients had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 1 and a histologic diagnosis of breast cancer; HER2 overexpression was allowed but not required. Prior chemotherapy was allowed but must have been completed ≥ 28 days before vaccination; concurrent endocrine and/or bisphosphonate therapy was allowed. Other requirements included cardiac ejection fraction ≥ 45%, adequate end-organ function, and negative testing for HIV and pregnancy. Stable treated CNS disease was allowed. Key exclusion criteria included a projected lifetime cumulative DOX dose ≥ 450 mg/m², past/current autoimmune disease, nonprotocol-specific treatment or parental steroids within 28 days of vaccination, and past/current second malignancy (except superficial melanoma, bladder cancer, or cervical carcinoma in situ).

**Study Plan and Intervention**

1. **Eligibility determination.** Written informed consent was obtained from each research participant. Baseline studies included computed tomography, bone scans, complete blood count with differential (CBC), chemistry profile, absolute eosinophil count, and echocardiogram or multiple gated acquisition scan.

2. **Treatment plan.** The intervention and data collection schedule is shown in Figures 1B and 1C. Six patients received vaccine alone, with three each receiving 5 × 10⁶ or 5 × 10⁷ cells. The remaining 22 patients received 5 × 10⁸ cells and chemotherapy, with CY given on day −1, vaccine on day 0, and DOX on day 7. This sequence was repeated every 4 to 6 weeks for three cycles, with a fourth cycle 6 to 8 months after cycle 1. Patients with evidence of disease progression were taken off study.

   **Vaccinations.** Vaccine development and manufacturing has been published. Twenty-eight patients were vaccinated.26 Briefly, the parent cell lines T47D (HER2low) and SKBR3 (HER2high) were genetically modified by plasmid DNA transfection to secrete GM-CSF. Clinical lots were prepared from two subcloned cell lines secreting bioactive levels of GM-CSF, ZT47D-V, and 35KBR3-7. On day 0, serum-free, cryopreserved, irradiated vaccine cells were thawed and mixed to create an HER2-positive vaccine that secreted GM-CSF levels of 305 ng/10⁶ cells/24 hours.26 Vaccine cells were injected intradermally, evenly distributed over three lymph node areas. Anesthetic lidocaine cream was applied to the injection sites before vaccination.

3. **End Points**

   **Toxicity assessment.** Toxicities were graded using the National Cancer Institute’s Clinical Trials Common Terminology Criteria for Adverse Events Version 3.0 (CTCAE v3.0). Toxicity monitoring included clinical assessment and complete blood counts weekly and on day 3 of each cycle; chemistry profiles were measured before and after each cycle and on day 7.

   **Pharmacokinetic assay of serum GM-CSF levels.** Serum was collected to measure GM-CSF levels on days 0, 1, 2, 3, 4, and 7 of each cycle, separated from whole blood by centrifugation, and frozen in 1-mL aliquots at −80°C.

   Serum GM-CSF levels were determined by enzyme-linked immunosorbent assay (Quantikine ELISA, R&D Systems, Minneapolis, MN). Serum GM-CSF levels were determined by using a recombinant GM-CSF standard calibrated against the WHO GM-CSF control standard.

**RESULTS**

**Patient Characteristics**

Twenty-eight eligible patients were enrolled, with an age range of 36 to 74 years (Table 1). All had estrogen receptor-positive and/or progesterone-positive disease; one patient also had HER2-positive breast cancer. The mean disease-free interval to relapse from first diagnosis was 29 months (range, 0 to 132 months); nine (32%) patients presented initially with metastatic disease. Eight patients (29%) received prior chemotherapy for metastatic disease. All (100%) were on concurrent endocrine therapy, and the majority (71%) received concurrent bisphosphonate therapy for skeletal metastasis.

Almost all dose combinations were evaluated in two or three patients (Fig 1A). All eligible patients (100%) received at least one vaccination, 25 (89%) received at least three vaccinations, and 16 (57%) received all four vaccinations. All off-study events before cycle 4 were due to progressive breast cancer except one; that patient was taken off-study to receive treatment for a preexisting, subclinical thyroid goiter.

**Toxicity**

No dose-limiting toxicities were observed. The most common adverse events were local vaccine site reactions, including erythema, induration, pruritus, and/or discomfort (Table 2). These self-limited local reactions occurred in all individuals, lasted up to 2 weeks, and...
developed urticaria or eczema distant from the vaccine site. Cardiac toxicities were fatigue and flu-like symptoms. Small numbers of patients (7%) received oral bisphosphonate for bone health in the absence of skeletal disease.

Typically increased in intensity but not duration with subsequent vaccinations. The most common vaccine-related systemic adverse events were fatigue and flu-like symptoms. Small numbers of patients (7%) developed urticaria or eczema distant from the vaccine site. Cardiac toxicities were fatigue and flu-like symptoms. Small numbers of patients (7%) developed urticaria or eczema distant from the vaccine site.

Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of Patients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total patients</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Range</td>
<td>36-74</td>
<td></td>
</tr>
<tr>
<td>ER-positive or PR-positive tumor</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td>HER2-positive tumor</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Metastatic disease at diagnosis</td>
<td>9</td>
<td>32</td>
</tr>
<tr>
<td>Disease-free interval to relapse, months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0-132</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>28.8</td>
<td></td>
</tr>
<tr>
<td>Prior chemotherapy for metastatic disease*</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>Concurrent endocrine therapy</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td>Concurrent bisphosphonate therapy†</td>
<td>20</td>
<td>71</td>
</tr>
</tbody>
</table>

Abbreviations: ER, estrogen receptor; PR, progesterone receptor.

*Two patients received consolidation chemotherapy after surgical resection to no evidence of disease, followed by endocrine therapy; three patients received first-line chemotherapy for metastatic disease followed by endocrine therapy; two patients received one or more regimens of salvage chemotherapy after initial endocrine therapy.

†Bisphosphonate therapy for skeletal metastasis; one additional patient received oral bisphosphonate for bone health in the absence of skeletal disease.

Table 2. Summary of Treatment-Related Adverse Events

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>All Grades</th>
<th>Grade 3 or 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Patients</td>
<td>%</td>
</tr>
<tr>
<td>Local vaccine site reactions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythema/induration</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td>Pruritus</td>
<td>27</td>
<td>96</td>
</tr>
<tr>
<td>Pain/soreness/tenderness</td>
<td>27</td>
<td>96</td>
</tr>
<tr>
<td>Blister formation</td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>Hyperpigmentation</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Ecchymosis</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Local desquamation</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Systemic toxicities</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatigue/malaise</td>
<td>11</td>
<td>39</td>
</tr>
<tr>
<td>Flu-like symptoms/myalgia</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>Fever</td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>Chills</td>
<td>6</td>
<td>21</td>
</tr>
<tr>
<td>Headache</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>Urticaria</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Eczema</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Lymph node pain</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Pruritus (distant from vaccine site)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Rash (distant from vaccine site)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Anorexia</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Nausea</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Anxiety</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

NOTE. Data are given as any incident per patient, for a maximum of 28 counts per event.

Serum GM-CSF Pharmacokinetics

Serum GM-CSF levels were measured as an indicator of the vaccine’s life span following injection. With vaccination alone, GM-CSF levels peaked by 48 hours regardless of cell dose or cycle; for the high vaccine cell dose, the peak amplitude decreased with each subsequent cycle (P = .0001) (Figs 2A and 2B). The addition of low-dose chemotherapy to the vaccine did not alter the timing of peak GM-CSF levels, but the peak GM-CSF level did not decline with subsequent vaccination (P = .99; Fig 2C). There was no statistically significant difference in peak GM-CSF level with time across the doses of CY and DOX tested (data not shown).

HER2-Specific CD4⁺ T-Cell–Dependent Immunity

De novo HER2-specific DTH was observed in five (83%) of six patients receiving vaccine alone, and in seven (32%) of 22 patients receiving vaccine with chemotherapy (P = .034; Table 3). The addition of 200 mg/m² CY had no impact on the rate of DTH development (P = .336), but CY doses higher than 200 mg/m² suppressed vaccine-induced DTH compared with vaccine alone (P = .007) or combined with 200 mg/m² CY (P = .03). The addition of 15 mg/m² DOX suppressed the rate of DTH development (P = .016), whereas higher doses of DOX preserved the vaccine-induced DTH response (P = .15 to .18). Significant HER2-specific humoral immunity (≥ 1.13 µg/mL) developed in one (17%) of six patients who received vaccine alone and in seven (32%) of 22 patients who received vaccine with any dose of chemotherapy (P = .329). The induction of HER2-specific humoral immunity was optimally enhanced by the addition of 200 mg/m² CY or 35 mg/m² DOX to vaccination (Figs 3A and 3B); antibody levels declined after the third vaccination but were restored with the fourth cycle.

Response Surface Analysis

The relationship between chemotherapy dose and antibody level is illustrated in a three-dimensional response surface generated by the model (Fig 3C). Canonical surface analysis showed eigenvalues of 0.68 and 0.46, indicating the stationary point is a saddle point. Ridge analysis estimated that the maximum HER2-specific antibody response is 0.739 µg/mL ± 0.37 µg/mL at 193 mg/m² CY and 35 mg/m² DOX. The closest dose combination formally tested is 200 mg/m² CY and 35 mg/m² DOX. A similar trend was observed for CY and DOX independently when antibody levels were analyzed as binary values by Fisher’s exact test. The agreement between the experimental and predicted dose values supports the suitability of the model.

This phase I factorial study of an allogeneic HER2-positive GM-CSF-secreting breast tumor vaccine with low-dose CY and DOX supports the following five conclusions. First, up to four sequential vaccine

Discussion

This phase I factorial study of an allogeneic HER2-positive GM-CSF-secreting breast tumor vaccine with low-dose CY and DOX supports the following five conclusions. First, up to four sequential vaccine
Factorial Study of Chemotherapy and a Breast Tumor Vaccine

A major difficulty in optimizing tumor cell vaccine-based therapies is the lack of biomarkers for assessing multidrug interactions. This study demonstrates the feasibility of using HER2-specific DTH and antibody responses pre- and postvaccination as immune response vaccines.
tumor immunity in preclinical models by a variety of mechanisms.\textsuperscript{35} It upregulates type I interferons, facilitating the evolution of a CD4\textsuperscript{hi} memory T-cell response.\textsuperscript{36} It also reverses immunologic skew, promoting the T-helper type I response.\textsuperscript{16} Several groups have shown that depletion of Tregs in animal models augments vaccine-induced antitumor immune responses.\textsuperscript{17,37-39} In tolerant neu-N transgenic mice, Treg depletion with low-dose CY enables the recruitment of latent, high-avidity CD8\textsuperscript{+} T cells to the antitumor immune response, resulting in tumor rejection in some mice.\textsuperscript{16,17} We are currently characterizing changes in peripheral Tregs and HER2-specific CD8\textsuperscript{+} T-cell responses in vaccinated patients.

Historically, clinical trials have used 250 to 300 mg/m\textsuperscript{2} CY 3 days before vaccination to alleviate immune suppression.\textsuperscript{6,40-45} One melanoma vaccine study\textsuperscript{46} tested CY doses of 75, 150, and 300 mg/m\textsuperscript{2}. CY 300 mg/m\textsuperscript{2} most effectively depleted CD8\textsuperscript{+} suppressor T cells; antigen-specific immunity was not evaluated.\textsuperscript{46} Here we report the first study to optimize CY dose on the basis of the antigen-specific immune response. Our finding that CY doses higher than 200 mg/m\textsuperscript{2} were detrimental to the immune response suggests that previous phase III vaccine trials incorporating 300 mg/m\textsuperscript{2} CY could have used immunosuppressive doses.\textsuperscript{36,44} However, our study tested CY given 1 day before vaccination and also included DOX in the vaccination sequence. The additional drug and distinct CY schedule are alternative explanations for the differences in our results compared with those previously reported.

In conclusion, this allogeneic GM-CSF–secreting breast tumor vaccine is safe and bioactive given alone or sequenced with low-dose CY and DOX. Further, it can induce HER2-specific immunity in breast cancer patients, and this can be augmented by low-dose CY and DOX. Finally, factorial design is an efficient, effective method for identifying the most active dose combination of interacting drugs in patients. This small study examining vaccine safety and immune activation in patients with stable metastatic breast cancer was not designed to determine whether these promising effects on immune activation translate into a clinical benefit. A vaccine safety and efficacy trial of the optimal chemotherapy dose combination is currently being designed.

**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 with a “U” are those for which no compensation was received; those relationships marked with a “C” were compensated. For a detailed description of the disclosure categories, or for more information about ASCO’s conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Administrative support: Leisha A. Emens

Provision of study materials or patients: Leisha A. Emens, Barry J. Kobrin, Antonio C. Wolff, Vered Stearns, John H. Fetting, Nancy E. Davidson

REFERENCES


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**JCO Announces New Requirement for Phase III Studies**

Effective this month, *JCO* requires authors of phase III reports to include protocol information in their submissions. *JCO* believes that for the editors and reviewers to properly peer review a submission, a redaction of the protocol for all phase III studies must be provided.

Protocol information must include the eligibility criteria, study schema and dose modifications, and a statistical section (including end points). This file will only be available to the editors and reviewers during the peer review process.

For more information about this new requirement, see the Submission Requirements section of the Information for Contributors page, at jco.ascopubs.org/ifc/requirements.dtl