

# Immunization by Particle-Mediated Transfer of the Granulocyte-Macrophage Colony-Stimulating Factor Gene into Autologous Tumor Cells in Melanoma or Sarcoma Patients: Report of a Phase I/IB Study

D.M. MAHVI,<sup>1,2</sup> F.-S. SHI,<sup>1</sup> N.-S. YANG,<sup>2</sup> S. WEBER,<sup>1</sup> J. HANK,<sup>2</sup> M. ALBERTINI,<sup>2,3</sup> J. SCHILLER,<sup>2,3</sup> H. SCHALCH,<sup>2</sup> M. LARSON,<sup>2</sup> L. PHARO,<sup>2</sup> J. GAN,<sup>2</sup> D. HEISEY,<sup>1</sup> T. WARNER,<sup>2,4</sup> and P.M. SONDEL<sup>2,5</sup>

## ABSTRACT

The primary objective of this phase I study was to determine the safety of an autologous tumor vaccine given by intradermal injection of lethally irradiated granulocyte-macrophage colony-stimulating factor (GM-CSF) gene-transfected autologous melanoma and sarcoma cells. Secondary objectives included validation of the gene delivery technology (particle-mediated gene transfer), determining the host immune response to the tumor after vaccination, and monitoring patients for evidence of antitumor response. Sixteen patients were treated with either of two different doses of GM-CSF-treated tumor cells. One patient received treatment with both doses of tumor cells. No treatment-related local or systemic toxicity was noted in any patient. Patients administered 100% treated cells (i.e., with a preparation of tumor cells that had all been exposed to GM-CSF DNA transfection) had a more extensive lymphocytic infiltrate at the vaccine site than did patients given 10% treated cells (a preparation of tumor cells in which 10% had been exposed to GM-CSF transfection) or non-treated tumor. The generation of a systemic immune response to autologous tumor by a delayed-type hypersensitivity response to the intradermal placement of nontransfected tumor cells was noted in one patient. One patient had a transient partial response of metastatic tumor sites. The entire procedure, from tumor removal to vaccine placement, was accomplished in less than 6 hr in all patients. Four of 17 patient tumor preparations produced greater than 3.0 ng of GM-CSF per 10<sup>6</sup> cells per 24 hr *in vitro*. The one patient with greater than 30 ng of GM-CSF per 10<sup>6</sup> cells per 24 hr *in vitro* had positive DTH, a significant histologic inflammatory response, and clinically stable disease. This technique of gene transfer was safe and feasible, but resulted in clinically relevant levels of gene expression in only a minority of patients.

## OVERVIEW SUMMARY

We investigated in a phase I/IB clinical trial the safety of vaccination with autologous tumor cells transfected with GM-CSF cDNA by particle-mediated gene transfer. No treatment-related toxicity was noted. A dose-dependent inflammatory response occurred at the vaccination site. A systemic immune response was detected in the patient treated with the tumor preparation showing the highest level of GM-CSF protein expression in this study.

## INTRODUCTION

GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF) has been widely used as a myelostimulatory agent (Metcalf, 1986). It also induces an antitumor effect in some cancer patients treated with GM-CSF protein (Rini *et al.*, 1998; Leong *et al.*, 1999). The mechanism of this effect is incompletely understood but is probably due to activation of macrophages as measured by the production of greater levels of membrane-bound and secreted interleukin 1 (IL-1), greater lev-

<sup>1</sup>Department of Surgery, University of Wisconsin School of Medicine, Madison, WI 53792.

<sup>2</sup>University of Wisconsin Comprehensive Cancer Center, Madison, WI 53792.

<sup>3</sup>Department of Medicine, University of Wisconsin School of Medicine, Madison, WI 53792.

<sup>4</sup>Department of Pathology, University of Wisconsin School of Medicine, Madison, WI 53792.

<sup>5</sup>Department of Pediatrics, University of Wisconsin School of Medicine, Madison, WI 53792.

els of Fc receptor (FcR) expression, and an increase in their ability to phagocytose opsonized sheep red blood cells *in vitro* (Kleinerman *et al.*, 1988; Morrissey *et al.*, 1988). Macrophages and monocytes stimulated by GM-CSF also produce secondary cytokines such as tumor necrosis factor, IL-1, and IL-6, which may result in local stimulation of other immune effector cells and enhancement of their ability to present antigen (Grabstein *et al.*, 1986; Thomassen *et al.*, 1991).

Preclinical comparisons indicate that the cDNA for GM-CSF is also effective when transfected into autologous tumor cells and utilized as a vaccine (Dranoff *et al.*, 1993). Multiple methods of tumor cell transfection with DNA for immunostimulatory proteins, such as GM-CSF, have been tested. Particle-mediated gene transfer (PMGT) can effectively induce expression of transfected genes, even in nondividing cells, and does not use any potentially infectious agent. Immunization of mice with irradiated murine melanoma cells transfected (by PMGT) with GM-CSF cDNA protected 58% of vaccinated animals from tumor development after subsequent challenge with parental non-irradiated tumor cells. In contrast, only 4% of mice similarly challenged with parental tumor after immunization with control transfected (luciferase cDNA) tumor survived the same tumor challenge (Mahvi *et al.*, 1996).

Other than surgical resection, there is no standard therapy for patients with metastatic melanoma and soft tissue sarcoma (Herrmann, 1995). Despite the many chemotherapy and biologic trials involving melanoma patients, no single therapeutic approach is presently considered the "standard of care," except in the case of isolated regional node disease. Immunotherapy has become a major focus of investigational treatment of metastatic melanoma as evidence has accumulated that the immunostimulatory agent IL-2, or vaccination with tumor-associated antigens, can cause tumor regression in some patients (Balch *et al.*, 1993; Brichard *et al.*, 1993; Crowley and Seigler, 1993; Hellman, 1994; Kawakami *et al.*, 1994a,b; Rosenberg *et al.*, 1994).

The results of nonsurgical treatment of sarcoma patients have been even more discouraging. Forty to 60% of soft tissue sarcomas are predicted to recur systemically irrespective of effective local-regional control, and will result in death from metastatic disease (Storm and Mahvi, 1991; Ezzell, 1995). A multitude of promising chemotherapy agents have been subjected to postoperative adjuvant therapy trials over the past two decades. Several of these agents have shown substantial activity in adults with metastatic soft tissue sarcoma (20–40% tumor stabilization or regression). However, these agents (alone or in combination) have not reduced the incidence of metastases, or improved survival for adult patients with intermediate or high-risk (grade II and III) tumors (Zalupski *et al.*, 1993).

PMGT was selected for this study for several reasons potentially important for the application of gene therapy to more widespread clinical use. These include the ability to (1) physically target gene expression to a tissue, (2) transfect resting, nondividing cells, irrespective of cell cycle stage or lineage, (3) circumvent the need to culture tissue before transfection, (4) potentially enable direct *in vivo* gene delivery at many clinical locations, using gene transfer materials readily stored and delivered with simple reagents and equipment, and (5) allow multiple genes to be inserted into the same cell (McCabe *et al.*, 1988; Yang *et al.*, 1990; McCabe and Martinell, 1992; Burk-

holder *et al.*, 1993; Cheng *et al.*, 1993; Thompson *et al.*, 1993; Yang and Sun, 1995; Albertini *et al.*, 1996). Thus, PMGT potentially overcomes many application problems currently associated with other methods of gene transfer.

The primary objective of this phase I study was to determine the safety of a GM-CSF gene-transfected autologous tumor vaccine given by intradermal injection of lethally irradiated GM-CSF gene-transfected autologous melanoma and sarcoma cells. Secondary objectives included validation of the gene delivery technology, monitoring of patients for evidence of antitumor immune responses, characterization of infiltrating immune effector cells in the vaccine sites by histology, and monitoring of patients for evidence of an objective antitumor response.

## MATERIALS AND METHODS

### *Patient eligibility*

Patients with a histologically confirmed diagnosis of metastatic melanoma or soft tissue sarcoma, which was surgically or medically incurable, were eligible for this study. Patients were eligible if they met the following criteria: they were 18 years of age or older, they were not pregnant, they were not currently treated with steroids, they had not been treated by cytotoxic chemotherapy or biologic therapy for 1 month, and they had at least a 1-cm tumor nodule that could be surgically excised for vaccine preparation. Patients with melanoma were required to have disease distant from the primary site and ipsilateral regional nodes. Any patient with soft tissue sarcoma was eligible. No requirement for residual measurable disease after resection for vaccine preparation was necessary; thus, some patients were rendered NED (no evidence of disease) by the tumor resection for vaccine preparation.

### *Study design*

Sixteen patients were entered into this clinical trial. One patient was entered into treatment group 1 as well as treatment group 3 at the time of subsequent disease progression at a different site (i.e., a total of 17 tumor preparations were done). Six patients were entered into group 1, six patients were entered into group 2, and five patients were enrolled into group 3. A planned sixth patient was not enrolled in group 3 because of unavailability of the gene preparation. For patients in groups 1 and 2, vaccination consisted of two intradermal injections ( $1 \times 10^7$  cells per site) of GM-CSF-transfected tumor cells into the upper extremity on the day of tumor biopsy. The GM-CSF dose was controlled by altering the percentage of transfected cells in the vaccine. Patients in group 1 received a vaccine consisting of 10% tumor cells exposed to PMGT with GM-CSF cDNA, mixed with 90% nontransfected tumor cells. Group 2 patients received a vaccine consisting of tumor cells, all of which had been treated with GM-CSF DNA plasmid by PMGT. The local dose of GM-CSF in group 1 patients was thus 10% of the dose in group 2 patients, but the dose of autologous tumor was held constant. This second treatment group received GM-CSF doses in this vaccine predicted to be comparable to an efficacious dose as defined in murine trials of a melanoma cell vaccine transfected with the GM-CSF gene *ex vivo* by PMGT (Dranoff *et al.*, 1993; Mahvi *et al.*, 1996). The percentage of cells

treated with GM-CSF DNA by PMGT, rather than the amount of DNA plasmid delivered, was the dosing variable, so that the number of tumor cells was held constant between groups 1 and 2. Group 3 patients received the same initial treatment as group 2 and were eligible to receive up to two more monthly treatments at the higher dose (using cryopreserved tumor from the original day 0 biopsy), if no progressive disease was noted.

#### *Vaccine preparation and placement*

Surgical tumor specimens were processed and vaccination was performed on the same day of biopsy or resection for all group 1 and 2 patients. Freshly resected tumor specimens were taken to the vaccine preparation laboratory and immediately dispersed in an enzyme solution consisting of collagenase, hyaluronidase, and DNase I after mincing the specimen with scissors into small fragments (less than 1 mm in diameter) in the presence of the enzyme solution, and incubated with gentle stirring. Undigested tissue and debris were separated from single cells and small cell clumps by use of a 30-gauge mesh stainless steel screen. This procedure was repeated up to three times if necessary. The cell suspension was then passed through a sterile 70- $\mu$ m nylon cell strainer to generate a single-cell population. The suspension was then counted. An aliquot of the tumor preparation was placed on a slide, fixed, and stained. The percentage of tumor cells was determined by an experienced cytopathologist. The pooled cells were then irradiated (10,000 cGy) in a  $^{137}\text{Cs}$   $\gamma$  irradiator. Immediately after irradiation the cells were subjected to gene transfer by particle-mediated gene transfer in groups 1 and 2. The surgical specimens of group 3 patients were processed to the stage of a single-cell suspension, aliquoted, and then frozen in liquid nitrogen before the irradiation step.

The cDNA for human GM-CSF was inserted into a human expression vector as previously described (Mahvi *et al.*, 1997). The plasmid DNA was precipitated onto 0.95- $\mu$ m-diameter gold particles and the DNA-gold particle preparation was coated onto the inner surface of Tefzel tubing. The GM-CSF DNA-coated gold particles were delivered to tumor cells with a hand-held helium gas-driven particle-mediated gene delivery device (PowderJect [Madison, WI] gene gun delivery device). Tumor cell samples in suspension were immediately transfected with a 250-lb/in<sup>2</sup> helium pulse. The treated vaccine cells were washed twice and resuspended in 0.2 ml of saline, split into two aliquots, and injected into two separate intradermal sites. Group 1 patients received two intradermal injections of a vaccine, each consisting of  $10^6$  cells treated by PMGT and  $9 \times 10^6$  nontransfected cells (2 mg of gold and 2.5  $\mu$ g of DNA). Group 2 patients received at each site two intradermal injections of  $10^7$  autologous tumor cells, all of which had been treated by PMGT (20 mg of gold and 25  $\mu$ g of DNA). Within 6 hr of surgical removal, the treated cells were injected intradermally for all patients in groups 1 and 2. On the day of vaccination for group 3 patients, their cryopreserved tumor cells were thawed, washed, irradiated, and then transfected. Each treatment for group 3 patients consisted of one intradermal vaccination with  $10^7$  autologous tumor cells, all of which had been treated by PMGT (10 mg of gold and 12.5  $\mu$ g of DNA). Group 3 patients without tumor progression were eligible for an identical second and third vaccination after 4 and 8 weeks. One of the five group 3 pa-

tients progressed within 4 weeks and two of five progressed within 8 weeks. One group 3 patient thus received two vaccine treatments and three received three vaccine treatments.

The efficiency of gene expression after cDNA delivery by this method is dependent on several factors, including the degree of gold particle adhesion to the tubing, the acceleration of the beads by the gas pulse, the ability to traverse the cell membrane, and the ability of the cells to produce and secrete the protein. A total of four batches of DNA-gold bead-tube preparations were manufactured for this trial. These DNA preparations were monitored by *in vitro* transfection of Chinese hamster ovary (CHO) cells and autologous tumor cells with determination of GM-CSF protein levels in the supernatant by enzyme-linked immunosorbent assay (ELISA), using a commercially available kit (R&D Systems, Minneapolis, MN).

#### *Posttreatment evaluation*

The local and systemic toxicity of the vaccination was evaluated on days 1, 2, 3, 8, 15, 25, and 28, and at 3, 6, and 12 months, and then yearly postvaccination by laboratory monitoring and physical examination focused on the vaccine site. Patients in group 3 had additional evaluation at the time of subsequent vaccine placement. An added potential safety benefit of this method of gene therapy was the ability to potentially excise the vaccine site in patients who might develop life-threatening toxicity. No patient required vaccine site removal except as scheduled for monitoring of the local inflammatory response to the vaccine. The effect of vaccine cell delivery to the local site was measured by evaluation of vaccine site biopsy on days 3 and 14 postvaccination. Biopsy specimens were divided into two pieces of equal size and snap frozen. One part of the biopsy was prepared for hematoxylin-eosin (H&E) staining and evaluated by microscopy. Quantitation of the local response in the biopsy specimen was performed semiquantitatively by the same technique used to quantitate inflammatory cells found in cardiac transplant biopsy specimens (McAllister, 1995). The number of lymphocytes in one microscopic section through the vaccine site was recorded without knowledge of the patient or vaccine site treatment. Results were analyzed with repeated measured analysis of variance on the log-transformed data. Lymphocytes were responsible for distant antitumor responses in preclinical murine depletion models and thus were the focus of the examination (Mahvi *et al.*, 1996). Protein was extracted from the remaining portion of the biopsy tissue after homogenization. GM-CSF levels in serum, homogenized tissue sites, as well as *in vitro* tissue culture supernatants were determined by ELISA. Serum GM-CSF levels were obtained on days 1, 3, 7, and 14 postvaccination.

On days 3 and 14 postvaccination, patients in groups 1 and 2 underwent complete surgical excision of the two vaccination sites to assess GM-CSF production and infiltration of immune effector cells. In the group 2 patients, an aliquot of  $10^7$  irradiated, nontransfected, freshly prepared tumor cells was also injected intradermally at the time of vaccine placement, and these injection sites were also completely removed on either day 3 or day 14. This provided a nontransfected control site. On day 25, all patients underwent delayed-type hypersensitivity (DTH) testing by intradermal injection in their opposite extremity of  $5 \times 10^6$  irradiated nontransfected autologous tumor cells that

had been cryopreserved at the time of vaccine preparation. DTH testing also included intradermal injection of  $5 \times 10^6$  peripheral blood autologous lymphocytes (exposed to enzyme treatment and cryopreserved on day 0), as well as standard preparations of mumps antigen and *Candida* antigen. These injection sites were assessed on day 28 postvaccination. Restaging of the patients' disease in addition to long-term toxicity evaluation was performed at 1, 3, 6, and 12 months and then yearly.

## RESULTS

### Tumor vaccine preparation

Nineteen eligible patients were identified. Three of the 19 underwent biopsy, but processing of their tumor biopsies yielded an inadequate number of tumor cells for vaccine preparation (fewer than  $3.2 \times 10^7$  cells after enzymatic dispersal) and thus had no vaccine placement. The other 16 patients all had placement of transfected tumor cells. The yield of tumor cells after *in vitro* processing varied widely, as did the concentration of viable tumor and inflammatory cells in the tumor cell preparations (Table 1).

The GM-CSF expression of CHO cells after PMGT with GM-CSF cDNA served as a means of quality control between five lots of GM-CSF DNA plasmid (Table 2). The first two lots (patients 1–5) resulted in comparable GM-CSF expression. The last three lots (patients 6–17) generated somewhat higher (not statistically significant) GM-CSF levels in the CHO cell assay.

The ability of cDNA-treated tumor cells to express the transgenic protein was evaluated by *in vitro* culture of an aliquot of

GM-CSF cDNA-treated tumor cells. The amount of GM-CSF protein expressed after *in vitro* (24 hr) culture of an aliquot of the transfected tumor varied widely, with lower levels generally in the group 1 patients. The increase in the expression of GM-CSF for tumor specimens from patients 6–12 is only partially explained by lower GM-CSF expression found in the CHO transfectants in the first two lots (patients 1–5). Group 3 patients, however, had lower levels of GM-CSF expression than group 2 patients in the 24-hr *in vitro* tumor culture despite good expression in the CHO cell assay. When the percentage of tumor cells in the tumor preparation was less than 50%, GM-CSF expression was always less than 1 ng/ $10^6$  cells per 2 hr.

### Evaluation after vaccine placement

The amount of GM-CSF protein detected at the vaccine site was also monitored by ELISA of the homogenized tissue extract obtained at the biopsy site. The amount of GM-CSF detected in group 1 patients (10% PMGT-treated tumor mixed with 90% nontreated tumor) was similar on days 3 and 14 and did not correlate with the *in vitro* culture data (Table 2). Group 2 patients (100% PMGT-treated tumor) also received a single intradermal injection of  $10^7$  irradiated autologous tumor cells not treated by PMGT in an effort to determine the background level of GM-CSF expression detectable at the vaccine site of nontransfected tumor cells. Among the group 2 patients, local GM-CSF expression at the site of GM-CSF-transfected cells was always higher on day 3 than on day 14. The background expression of GM-CSF in the nontransfected tumor vaccine sites varied, but more GM-CSF was always detected at the site of the transfected tumor cell vaccine. The amount of GM-CSF

TABLE 1. VACCINE PREPARATION<sup>a</sup>

Patient	Tumor weight (g)	Cell yield ( $\times 10^6$ )	Tumor cells (%)	Tumor type	Site of tumor removed for vaccine preparation
1	6.02	37	68	Melanoma	Axillary lymph node
2	8.87	710	74	Leiomyosarcoma	Supraclavicular lymph node
3	4.33	177	76	Melanoma	Axillary lymph node
4	4.50	286	81	Melanoma	Subcutaneous mass
5	18.8	62	95	Leiomyosarcoma	Retroperitoneal mass
6	10.0	51	95	Liposarcoma	Retroperitoneal mass
7	11.4	210	89	Melanoma	Small bowel
8	3.1	285	85	Melanoma	Small bowel
9	12.4	1700	96	Leiomyosarcoma	Retroperitoneal mass
10	4.58	174	97	Melanoma	Subcutaneous mass
11	2.23	204	10	Melanoma	Supraclavicular mass
12	0.65	65	74	Melanoma	Solitary lung nodule
13	17.0	348	45	Sarcoma	Intraabdominal
14	3.64	113	7	Leiomyosarcoma	Retroperitoneal
15	3.0	160	36	Melanoma	Subcutaneous
16	4.6	564	25	Melanoma	Iliac node
17	4.3	106	44	Melanoma	Neck node

<sup>a</sup>All tissue obtained for vaccine preparation was weighed, physically and enzymatically dispersed, and the number and type of cells determined by cell counting, and by cytological analysis of cytocentrifuge preparations. Three patients (not shown) did not have a sufficient number of tumor cells (greater than  $3.2 \times 10^7$ ) and thus did not receive vaccination. Patient 2 and patient 14 are the same patient treated with cells derived from different sites 15 months apart.

TABLE 2. GM-CSF EXPRESSION<sup>a</sup>

Patient	Vaccine site (pg/site)		Serum (pg/ml)	Tumor <i>in vitro</i> (ng/10 <sup>6</sup> cells)	CHO <i>in vitro</i> (ng/10 <sup>6</sup> cells)
	Day 3	Day 14			
1	5.8	3.5	0	0.40	127
2	0	0	0	0.07	49
3	34.3	12.4	0	3.22	169
4	48.6	99.8	10.7	0.49	286
5	107.6	107.9	0	0.06	314
6	111.7	94.5	0	0.13	388
7	243.9	37.2 (29.0)	0	1.90	364
8	185.5 (30.9)	42.0	0	32.8	979
9	3888	33.5 (2.7)	0.4	8.62	723
10	88.1 (33.5)	37.2	3.4	3.50	628
11	55.8	22.2 (12.4)	0.9	0.34	901
12	839.2 (50.4)	307.3	0	3.56	599
13				0.64	794
14				0.5	1148
15				0.58	727
16				0.07	1014
17				0.02	545

<sup>a</sup>The expression of GM-CSF protein was determined at the site of vaccine placement, in the serum (24 hr after vaccine placement), in the supernatant of 10<sup>6</sup> cultured GM-CSF transfected autologous tumor cells (after 24 hr of culture), and in the supernatant of cultured GM-CSF-transfected CHO cells (after 24 hr of culture). For patients in group 2 (patients 7–12), each patient also received one injection of 10<sup>7</sup> nontransfected autologous tumor cells. These were biopsied either on day 3 or day 14, and these specimens were processed as negative controls. The GM-CSF values for these negative control sites are shown in parentheses at the time they were evaluated for each of patients 7–12. No biopsies or serum samples were obtained in group 3 patients.

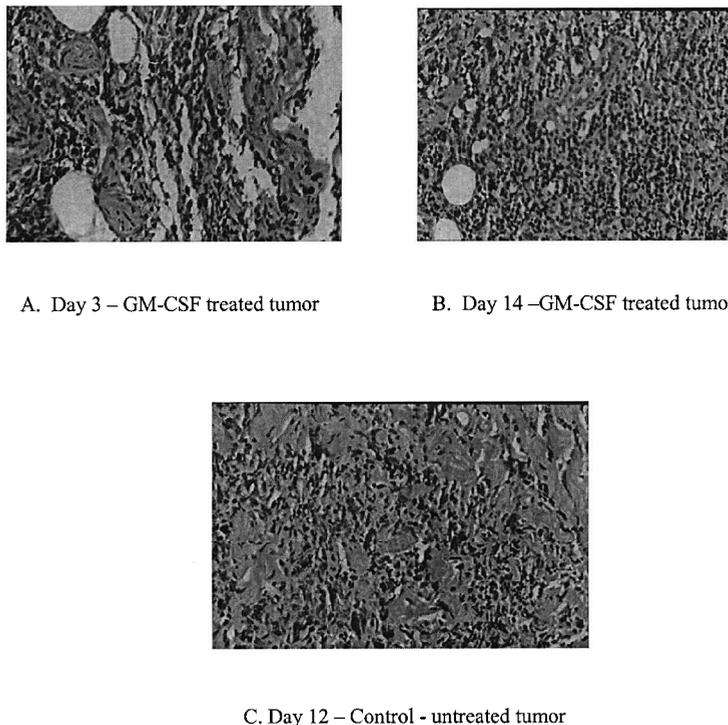
expression at the vaccine site did not, however, correlate with the *in vitro* cell assay. This may be due to division of the biopsy sample for both histology and ELISA. Group 3 patients did not undergo vaccine site biopsy.

The safety of this approach to gene therapy was the major end point of this study. No local or systemic vaccine-related toxicity was noted during the 14 days the vaccination was in place in any patient in groups 1 and 2. No local or systemic vaccine-related toxicity was noted in group 3 patients with a minimum of 3 months of follow-up. A transient decrease in circulating lymphocyte levels meeting criteria for grade II or III toxicity (National Cancer Institute [NCI, Rockville, MD] common toxicity criteria) was noted in 6 of 12 patients. These were not reflected in decreased total white blood cell counts. No infections were noted, and no correlation with GM-CSF levels detected in the *in vitro* autologous tumor cell preparation was noted. One patient developed an elevated anti-double-stranded DNA antibody at the 3-month time point, which had previously been negative. The patient was asymptomatic, with no signs of autoimmune or other toxicity. No other significant change was noted in anti-nuclear antibody titer, anti-single-stranded DNA antibody titer, rheumatoid factor, or anti-thyroid antibody titer in the patient with an abnormal double-stranded DNA value or any other patient.

The local immune response to the vaccine in group 1 and 2 patients was monitored by examination of the tumor site for erythema and by biopsy of all vaccine sites at two time points (day 3 and day 14). In group 2 patients, a third biopsy of non-

transfected tumor was also performed (Figs. 1 and 2). One patient had a local inflammatory response at a GM-CSF-treated tumor vaccine site (erythema greater than 5 mm) on day 3 and one patient showed inflammation on day 14 (Table 3). The number of lymphocytes in the biopsy site was greater in group 2 patients compared with group 1 patients. Because of the small sample size, all the group 1 biopsies (3 and 14 day) were compared with all group 2 biopsies (3 and 14 day) of transfected cell vaccine sites. Overall, group 2 patients had a more extensive lymphocytic infiltrate than group 1 patients ( $p = 0.0458$ ). For the six biopsies from control (non-PMGT-treated) vaccination sites, the degree of lymphocytic infiltration was significantly lower than the lymphocytic infiltration seen at the PMGT-treated vaccine sites ( $p = 0.0313$ ). This indicates that the local dose of GM-CSF protein in the patients treated with 100% PMGT-treated tumor generated a greater inflammatory response than in patients treated with 10% PMGT-treated tumor or nontreated control tumor. Routine T cell monitoring was not performed in this study. The primary goal of the study was to determine safety and feasibility. Subsequent studies of cytokine gene therapy will be routinely monitored with intracellular cytokine expression assays.

The generation of a systemic antitumor response was also examined by intradermal injection of nontransfected tumor cells on day 25 (Table 4). Erythema exceeding 5 mm in response to tumor cell DTH injection, without a similar reaction to intradermal autologous peripheral blood lymphocyte (PBL) DTH injection, was noted in one patient (patient 8). Three patients (pa-



**FIG. 1.** Photomicrograph of three separate hematoxylin and eosin stained vaccine site biopsies in patient 7. Day 3, GM-CSF-treated tumor; day 14, GM-CSF-treated tumor; day 12, control (untreated tumor). The lymphocytic infiltrate is greater on day 14 and more intense at the site of GM-CSF-treated tumor. Residual pigment confirms that the site of biopsy previously contained tumor cells.

tients 3, 9, and 13) did demonstrate erythema in response to tumor cells but also to PBLs, indicating the importance of a control preparation to account for potential reactivity to the enzymes used in tumor disaggregation in evaluating DTH results.

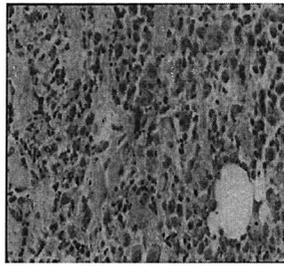
Clinical evaluation was also performed at 1, 3, 6, and 12 months and yearly after vaccine placement (Table 5). At the 1-month evaluation, 5 of 10 patients with measurable disease showed stable disease. One patient (patient 2) had a transient decrease in paraaortic nodal disease, although this eventually progressed after 12 months. At the time of disease progression (15 months after initial treatment) patient 2 was reevaluated, met the criteria for study entry, and was retreated (patient 14). No clinical response was detected after the second treatment. One patient (patient 8) with rapidly progressive disease before this treatment (brain metastasis requiring resection with residual disease after resection, and two resected small bowel metastatic lesions) has not developed progressive disease and currently has stable disease 5 years after vaccination. Interestingly, this patient had the highest GM-CSF protein expression in the *in vitro* autologous tumor cell assay and was the only patient with a positive DTH response to tumor. With an average of more than 5 years of follow-up (minimum time since treatment is 4.5 years), 14 patients have died, 1 has progressive disease, and one remains NED.

## DISCUSSION

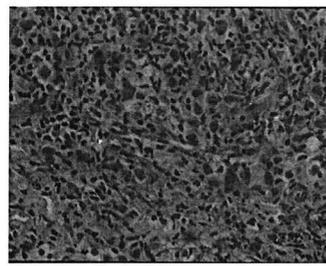
The delivery of cytokine genes into autologous tumor cells has resulted in a vaccine that provides protection from subse-

quent tumor challenge in murine model systems (Dranoff *et al.*, 1993; Yang and Sun, 1995). This study was focused on GM-CSF, based on the superior protection from subsequent B16 melanoma challenge of mice vaccinated with B16 melanoma cells transduced with GM-CSF compared with several other cytokine genes (Sun *et al.*, 1995). This method of generating high local cytokine concentrations is more cumbersome than is administration of systemic cytokine, but expression of the cytokine gene by the tumor cell provides superior vaccine-induced immunization than does direct injection of the cytokine protein at the vaccine site, as well as a lack of systemic toxicity (Shi *et al.*, 1999). The extrapolation of this interesting method of generating an antitumor immune response in mice to the potential treatment of patients with cancer has led to several human trials (Plautz *et al.*, 1993; Berns *et al.*, 1995; Cassileth *et al.*, 1995; Freeman *et al.*, 1995; Mackiewicz *et al.*, 1995; Soifer *et al.*, 1998; Dranoff, 1999; Mastrangelo *et al.*, 1999; Simons *et al.*, 1999; Nelson *et al.*, 2000). For cytokine gene therapy to be clinically effective, the cytokine gene must be expressed by the target cell at a level that results in a systemic immune response, with acceptable toxicity, and a clinically meaningful antitumor effect (shrinkage of measurable tumor, prevention of relapse, or prolongation of survival) should be achieved.

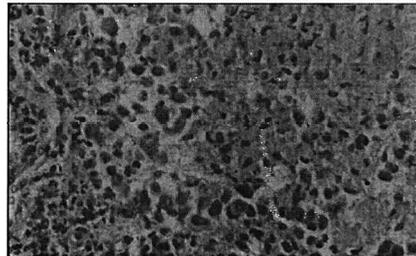
The method of "immunization" utilized in this study (*ex vivo* insertion of GM-CSF cDNA into autologous tumor) has both advantages and disadvantages. By utilizing autologous tumor cells as the vaccine, unique tumor antigens in the context of self-MHC may be presented to the host immune system with the additional stimulation of GM-CSF. The use of autologous



A. Day 3 – GM-CSF treated tumor



B. Day 3 –Control untreated tumor



C. Day 14 – GM-CSF treated tumor

**FIG. 2.** Photomicrograph of three separate hematoxylin and eosin stained vaccine site biopsies in patient 8. Day 3, GM-CSF-treated tumor; day 3, Control (untreated tumor); day 14, GM-CSF-treated tumor. The lymphocytic infiltrate is also greater on day 14. The control tumor has fewer lymphocytes than the GM-CSF-treated tumor.

tumor, however, does not lend itself to precise assays of a cell-mediated response to a specific tumor antigen. Several reports have utilized peptide either alone or in combination with professional antigen-presenting cells in concert with cytokine as a vaccine. The use of peptides allows monitoring the host response to peptide and the ability to select either a self or non-self antigen-presenting cell. This method of immunization results in a measurable response to the peptide, but no dramatic antitumor effect has been demonstrated (Scheibenbogen *et al.*, 2000). The lack of an antitumor effect may be at least partially related to the lack of expression of the immunogen on antigenically heterogeneous human tumors. The utilization of autologous tumor may avoid the escape of a heterogeneous population from immune-mediated destruction.

The utilization of “naked” DNA does not require cell division and thus can be done expeditiously, as we demonstrate in this article. The ability to accomplish gene transfer quickly, however, is of little value if the transgene is expressed at levels inadequate to generate an immune response (as in the majority of our patients).

Safety was the primary end point of this phase I study. The potential toxicities evaluated included systemic toxicity from the cytokine, local toxicity at the vaccine site, and toxicity related to the insertion of foreign DNA. The systemic levels of GM-CSF achieved were below the threshold of toxicity, as expected, and thus no clinical manifestations of systemic GM-

CSF administration were noted. No treatment-related systemic toxicity was noted in any patient. Transient decreases in lymphocyte counts did occur in 6 of 12 patients, but white blood cell counts remained within the normal range and the lymphocyte count abnormalities were not related to dose or timing of the GM-CSF-treated tumor vaccine. No local toxicity at the site of intradermal vaccine placement was observed at any time point. One patient was noted to have an asymptomatic elevation in anti-double-stranded DNA antibody after 3 months of follow-up. The level subsequently (12 months) returned to normal. No other autoimmune laboratory values were abnormal. This method of vaccination thus was not associated with any significant measurable toxicity. The lack of toxicity that we report in this study is confirmed by similar GM-CSF gene therapy trials using GM-CSF DNA transfection by retrovirus into cultured autologous renal carcinoma cells. A pilot human clinical trial testing vaccination with autologous GM-CSF-transduced (by retrovirus), irradiated melanoma cells reported marked erythema and induration at the injection sites but no other toxicity (Dranoff and Mulligan, 1995).

Although safety and evaluation of treatment-related toxicity were the primary goals of this study, patients were also evaluated for an antitumor immune response. One critical factor in the development of an effective systemic antitumor immune response is the generation of a local inflammatory response at the site of the transfected tumor vaccine (Colombo *et al.*, 1992).

TABLE 3. LOCAL INFLAMMATORY RESPONSE<sup>a</sup>

Patient	Day 3		Day 14	
	Erythema (mm)	Lymphocytes/site	Erythema (mm)	Lymphocytes/site
1	0	75	0	350
2	0	0	0	0
3	0	15	0	300
4	Tr	2,000	0	250
5	0	30	0	130
6	0	30	0	50
7	0 (0)	1,400	0 (0)	10,000 (2,200)
8	Tr (Tr)	1,500 (100)	0	10,000
9	Tr (Tr)	80	0 (0)	55 (40)
10	15 (0)	900 (100)	0	420
11	0 (0)	220	0 (0)	100 (0)
12	0 (0)	270 (0)	10	4,000

<sup>a</sup>The local inflammatory response observed on day 3 and day 14 after vaccination was determined by measuring both the amount of erythema at the vaccination site and by semiquantitative counts of the number of lymphocytes per biopsy (Tr, trace). The control vaccination site (in group 2) is depicted in parentheses.

Differences in lymphocyte infiltration at irradiated tumor vaccine sites in the present study were noted and dependent on the presence of GM-CSF DNA. That GM-CSF production by the transfected tumor cells was the cause of the local inflammatory response is confirmed by the relative lack of inflammatory response to nontransfected tumor cells at the control vaccine site.

The local inflammatory response generated by local GM-CSF, however, resulted in a detectable systemic antitumor im-

mune response as measured by DTH testing with nontransfected tumor in only one patient. The lack of a memory antitumor response in the other patients may be due to either intrinsically poor tumor immunity, the lack of a functional immune system (suggested by anergy to mumps and *Candida* in several patients), the ineffective nature of exposure to a single vaccine, or because the dose of GM-CSF expressed locally was inadequate to generate a systemic response. The murine preclinical

TABLE 4. LOCAL ERYTHEMA AFTER DTH TESTING<sup>a</sup>

Patient	Tumor (mm)	PBLs (mm)	Mumps (mm)	<i>Candida</i> (mm)
1	0	0	0	13
2	0	5	30	0
3	20	22	25	0
4	0	0	2	5
5	0	0	5	0
6	0	0	0	13
7	0	0	0	16
8	7	0	0	16
9	10	20	28	30
10	0	0	25	0
11	0	0	50	0
12	0	0	0	0
13	0/8/10	0/10/12	0	0
14	0/0/0	0/0/0	25	8
15	0/0	0/0	5	0
16	0/0/0	0/0/0	24	0
17	0	0	0	0

<sup>a</sup>Delayed-type hypersensitivity to  $5 \times 10^6$  nontransfected autologous tumor cells,  $5 \times 10^6$  peripheral blood leukocytes (prepared identically to autologous tumor), mumps antigen, and *Candida* antigen was determined on day 25 after vaccination. All measurements were performed 3 days after intradermal injection. Patients 13–17 (group 3) had placement of tumor and PBLs after each course of treatment (course 1/course 2/course 3).

TABLE 5. ANTITUMOR RESPONSE<sup>a</sup>

Patient	Tumor type	Tumor response	Current status
1	Melanoma	PD	PD/Exp
2	Sarcoma	MR	PD/Exp
3	Melanoma	PD	PD/Exp
4	Melanoma	PD	PD/Exp
5	Sarcoma	N/A	PD/Exp
6	Sarcoma	N/A	PD/Exp
7	Melanoma	N/A	PD/Exp
8	Melanoma	SD	SD
9	Sarcoma	N/A	PD/Exp
10	Melanoma	PD	PD/Exp
11	Melanoma	SD	PD/Exp
12	Melanoma	N/A	PD/Exp
13	Sarcoma	N/A	PD/Exp
14	Sarcoma	SD	PD/Exp
15	Melanoma	PD	PD/Exp
16	Melanoma	N/A	PD
17	Melanoma	PD	PD/Exp

<sup>a</sup>Tumor response (SD, stable disease; PD, progressive disease; MR, mixed response; N/A, not applicable due to absence of evaluable disease; NED, no evidence of disease; Exp, expired) was assessed for all patients. Minimal time since treatment is 4.5 years.

model was specifically designed to mimic the clinical situation by utilizing a poorly immunogenic tumor, although the human tumors studied may be even less immunogenic. We proposed to study the effect of multiple vaccinations in the third group of patients. GM-CSF expression (as measured *in vitro* after transfection of autologous tumor) in the four patients treated with multiple vaccinations was less than in group 2 patients and did not correspond to an effective dose in preclinical animal models (greater than 3 ng after 24 hr of *in vitro* culture of  $10^6$  cells). No other patients were added to this group because of the lack of available plasmid/bead preparations. The lack of a DTH response in this small number of treated patients with relatively low gene expression does not establish that multiple vaccinations are inferior to single vaccination.

One factor that can be controlled in vaccination studies with transfected autologous tumor is gene expression in the tumor cell. The rejection of subsequent tumor challenge in mice is dependent on achieving enough local GM-CSF to stimulate an immune response (Mahvi *et al.*, 1997). Others and we have confirmed that the local delivery of tumor cells that can generate 30 ng of GM-CSF per  $10^6$  cells (measured by *in vitro* culture of cells for 24 hr) to the tumor microenvironment was sufficient to protect the animal. No patient in group 1 had a GM-CSF level equivalent to an effective local dose (greater than 30 ng after 24 hr of *in vitro* culture of the dose of cells placed at the vaccine site— $10^6$  in the murine system,  $10^7$  in the present study). In contrast, four of the six patients in group 2 had expression equivalent to an effective local dose greater than 30 ng after 24 hr of *in vitro* culture of the cells placed at vaccine site— $10^6$  in the murine system,  $10^7$  in this study. The increase in expression was due to higher transfection efficiency in the group as measured by GM-CSF expression in cultured tumor cells and by the use of  $10^7$  cells, all exposed to PMGT, as com-

pared with 10% (or  $10^6$  cells) in group 1. The lack of a detectable antitumor DTH response in five of six group 2 patients suggests that the murine data cannot be directly translated to the human. Although the local cytokine concentration was similar in group 2 to that achieved in murine models, stimulation of a detectable systemic immune response as measured by DTH did not occur, except in patient 8, whose GM-CSF production level was 10-fold higher than the effective murine dose.

The lack of a DTH response in most patients thus may be related to the ability of the tumor cells transfected with GM-CSF cDNA to generate adequate local cytokine concentrations at the site of vaccination. Prostate cancer cells transfected with GM-CSF cDNA *ex vivo* were able to generate a positive DTH response in seven of eight treated patients (Simmons *et al.*, 1999). The transfected tumor cells *in vitro* generated 140–1400 ng of GM-CSF protein per  $10^6$  cells per 24 hr, which was greater than in any of our preparations. The threshold level of GM-CSF protein may be greater than that necessary in the murine system. Similar results were noted in an autologous melanoma *ex vivo* vaccine study. Potent immunity was generated, although only one partial response was noted (Soiffer *et al.*, 1998). The level of local GM-CSF protein in that report as measured by *in vitro* culture was 84–965 ng/ $10^6$  cells per 24 hr, again indicating that a threshold level of greater than 30 ng is necessary for the generation of an antitumor immune response. When the local concentration was lower no immune response or antitumor effect was noted (Jaffee *et al.*, 1998; Mastrangelo *et al.*, 1999). In this study we generated an amount of transgenic GM-CSF adequate to generate a detectable DTH immune response in one patient. This patient with rapidly progressive disease (brain and visceral metastases over a 6-month period) has remained disease free for 5 years. This patient was different from other study patients in two ways: he had a nearly

complete tumor debulking, and he generated a DTH response after vaccination.

A separate important factor in the application of gene therapy to clinical use is the transferability of the technology to clinical cancer treatment centers. The feasibility of this method (PMGT) of *ex vivo* gene transfer has been established by this study. The entire procedure, from tumor removal to vaccine placement, was accomplished in less than 6 hr in all patients. This precluded the need for long-term selection of transfected cells *in vitro*, and allowed the technology to be applied to non-dividing tumor cell populations. The materials needed to provide this form of treatment could easily be exported to clinical treatment locations without a large biotechnology research infrastructure. Unfortunately, the ability of this technique (PMGT) to consistently generate an effective local cytokine level was not demonstrated in this study.

In summary, this technique of gene transfer is safe and feasible, but resulted in clinically relevant levels of gene expression in a minority of patients. A measurable inflammatory response that is dose dependent occurred at the site of vaccine placement. Higher local doses of cytokine protein than consistently possible with this technique appear essential for a more effective tumor vaccine.

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Address reprint requests to:

Dr. David Mahvi  
H4/726 University of Wisconsin Hospital and Clinics  
600 Highland Avenue  
Madison, WI 53792

E-mail: mahvi@surgery.wisc.edu

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