Induction of Immunity to Prostate Cancer Antigens: Results of a Clinical Trial of Vaccination with Irradiated Autologous Prostate Tumor Cells Engineered to Secrete Granulocyte-Macrophage Colony-stimulating Factor Using ex Vivo Gene Transfer


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ABSTRACT

Vaccination with irradiated granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting gene-transduced cancer vaccines induces tumoricidal immune responses. In a Phase I human gene therapy trial, eight immunocompetent prostate cancer (PCA) patients were treated with autologous, GM-CSF-secreting, irradiated tumor vaccines prepared from ex vivo retroviral transduction of surgically harvested cells. Expansion of primary cultures of autologous vaccine cells was successful to meet trial specifications in 8 of 11 cases (73%); the yields of the primary culture limited the number of courses of vaccination. Side effects were pruritis, erythema, and swelling at vaccination sites. Vaccine site biopsies manifested infiltrates of dendritic cells and macrophages among prostate tumor vaccine cells. Vaccination activated new T-cell and B-cell immune responses against PCA antigens. T-cell responses, evaluated by assessing delayed-type hypersensitivity (DTH) reactions against untransduced autologous tumor cells, were evident in two of eight patients before vaccination and in seven of eight patients after treatment. Reactive DTH site biopsies manifested infiltrates of effector cells consisting of CD45RO+ T-cells, and degranulating eosinophils consistent with activation of both Th1 and Th2 T-cell responses. A distinctive eosinophilic vasculitis was evident near autologous tumor cells at vaccine sites, and at DTH sites. B-cell responses were also induced. Sera from three of eight vaccinated men contained new antibodies recognizing polypeptides of 26, 31, and 150 kDa in protein extracts from prostate cells. The 150-kDa polypeptide was expressed by LNCaP and PC-3 PCA cells, as well as by normal prostate plastic prostate cells in men with PCA, we conducted a clinical trial of this treatment approach for malignant melanoma, both T-cell and B-cell immune responses against melanoma antigens were detected (26).

INTRODUCTION

New PCA treatments are needed urgently for the more than 40,000 men in the United States who die from metastatic PCA annually. Several new PCA treatment approaches aim to eradicate PCA cells by inducing systemic immunity to antigens expressed by PCA cells (1–11). Because many such antigens may also be present in normal prostate epithelial cells as well as PCA cells, one major therapeutic challenge for induction of anti-PCA immune responses may be the need to overcome immune tolerance against normal prostate antigens.

GM-CSF-secreting cancer cell vaccines, generated from cancer cells by ex vivo gene transfer, have been shown to elicit tumoricidal antitumor immune responses in a variety of animal tumor models (12–23), including preclinical models of PCA (24, 25), and in human clinical trials (26, 27). Irradiated GM-CSF-secreting cancer cell vaccines induce antitumor immune responses by recruiting antigen-presenting cells, such as DCs, to immunization sites. DCs, the most potent immunostimulatory antigen-presenting cells known, activate antigen-specific CD4+ and CD8+ T-cells by priming them by oligopeptides processed from the dying cancer cells (28). Recent preclinical studies have suggested that CD4+ T-cells activated by GM-CSF-secreting cancer cell vaccines do not merely facilitate cancer cell destruction by CD8+ T-cell responses (28). Rather, vaccination simultaneously elicits both Th1 and Th2 cytokine responses, leading to cancer cell killing by a variety of mechanisms (28). In a clinical trial of this treatment approach for malignant melanoma, both T-cell and B-cell immune responses against melanoma antigens were detected (26).

To determine whether GM-CSF-secreting PCA vaccines might elicit T-cell and B-cell immune responses against normal and neoplastic prostate cells in men with PCA, we conducted a clinical trial of this treatment strategy. The clinical translation involved tumor resection by radical prostatectomy, establishment of primary PCA cultures, and ex vivo gene transfer. This was the first NIH Office of Recombinant DNA Activities-approved trial of human gene therapy for PCA. Eight men with PCA were treated by intradural injections of irradiated PCA vaccine cells, and then monitored for treatment-associated side effects, for signs of PCA progression after vaccination, and for evidence of induction of T-cell and B-cell immune responses. Results indicated that this new PCA immunotherapy treatment approach, featuring vaccination with GM-CSF-secreting autologous prostate tumor cells, was feasible, safe, and capable of eliciting systemic immune responses against PCA antigens.

MATERIALS AND METHODS

Selection of Patients for Treatment with Genetically Modified PCA Vaccines. Candidates for enrollment into the Phase I clinical trial included men with adenocarcinoma of the prostate who had undergone a radical pros-
The rationale for choosing dose level 1 at $1 \times 10^7$ cells was based on four facts. First, it was based on preclinical studies showing efficacy against preestablished tumors in this vaccine cell dose range in the hormone-refractory Dunning rat (PCA) model (24, 25). Second, the cell dose range was found to be safe in renal cell carcinoma patients in a Phase I study using MFG-GM-CSF gene-transduced tumor vaccines (27). Third, in clinical trial simulations, autologous prostate cell vaccine could be generated consistently in the dose level 1 range following gene transfer with the MFG-GM-CSF vector in patients undergoing anatomical radical prostatectomy. (24). Fourth, this vaccine cell dose range secreting GM-CSF conferred an objective clinical response in metastatic renal cell carcinoma patients (27). The Phase I clinical study was reviewed and approved by The Johns Hopkins Joint Committee on Clinical Investigation, by the Johns Hopkins University School of Medicine Biosafety Committee, by the Food and Drug Administration, and by the NIH Office of Recombinant DNA Activities.

Preparation of GM-CSF-secreting Autologous PCA Vaccine Cells Using Retroviral Gene Transfer. Excised prostate tumors were mechanically dissociated into 0.1–0.5-cm fragments, suspended in a transport medium, sealed, and shipped in a thermally secure container to Somatix Therapy Corp. (Alameda, CA). Tumor fragments were mechanically disaggregated into a suspension and then cultivated in serum-free medium (24). At the time of initial plating, cell viability was consistently 70–80% by trypan blue exclusion. The expansion rate in primary culture was consistent with that described previously by Sanda et al. (24) using serum-free media. The doubling times ranged from 4 to 12 days. No fetal bovine serum or collagenase was used in cell disaggregation, shipping, or cryopreservation. Primary cultures were transduced with the replication-defective retrovirus containing cDNA encoding GM-CSF (MFG-GM-CSF) as described previously (24, 27, 30). Genetically modified PCA vaccine cells (GVAX, Cell Genesys, Inc., Foster City, CA) were then lethally irradiated (with 15 Gy), assayed for GM-CSF secretion by ELISA (R&D Systems, Minneapolis, MN), evaluated for MFG-GM-CSF integration by quantitative Southern blot analysis (24), tested for microbial contaminants and for RCR (27, 30), and stored in liquid nitrogen. Immediately before administration as vaccines, the cells were thawed, washed three times with HBSS, and checked for viability by trypan blue exclusion.

Analyses of Immune Responses Elicited by Vaccine Treatment. Assessment of T-cell and B-cell immune responses induced by vaccination with irradiated GM-CSF-secreting PCA cells included histopathological and immunohistochemical studies of vaccine site biopsies, DTH testing using unmodified autologous PCA cells, and immunoblot analyses of serum antibodies appearing after vaccination.

For each man treated with PCA vaccines, a skin biopsy was obtained before vaccination, and then vaccine site biopsies were performed 3 days following the first and final vaccine inoculations. Biopsy specimens were dissected such that samples could be formalin fixed and paraffin embedded and also snap frozen without formalin fixation. Tissue sections cut from formalin-fixed samples were stained with H&E. Vaccine cells were detected by immunohistochemical staining for cytokeratins (using antikeratin antibodies AE1 and AE3; Roche Molecular Biochemicals, Indianapolis, IN; Ref. 31), for PSA (using antibody 5/26, Immunotech, Inc., Westbrook, ME), and for prostate-specific acid phosphatase (using rabbit antiserum A0627, Dako Corp., Carpinteria, CA). Infiltrating inflammatory cells were detected and characterized by immunohistochemical staining for CD68 (a macrophage marker; antibody KP1, Dako; Ref. 32), for CD1a (a Langerhans cell marker; antibody O10, Immunotech; Ref. 33), for S-100 (a Langerhans cell and melanocyte marker; rabbit antiserum Z311, Dako), and for S-100 (a natural killer cell marker; antibody 123C3 against NCAM, Zymed Laboratories, Inc., South San Francisco, CA; Refs. 34 and 35), for leukocyte common antigen (LCA; antibodies PD7/26/16 and 2B11; Dako; Ref. 36), for CD3 (a T-cell marker; rabbit antiserum A0452, Dako; Ref. 37), for CD4 (a helper T-cell marker, antibody 1F6, Novocastra, Vector Laboratories, Inc., Burlingame, CA), for CD8 (a cytotoxic T-cell marker; antibody C8 144B, Dako; Ref. 38), for CD56 (a marker for activated or memory T-cells; antibody UCHL1, Dako; Ref. 39), for CD20 (a B-cell marker; antibody L26, Dako; Ref. 40), for Ki-67 (a marker of proliferation; antibody MB1-1, Immunotech; Ref. 41), and with DIF Quick (a stain that detects eosinophils and neutrophils; Alogenica, Columbus, MD). Frozen sections were stained for eosinophil major basic protein using an immunohistochemical technique (antibody BMK13, Research Diagnostics, Inc., Flanders, NJ).
NJ; Ref. 42). Evaluation of the stained vaccine site biopsy sections was assessed by three observers (B. M., J. W. S., and A. M. D.).

DTH testing was accomplished by intradermal administration of irradiated (15 Gy) autologous prostate tumor cells (1 × 10^6 in 0.2 ml of HBSS) that had been propagated ex vivo in serum-free medium but had not been exposed to the MFG-GM-CSF retrovirus or fetal bovine serum in handling. DTH target cells were derived at the second passage of primary cultures of autologous prostate tumor cell explants using identical methods of primary cell culture as described above; they were not permanently established cell lines (24, 27). These DTH target cells were cryopreserved in a buffer containing human serum albumin. DTH reactivity was assessed by measuring the extent of induration at 48 h at the DTH injection site as described previously (27). DTH site biopsies (5 mm) were obtained at 48 h and processed in a manner similar to the vaccine site biopsies. In addition to DTH testing using autologous prostate tumor cells, DTH testing using seven defined common recall antigens was also undertaken (Multitest CMI panel, Pasteur-Merieux-Connaught, Swiftwater, PA).

Sera obtained from vaccinated men were analyzed for the presence of induced antibody responses against PCAs antigens by using the sera to stain immunoblots containing protein extracts from cultured PCA cells as well as from other cultured human cells. LNCaP PCA cells (43), PC-3 PCA cancer cells (44), DU 145 PCA cells (45), A549 lung carcinoma cells (46), LS-174T colon carcinoma cells (47), KLE endometrial carcinoma cells (48), Jurkat T-cell leukemia cells (49), and MDA-MB-435 breast carcinoma cells (50, 51) were propagated in vitro in DMEM or Ham’s F-12 growth medium (JHR Bioscience, Lenexa, KS) containing 10% FCS. Primary cultures of human prostate epithelial cells, prostate stromal cells, prostate smooth muscle cells, and lung fibroblasts were obtained from the Clonetics Corp. (Walkersville, MD). To prepare protein extracts, cultured cells were harvested, collected by centrifugation at 1000 rpm for 10 min using a Beckman CS-6R centrifuge (Beckman, Palo Alto, CA), washed extensively with PBS, and then subjected to lysis in 10 mM Tris-HCl at pH 7.4, 1 mM EDTA, 10% glycerol, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail set III (Calbiochem, La Jolla, CA) for 1 h at 4°C. The cell lysates were then clarified by centrifugation at 600 g and subjected to protein quantification using the BCA assay (Pierce, Rockford, IL). Lysates containing 25–35 g of protein were electrophoresed on 4–20% gradient polyacrylamide gels (Norvex, San Diego, CA) in the presence of SDS and transferred electrophoretically to nitrocellulose membranes (Norvex). The resultant nitrocellulose membrane blots were first treated with a blocking solution (10% nonfat dry milk and 0.5% Tween 20 in PBS) overnight at 4°C, next exposed to sera recovered from vaccinated men (at a 1:100 dilution in PBS containing 0.05% Tween 20) for 2 h at room temperature, and then washed extensively with PBS containing 0.1% Tween 20. Human serum antibodies adhering to blotted proteins were detected by incubation of the blots with horseradish peroxidase-conjugated goat anti-human IgM + IgG + IgA (Zymed) at a dilution of 1:3000 in PBS containing 0.05% Tween 20 for 1 h at room temperature. After further washing in PBS (3 × 20), horseradish peroxidase activity was detected using an ECL kit (Amersham Pharmacia Biotech, Arlington Heights, IL). Human serum antibodies directed specifically against PSA were assayed using immunoblots containing purified PSA (Calbiochem) in an identical manner.

RESULTS
Generation of GM-CSF-secreting Autologous Prostate Tumor Cell Vaccines Using ex Vivo Gene Transfer. After radical prostatectomy, 11 men with PCA were found to have metastatic cancer and were eligible for participation in the Phase I clinical trial. For each of the 11 men, autologous prostate tumor cells were placed in primary cultures using 2–5 grams of prostate tumor tissue dissected from radical prostatectomy resection specimens. The accrual of the Phase I trial was 8 patients evaluated for toxicities after a minimum of three vaccinations given every 2 weeks. Primary cultures were successfully established in 8 of 11 patients (73%; see Table 1). Prostate tumor cell expansion via propagation in vitro to cell yields adequate for treatment at the assigned dose level was somewhat less successful. For patients accrued in the trial at dose level 1 (1 × 10^7 cells/vaccination), sufficient cells were expanded in three of four primary culture attempts (75%); one primary culture failed to be established at dose level 1. When dose level 1 was found to be clinically safe in three treated patients, a total of seven patients were accrued for dose level 2 (5 × 10^7 cells/vaccination). Sufficient cells were recovered in only three of seven patients’ primary cultures to allow three vaccinations at dose level 2. Thus, only three patients were treated at dose level 2. Two patients had tumors that failed to be established in primary culture, and the remaining two patients accrued at dose level 2 had sufficient vaccine cell yields to specifications to be treated at dose level 1. As a result, primary prostate tumor cell cultures in this trial permitted five patients to be treated at dose level 1 and three to be treated at dose level 2 (Table 2). On average, >90% of the entire prostate tumor tissue was placed into primary culture, making it unlikely that total cell yield could be significantly increased by procuring more primary tumor at surgery (Table 2).

In contrast to the suboptimal success rate in expanding prostate tumor cell numbers in primary cultures, genetic modification of PCA cells to achieve high level secretion (>140 ng/10^6 cells over 24 h) of GM-CSF using ex vivo retroviral gene transfer was remarkably effective (eight of eight attempts, or 100%; see Table 1). Previous studies using preclinical models have suggested a minimum threshold of >35 ng of GM-CSF/10^6 cells over 24 h secreted by vaccine cells might be required to elicit therapeutic antitumor immune responses (12). Transduction of the prostate tumor cells by the replication-defective retrovirus MFG-GM-CSF resulted in viral integration to a copy number of between 1 and 4 per haploid genome as assessed by quantitative Southern blot analysis (not shown; see Ref. 24). Resultant GM-CSF secretion by the prostate tumor cells increased from <0.2 ng/10^6 cells over 24 h to between 143 and 1403 ng/10^6 cells over 24 h (Table 2).

Vaccine Administration. Eight men were treated with irradiated GM-CSF-secreting prostate tumor cell vaccines at 2 dose levels (five at dose level 1 and three at dose level 2). Up to six intradermal vaccinations were administered every 3 weeks. Skin reactions (see Fig. 1), including discomfort, erythema, swelling, and pruritis, were common after vaccine treatment (Table 3). The maximum level of erythema and induration, up to 4 cm in diameter, typically appeared within 24–48 h of vaccination and resolved without intervention in 5–7 days. Mild low-grade fevers, chills, and malaise were noted by a few men as a consequence of vaccine inoculation (Table 3). No severe or dose-limiting cutaneous or systemic side effects were observed. No significant alterations in serum electrolyte and chemistry values or in hematology counts, including WBC counts, total eosinophil counts, and total neutrophil counts, were seen. Plasma GM-CSF pharmacokinetic analyses failed to show any vaccine-associated rise in plasma GM-CSF levels. No RCR was detected in any vaccine cell preparation or in blood from any of the vaccinated men at any time.

Assessment of PCA treatment efficacy was not a primary end point of the Phase I clinical trial of GM-CSF-secreting autologous prostate tumor cell vaccines. With eight fully evaluable patients enrolled and treated, the trial did not have adequate statistical power to estimate a treatment-associated response rate. The median serum PSA before radical prostatectomy was 28.85 ng/ml (with a range of 6.7–75 ng/ml), and the median serum PSA at first vaccination was 0.65 ng/ml (with a range of 0.1–30.4 ng/ml; see Table 1). Ultimately, all eight vaccinated patients manifested a rising serum PSA, showing PCA progression.

Recruitment of Immune Effector Cells to Vaccine Sites. Intra-dermal injection was chosen for the mode of vaccination because of the abundance of Langerhans cells, the skin DCs, which constitute the cell vaccines. With eight fully evaluable patients enrolled and treated, the trial did not have adequate statistical power to estimate a treatment-associated response rate. The median serum PSA before radical prostatectomy was 28.85 ng/ml (with a range of 6.7–75 ng/ml), and the median serum PSA at first vaccination was 0.65 ng/ml (with a range of 0.1–30.4 ng/ml; see Table 1). Ultimately, all eight vaccinated patients manifested a rising serum PSA, showing PCA progression.
IRRADIATED GM-CSF GENE-TRANSUDUCED PCA VACCINES

Table 1 Characteristics of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. enrolled in study (including those without metastatic PCA)</td>
<td>21</td>
</tr>
<tr>
<td>No. clinically eligible</td>
<td>11</td>
</tr>
<tr>
<td>No. treated with metastatic PCA</td>
<td>8</td>
</tr>
<tr>
<td>No. of successful PCA cell cultures (sufficient for vaccine preparation/no. of culture attempts)</td>
<td>8/11</td>
</tr>
<tr>
<td>No. of successful GM-CSF gene transductions/no. of transduction attempts</td>
<td>8/8</td>
</tr>
<tr>
<td>Median age of treated patients (range), yr</td>
<td>51 (46–64)</td>
</tr>
<tr>
<td>Mean age of treated patients, yr</td>
<td>53</td>
</tr>
<tr>
<td>Median PSA level presurgery for treated patients (range), ng/ml</td>
<td>28.85 (6.7–75)</td>
</tr>
<tr>
<td>Mean PSA level presurgery for treated patients, ng/ml</td>
<td>30.85</td>
</tr>
<tr>
<td>Median PSA level for treated patients at 1st vaccination (range), ng/ml</td>
<td>0.65 (0.1–30.4)</td>
</tr>
<tr>
<td>Mean PSA level for treated patients at 1st vaccination, ng/ml</td>
<td>6.08</td>
</tr>
</tbody>
</table>

*a* GM-CSF secretion before MFG-GM-CSF transduction, <0.2 ng/10⁶ PCA vaccine cells/24 h; average GM-CSF secretion after transduction, 461.5 ng/10⁶ PCA vaccine cells/24 h.

27). Autologous prostate tumor cells, characterized by positive immunohistochemical staining for cytokeratins, were present in all vaccine site biopsies evaluated (see Fig. 2B). Acanthosis, a thickening of the stratum spinosum of the epidermis often associated with autoimmune diseases or malignancy, was apparent in each of the vaccine site biopsies (Fig. 2A). The severity of acanthosis, graded by a pathologist (A. M. D.), appeared to increase from the first vaccine site biopsy to the last for each of the vaccinated men.

Inflammatory cell infiltrates were present in each of the vaccine site biopsies compared to pretreatment biopsies. The magnitude of the inflammatory cell response surrounding intradermal deposits of tumor vaccine appeared to be greater in dose level 2 patients compared to the dose level 1 cohort. Langerhans cells, the skin DCs, were evident at the junction of the epidermis and dermis and also in the dermis near vaccine cells in one vaccine site biopsy examined (Fig. 2C). Macrophages were also detected (Fig. 2D). Neutrophils and eosinophils, usually present near vaccine cells in the dermis, increased in abundance from <1 to 37.5% and from <0.5 to 15% of inflammatory cells, respectively, in skin biopsies from vaccine sites versus skin biopsies from similar locations obtained before vaccination. Eosinophils infiltrating the vaccine sites showed evidence of activation and massive levels of degranulation (Fig. 3). After repeated vaccinations, the fraction of eosinophils (assessed as the ratio of eosinophils to CD3+ T-cells) at vaccine sites increased. In areas of acanthosis, dural blood vessels manifest a striking eosinophilic vasculitis, with characteristic subendothelial eosinophils (Fig. 2F). Small blood vessels near vaccine cells were also typically surrounded by eosinophils, often accompanied by other mononuclear inflammatory cells, including CD3+ T-cells (Fig. 2E). CD3+ T-cells also appeared near DCs and macrophages recruited to vaccine sites. Frequent CD8+ T-cells were consistently present in the dermis following vaccination.

Induction of T-cell Immune Responses against Prostate Tumor Antigens. The T-cell epitopes that were recognized by infiltrating CD-3+ T-cells on newly induced DTH sites following vaccination with GM-CSF gene-transduced autologous prostate vaccines are not known at this time. Several new reports suggest that PCA patients may even have preexisting CD-4 and CD-8 T-cells against many potential PCA associated antigens, including PSA and PSMA (8, 52). In previous tumor vaccine studies, in which defined peptide antigens were not known, tumor antigen-specific T-cell responses were assessed using cutaneous DTH testing against antigens present in autologous tumor cells (26, 27, 53–58). For this study, irradiated autologous prostate tumor cells served as DTH target cells. These prostate tumor cells were propagated in serum-free media and cryopreserved in a buffer containing human serum albumin. Thus, the prostate tumor DTH cells were largely free of contaminating xenoproteins, such as collagenase or proteins present in bovine sera, which have been reported to confound interpretations of DTH skin tests in some other cancer vaccine trials (57). DTH reactivity was measured as the bidimensional extent of induration at the intradermal DTH injection site at 48 h.

Vaccinated men were subjected to prostate tumor DTH testing before vaccination, after three vaccine treatments, and at the end of the vaccine course. All vaccinated men were also subjected to DTH testing for reactivity against seven common recall antigens, as described previously (27). Each of the vaccinated men appeared immunocompetent, as evidenced by DTH reactivity against one or more of the recall antigens, before beginning treatment with PCA vaccines. In addition, all vaccinated men displayed normal levels of T-cell receptor κ chains in circu-

**Table 2 GM-CSF secretion by PCA vaccine cells following MFG-GM-CSF gene transfer**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Dose level</th>
<th>GM-CSF secretion ng/10⁶ PCA vaccine cells/24 h</th>
<th>Total vaccinations</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>143</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>197</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
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<td>349</td>
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<td>4</td>
<td>1</td>
<td>607</td>
<td>5</td>
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<tr>
<td>5</td>
<td>2</td>
<td>1403</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>240</td>
<td>3</td>
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<tr>
<td>7</td>
<td>2</td>
<td>520</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>233</td>
<td>3</td>
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</table>

*a* GM-CSF secretion before MFG-GM-CSF transduction, <0.2 ng/10⁶ PCA vaccine cells/24 h; average GM-CSF secretion after transduction, 461.5 ng/10⁶ PCA vaccine cells/24 h.

**Table 3 Toxicities accompanying vaccination with irradiated with GM-CSF-secreting autologous PCA cells**

<table>
<thead>
<tr>
<th></th>
<th>Dose level 1 (5 patients)</th>
<th>Dose level 2 (3 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully evaluable cycles</td>
<td>26</td>
<td>15</td>
</tr>
<tr>
<td>Systemic side effects</td>
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<tr>
<td>Low-grade fever (grade 2 or less)</td>
<td>0/26</td>
<td>2/15</td>
</tr>
<tr>
<td>Chills</td>
<td>0/26</td>
<td>3/15</td>
</tr>
<tr>
<td>Malaise</td>
<td>1/26</td>
<td>4/15</td>
</tr>
<tr>
<td>Acute vaccine site effects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection site erythema/swelling (grade 2 or less)</td>
<td>26/26</td>
<td>15/15</td>
</tr>
<tr>
<td>Site tenderness (1–3 days)</td>
<td>5/26</td>
<td>5/15</td>
</tr>
<tr>
<td>Pruritus (grade 2 or less)</td>
<td>3/26</td>
<td>10/15</td>
</tr>
<tr>
<td>Pain during injection</td>
<td>26/26</td>
<td>15/15</td>
</tr>
</tbody>
</table>

*a* Graded using NCI Common Toxicity Criteria.
lating CD3+ T-cells (not shown; see Ref. 59). Reactive prostate tumor DTH tests were present before vaccination in two of eight men. Following vaccination, seven of eight patients manifested positive DTH tests to challenge with irradiated, untransduced autologous prostate cell targets range (5–23 mm). Of the six of eight patients with no positive DTH to challenge with autologous prostate cells prior to vaccination, five of six became positive after treatment. In the case of the two of eight patients with a pretreatment positive DTH reaction to their autologous tumor prior to treatment, patient 3 (dose level 1) showed a 2-fold increase in induration (6 to 12 mm) following vaccination, whereas patient 5 (dose level 2) manifested no appreciable increase in DTH from 8 mm following vaccination. The study was too small to ascertain any statistically significant differences in DTH induction sizes associated with vaccine treatment at dose level 1 versus dose level 2. The magnitude of reactivity of DTH could not be clearly ascribed to total number of vaccinations or GM-CSF levels. The largest DTH conversion (3 to 25 mm) was observed in patient 8, who received only three vaccinations at dose level 2 (Table 2). Interestingly, the largest DTH reactivity appeared in patient 8, who had a history of clinical prostatitis.

Histopathological and immunohistochemical analysis of prostate tumor DTH site biopsies taken from two patients after vaccination revealed abundant inflammatory infiltrates not evident in skin biopsies obtained before vaccination. Perivascular cuffing by infiltrating lymphocytes, characteristic of DTH reactions, was present in all reactive DTH site biopsies (Fig. 4A). DTH site biopsies also disclosed ingress of macrophages (Fig. 4B) and of natural killer cells (Fig. 4C). Extensive eosinophil infiltrates and a subendothelial eosinophilic vasculitis, reminiscent of the vaccine site biopsies, were present in reactive DTH site biopsies taken after vaccination. T-cells were also present at DTH sites. In the DTH site biopsies, some 80% of the CD3+ T-cells expressed CD45RO, indicative of T-cell activation after vaccination (Fig. 4D). Similar to the vaccine site biopsies, the prostate tumor DTH site biopsies displayed an increasing abundance of eosinophils relative to T-cells as the vaccine treatment course proceeded. Few B-cells were evident at the DTH sites.

Pre- and postvaccine peripheral blood samples were also tested for proliferation and cytokine release (IL-2, GM-CSF, and γ IFN) in response to recombinant PSA, using candida as a positive control. All patients demonstrated responsiveness to candida, but none showed evidence of PSA-specific T-cell recognition. Due to low yields of

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Fig. 2. Vaccine site biopsy photomicrographs showing skin infiltration by immune effector cells 72 h after vaccination. Immunohistochemical staining for various antigens was accomplished as described in “Materials and Methods.” A, inflammatory cell infiltrates apparent after H&E staining; B, cytokeratin-positive vaccine cells; C, CD1a-positive DCs in the epithelium and dermis; D, CD68-positive macrophages; E, CD3-positive T-cells; F, eosinophils, neutrophils, and other mononuclear inflammatory cells evident after H&E staining.

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autologous tumor cells in early passage, autologous tumor cells were
not available to assess the priming of responses to undefined tumor-
associated antigens.

**Induction of B-Cell Immune Responses against Prostate Tumor Antigens.** Although few B-cells appeared to be present at prostate
tumor vaccine sites or at prostate tumor DTH sites, immunoblot
analyses using sera from the eight treated men, obtained before and
after vaccination, disclosed the appearance of increased titers of
antibodies recognizing prostate tumor antigens in sera from three of
the men as a consequence of vaccine treatment (Fig. 5, patients 1, 6,
and 7). New antibodies at 1:1000 titer were observed in patients 1 and
6 (dose level 1) and patient 7 (dose level 2), 2 weeks following final
vaccination (Fig. 5). All three of these patients had negative DTH skin
reactivity to challenge with autologous prostate cells prior to vacci-
nation with GM-CSF gene-transduced PCA vaccines. The induced
immunoglobulins recognized polypeptides of 26, 31, and 150 kDa in
extracts from LNCaP PCA cells (Fig. 5). The 150-kDa polypeptide
was expressed by both normal and neoplastic prostate epithelial cells
and by prostate smooth muscle cells, but not by prostate stromal cells,
lung fibroblasts, or WBCs (Fig. 6A). The 150-kDa polypeptide was
also expressed by a number of human cancer cell lines (Fig. 6B).
Characterization of the precise epitope(s) recognized by the induced
antibodies is ongoing. Using the same immunoblot analysis approach,
no antibodies against PSA were detected (not shown). Despite the
induction of new antibodies in three of eight patients, none of the eight
patients developed any clinically detectable lymphadenopathy.

**DISCUSSION**

The results of the Phase I clinical trial of irradiated GM-CSF-secreting
autologous prostate tumor vaccine therapy indicated that such treatment
was safe and induced both B-cell and T-cell immune responses against
PCA cell-associated antigens. A major difficulty for future clinical de-
velopment of this autologous treatment approach was the low yield of
autologous PCA vaccine cells recovered (often $5 \times 10^7$ or less) using cell
culture approaches to expand PCA cell numbers. This approach appears
clinically impractical for large Phase II studies to assess efficacy given
the cell culture technology of primary cultures used in this study. Retro-
viral transfer of GM-CSF cDNA to permit high level secretion of GM-
CSF was not limiting. Nonetheless, with limited numbers of autologous
PCA vaccine cells likely available from most men with localized PCA,
and no PCA vaccine cells likely available from most men with advanced
PCA, exploration of higher vaccine doses and different vaccination
schedules in future clinical trials will not be possible. Allogeneic PCA
vaccines, prepared from PCA cell lines genetically modified to secrete
high levels of GM-CSF, may offer a solution to this clinical development
problem. Preclinical studies have suggested that antigens from irradiated
GM-CSF-secreting cancer vaccine cells are presented to T-cells on MHC
molecules expressed by host antigen-presenting cells (see Refs. 60 and
61); such vaccine cells thus do not necessarily need to be MHC-matched
with host T-cells to be effective at eliciting potent antitumor immunity.
Rather, the vaccine cells need to express tumor antigens expressed by
metastatic PCA clones in patients, and they need to secrete sufficient
paracrine GM-CSF to recruit bone marrow-derived antigen-presenting
cells (DCs and macrophages) to sites of vaccination (60, 61). The PCA

![Fig. 3. Eosinophil degranulation at the vaccine site. Vaccine site biopsy obtained 72 h after vaccine inoculation was stained for eosinophil major basic protein (MBP) using an immunohistochemical technique (see Materials and Methods).](image)

![Fig. 4. Infiltration of immune effector cells at DTH reaction sites at 48 h. Immunohistochemical staining for immune effector cell antigens was accomplished as described in Materials and Methods. A, vasculitis evident by H&E staining; B, CD68-positive macrophages; C, CD56-positive natural killer cells; D, CD45RO-positive T-cells. These photomicrographs are representative of post-vaccination DTH cellular infiltrates against autologous prostate cell targets at both dose levels.](image)
cell lines LNCaP and PC-3, which contain a number of common PCAs, express the newly identified 150-kDa polypeptide recognized by sera from men treated with autologous PCA vaccines. Based on data from this Phase I trial, a Phase II clinical trial of irradiated GM-CSF-secreting LNCaP and PC-3 vaccines, using vaccine cell doses higher than that possible with autologous PCA vaccines, is currently under way. GM-CSF gene-transduced allogeneic PCA vaccines appear to permit long schedules of booster vaccinations, given the inexhaustible supply of the permanently established tumor cell lines.7

Tumor-specific cytotoxic CD8+ T-cells recognize tumor antigens presented on class I MHC molecules to target cancer cells for destruction. For many men with advanced PCA, class I MHC low or negative PCA cells may be present (62–65). Such cells would likely be difficult to eradicate with tumor-specific cytotoxic CD8+ T-cells alone. Fortunately, Hung et al. (28) have recently reported that vaccination with irradiated GM-CSF-secreting cancer vaccines activate tumor-specific CD4+ T-cells to simultaneously produce Th1 and Th2 responses. Both CD8+ T-cell effectors and non-MHC-dependent immune effectors of antitumor immunity are elicited by GM-CSF gene-transduced vaccine treatment. Immune response data collected during the conduct of the Phase I trial of irradiated GM-CSF-secreting PCA vaccines supports in man the conclusions Hung et al. (28) derived in mice. Notably, DCs and macrophages were recruited for antigen processing by paracrine GM-CSF secretion at tumor vaccination sites (Figs. 2 and 3). Following PCA antigen presentation in vivo, postvaccination reactive DTH site biopsies indicated the participation of degranulating eosinophils as well as circulating T-cells in vaccination-induced immune responses to autologous PCA cell DTH tar-

gets (Fig. 4). Architecturally, the concerted immunological response appears to include eosinophil accumulation and degranulation in the subendothelial space of small blood vessels immediately adjacent to depositions of DTH tumor antigen. Identical pictures of postvaccination DTH response to autologous untransduced tumor target cells have been reported in clinical trials of renal cell carcinoma and melanoma using GM-CSF gene-transduced autologous tumor vaccines (26, 27).

GM-CSF gene-transduced PCA vaccines increased antibody titers against prostate tumor cell line-associated antigens. This suggests that B-cells participated in the immune response following treatment. Increasing titers of antibodies to PCA antigens were detected among three of the men treated with irradiated GM-CSF-secreting autologous PCA cell vaccines. These antibody responses are reminiscent of antibody responses reported for a clinical trial of irradiated GM-CSF-secreting autologous melanoma cell vaccines (26). To our knowledge, this is the first report of induction of new antibody responses to PCA antigens in patients using cytokine gene-modified tumor vaccines, peptide-pulsed DCs, or any other strategy of immunotherapy. One of the antigen epitopes, present in a 150-kDa polypeptide, was expressed in normal prostate epithelial cells, as well as in PCA cells. However, it is unclear why the majority of patients (5 of 8) did not elicit detectable antibodies following treatment. The lack of yield of autologous PCA cells has not permitted testing of these antibodies against antigens expressed by each patient’s tumor. Of interest, the newly identified 150-kDa antigen was also expressed by a number of non-PCA human cancer cell lines. The molecular identification of the 150-, 31-, and 26-kDa polypeptides containing antigenic epitopes recognized by sera from vaccinated men is currently under way.8 It will be critical to define the PCA tumor-associated antigens for B-cells and T-cells in future clinical trials using new, sensitive techniques for antigen discovery.

GM-CSF gene-transduced PCA vaccines represent only one of several new approaches to active specific immunotherapy of PCA, which are in early clinical development. Examples of new approaches involve vaccinations with defined prostate-specific antigens, such as PSA (66), vaccinations with carbohydrate antigens (67), vaccinations with vaccinia vectors expressing prostate-specific peptides (69) or even defined prostate-specific peptides (69) or even amplified tumor RNA (70). Some of these approaches in Phase I studies have reported anecdotal evidence of clinical activity (67, 69). To our knowledge, this is the first report of new antibody responses to normal prostate epithelial antigens following immunotherapy. In addition, despite the limitations in autologous cell yields, which compromised dose escalation, the expression of GM-CSF in the context of autologous PCA vaccine cells did recruit antigen-presenting cells at the vaccination site and induce systemic T-cell responses at DTH sites histologically identical to those discovered in mice treated with GM-CSF gene-transduced vaccines. Extensive clinical efficacy testing of each of these approaches is required. Our data, and data from other approaches, now seem to suggest that systemic immune responses to human PCA can be generated against a tumor type that has been conventionally viewed as being nonimmunogenic, and refractory to immunotherapy. Efficacy testing appears particularly justified as adjuvant therapy, in which immunotherapy is most favored at effecting curative antitumor immune responses following surgery or radiation therapy.

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