Published Ahead of Print on December 22, 2003 as 10.1200/JCO.2004.06.043

VOLUME 22 · NUMBER 3 · FEBRUARY 1 2004

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

# Immunopharmacologic Analysis of an Autologous, Hapten-Modified Human Melanoma Vaccine

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Submitted June 11, 2003; accepted September 16, 2003.

Authors' disclosures of potential conflicts of interest are found at the end of this article.

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0732-183X/04/2203-1/$20.00

DOI: 10.1200/JCO.2004.06.043

A B S T R A C T

**Purpose**
We have previously reported a clinical trial of a human cancer vaccine consisting of autologous tumor cells modified with the hapten, dinitrophenyl (DNP), in patients with clinical stage III melanoma. Here we present a follow-up report expanded to 214 patients with 5-year follow-up.

**Patients and Methods**
Two hundred fourteen patients with clinical stage III melanoma (117 patients with stage IIIC and 97 patients with stage IIIB) who were melanoma-free after standard lymphadenectomy were treated with multiple intradermal injections of autologous, DNP-modified vaccine mixed with bacille Calmette-Guérin. Four vaccine dosage schedules were tested sequentially, all of which included low-dose cyclophosphamide. Patients were tested for delayed-type hypersensitivity (DTH) to autologous melanoma cells, both DNP-modified and unmodified, and to control materials.

**Results**
The 5-year overall survival (OS) rate of the 214 patients was 44%. DTH responses to unmodified autologous melanoma were induced in 47% of patients. The OS of this DTH-positive group was double that of DTH-negative patients (59.3% *v* 29.3%; $P < .001$). In contrast, positive DTH responses to DNP-modified autologous melanoma cells and to purified protein derivative developed in almost all patients but did not affect OS. Surprisingly, the OS after relapse was also significantly longer in patients who developed positive DTH to unmodified tumor cells (25.2% *v* 12.3%; $P < .001$). Finally, the development of DTH was dependent on the schedule of administration of the vaccine, specifically, the timing of an induction dose administered at the beginning of the treatment program.

**Conclusion**
This study underscores the importance of the immunopharmacology of the autologous, DNP-modified vaccine and may be relevant to other cancer vaccine technologies.

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## INTRODUCTION

Progress in the development of an effective therapeutic human cancer vaccine has been disappointingly slow. Despite a number of seemingly positive clinical trials published over the past 20 years [1-6], oncologists remain skeptical about the results, and no cancer vaccine is likely to obtain marketing approval in the United States in the near future.

Paradoxically, the theoretical and experimental bases for active immunotherapy of malignant tumors have become more firmly established as progress in the clinic has languished. Animal models continue to indicate that established, albeit small, tumors can be made to regress by active immunization [7]. A number of human tumor-associated antigens have been discovered, from which immunogenic peptides have been identified and synthesized [2,4]. T cells that can modulate antitumor immunity have been rediscovered as regulatory T cells with a characteristic phenotype and cytokine production profile [8].

A major impediment to progress in this area has been a dearth of information on what may be called the immunopharmacology of cancer vaccines. Little is known about optimum vaccine doses, schedules of administration, or routes of administration.

There are no standard, widely accepted immunologic tests for monitoring vaccine efficacy; as characterized in a recent editorial [9], it has not been easy to determine whether a vaccine works.

We have been conducting clinical trials with a cancer vaccine consisting of autologous, intact tumor cells modified with the hapten, dinitrophenyl (DNP). The rationale for this approach consists of the value of the autologous tumor cell as a source of tumor rejection antigens [10-12] and the ability of haptenization to render immunogenic proteins to which the host is otherwise unresponsive [13-15]. Previously we reported the preliminary immunologic and clinical results of clinical trials in which 77 patients with clinical stage III melanoma were treated postoperatively with the autologous, DNP vaccine [16]. Here we present an updated report expanded to 214 patients, almost all of whom have completed 5-year follow-up. We confirm our previous finding that patients who developed positive delayed-type hypersensitivity (DTH) to unmodified, autologous tumor cells had significantly longer durations of relapse-free survival (RFS) and overall survival (OS). In addition, we show that this response was a significant determinant of survival, even in patients who eventually experienced relapse, because it affected their pattern of relapse. Finally, we present evidence that the schedule of administration of the DNP vaccine, in particular, the timing of what we are calling an induction dose, affects both immunologic and clinical end points.

## PATIENTS AND METHODS

### *Patients*

The study population consisted of 214 patients with clinical stage III melanoma. Metastatic masses were ≥ 2.5 cm in diameter but were completely resectable. The clinical characteristics of these patients are listed in Table 1. One hundred seventeen patients (55%) had stage IIIC disease by virtue of one or more of the following characteristics: four or more positive nodes, in-transit metastases, gross extranodal extension, or ulceration of the primary lesion; the other 97 patients had stage IIIB disease [17]. Assessment of the pathology of the primary lesions and the lymph nodes was done at the hospitals where the surgeries were performed.

The protocols were approved by the institutional review board of Thomas Jefferson University. Patients had to be entered onto the protocol within 30 days of lymphadenectomy. The major entry criteria were as follows: completion of standard lymphadenectomy, including, when necessary, excision of in-transit metastases; no clinically evident metastases as determined by physical examination and imaging studies (computed tomography of the chest, abdomen, and pelvis); age ≥ 16 years; no systemic antimelanoma therapy within 8 weeks of study entry; and not taking corticosteroids or other immunosuppressive drugs.

After the completion of vaccine treatments, patients were evaluated by history and physical examination at the Thomas Jefferson University Hospital every 2 months in years 1 and 2, every 3 months in year 3, and every 6 months in years 4 and 5. No patients were lost during the 5-year follow-up period.

**Table 1.** Patient-Related Variables

| | No. of Patients |
|---|---|
| Total patients | 214 |
| Sex | |
| Male | 122 |
| Female | 92 |
| Age, years | |
| Median | 52 |
| Range | 16–83 |
| Site of primary melanoma | |
| Extremity | 74 |
| Trunk, head/neck, acrolentiginous | 129 |
| Unknown | 11 |
| Ulceration of primary melanoma | |
| Positive | 52 |
| Negative | 128 |
| No data or no primary | 34 |
| Time from primary to nodal metastasis, months | |
| ≤ 3 | 45 |
| 4-11 | 59 |
| 12-36 | 52 |
| > 36 | 47 |
| Metastases to one nodal site | |
| Axillary | 99 |
| Inguinal | 58 |
| Neck | 15 |
| Other | 2 |
| Total | 174 |
| Metastases to two nodal sites | |
| Axillary + supraclavicular | 8 |
| Inguinal + pelvic | 15 |
| Bilateral axillary | 7 |
| Bilateral neck | 8 |
| Other | 2 |
| Total | 40 |
| No. of positive nodes | |
| Mass only | 74 |
| Mass and 1-2 microscopic, + | 62 |
| Mass and ≥ 3 microscopic, + | 68 |
| Mass, no node count | 10 |
| Gross extranodal extension | 21 |
| In-transit metastases | 20 |
| Stage | |
| IIIB | 97 |
| IIIC | 117 |
| Prior systemic therapy | |
| Chemotherapy | 12 |
| Interferon | 5 |
| Immunotherapy | 5 |

Because of early relapse and the need for additional antitumor therapy, 24 patients did not complete the intended course of vaccine administration. However, because all patients who received at least one vaccine injection were considered assessable, these 24 patients were included in the analysis. There were no drop-outs from the trial.

### *Tumor Processing and Vaccine Preparation*

Autologous, DNP-modified vaccines were prepared as previously described [18]. In brief, tumor cells were extracted by enzy-

**Table 2.** Summary of Vaccine Schedules

| Schedule | No. of Patients | No. of Doses of Cyclophosphamide | DNFB Sensitization | Vaccine Schedule | Vaccine Dosage Range ($\times 10^6$) | BCG Mixed With |
|---|---|---|---|---|---|---|
| A | 47 | 3 | + | Every 28 days × 8 | 5.0-25.0 | Every dose |
| B | 30 | 3 | + | Weekly × 12 | 5.0-25.0 | 1/3 of doses |
| C | 50 | 3 | + | Weekly × 12 | 5.0-25.0 | Every dose |
| D | 87 | 1 | − | Weekly × 6 | 2.5-7.5 | Every dose |

NOTE. Schedule A, cyclophosphamide 300 mg/m$^2$ administered intravenously 3 days before DNFB and 3 days before first two doses of vaccine; Schedule B, cyclophosphamide 300 mg/m$^2$ administered intravenously 3 days before DNFB and 3 days before each six-week series of vaccine (half of the vaccines were dinitrophenyl-modified and half unmodified); Schedule C, cyclophosphamide 300 mg/m$^2$ administered intravenously 3 days before DNFB and 3 days before each six-week series of vaccine (all vaccines were dinitrophenyl-modified); Schedule D, cyclophosphamide 300 mg/m$^2$ administered intravenously 3 days before first vaccine only (booster injections of vaccine were given at 6 and 12 months).

Abbreviations: DNFB, dinitrofluorobenzene; BCG, bacille Calmette-Guérin.

matic dissociation of freshly obtained metastatic masses with collagenase and DNAse, aliquotted, frozen in a controlled-rate freezer, and stored in liquid nitrogen in a medium containing 2.5% human albumin and 10% dimethylsulfoxide until needed. Aliquots of tumor cells were obtained by mechanical dissociation (mincing with a scalpel) for use as controls in DTH testing.

On the day that a patient was to be treated, an aliquot of cells was thawed, washed, and irradiated to 25 Gy. Then the cells were washed again and modified with DNP by the method of Miller and Claman [19]. This involves a 30-minute incubation of tumor cells with dinitrofluorobenzene (DNFB), followed by washing with saline.

Each vaccine fell within a specified dose range of live (trypan blue–excluding) tumor cells suspended in 0.2 mL of Hanks solution with human serum albumin. In addition, there were variable numbers of lymphocytes and dead cells in all specimens [18].

### *Vaccine Administration*

Vaccine cell suspensions were mixed with 0.1 mL of bacille Calmette-Guérin (BCG; Tice strain obtained from Organon Teknika Corporation, Durham, NC) just before injection. The dose of BCG was progressively attenuated to produce a local reaction consisting of an inflammatory papule without ulceration, as previously described [6]. The vaccine plus BCG suspension was injected intradermally into three adjacent sites, usually on the upper dorsal arm, excluding sites ipsilateral to a lymph node dissection.

### *Vaccine Dosage Schedules*

Four vaccine dosage schedules were tested sequentially: Schedule A, October 1988 to March 1993; Schedule B, April 1993 to March 1994; Schedule C, April 1994 to August 1995; Schedule D, September 1995 to June 1998. These are summarized in Table 2. All of the schedules incorporated low-dose cyclophosphamide (300 mg/m$^2$).

For Schedules A, B, and C, patients received baseline skin testing and then were sensitized with DNFB by topical application of a 1% solution in acetone corn oil on 2 consecutive days in the same site on the ventral upper arm; cyclophosphamide 300 mg/m$^2$ was administered by intravenous rapid infusion 3 days before DNFB application. The first vaccine injection was given 2 weeks later. For Schedule D, DNFB sensitization was omitted.

For Schedule A, DNP vaccine mixed with BCG was administered every 4 weeks for a total of eight doses; cyclophosphamide 300 mg/m$^2$ was administered 3 days before the first and second doses. All vaccine injections were given into the same site. For Schedule B, vaccine was administered weekly for 6 weeks; after a 4-week re-evaluation period, vaccine was again administered weekly for 6 weeks. The first three vaccines of each course were DNP-modified and the last three were unmodified. BCG was admixed only with the first and fourth vaccine of each course. All of the DNP vaccine injections were given into one area, and all of the unmodified vaccine injections were given into a second area. Cyclophosphamide 300 mg/m$^2$ was administered 3 days before the start of each vaccine course. Schedule C was identical to Schedule B, except that all vaccines were DNP-modified and all were mixed with BCG. Schedule D was a simplified regimen in which DNFB presensitization was omitted, and only one series of six weekly DNP-modified vaccines was administered.

### *Rationale for Dosage Schedules*

The first dosage schedule, A, was chosen for patients with resected stage III disease after the observation of positive immunologic and clinical results in patients with measurable metastases [6]. The development of schedules B and C was based on the following hypotheses: first, multiple frequent vaccine injections (ie, weekly rather than monthly) would induce more potent T-cell responses; and second, the immune response to unmodified tumor cells could be augmented by sequential administration of DNP-modified tumor cells followed by injection of unmodified tumor cells. Schedule D resulted from a rethinking of the immunologic basis of hapten-conjugated vaccines stimulated by the publications of Weltzien's group [20]. Their data suggested that presensitization with DNFB might be superfluous, because it could prime a response to hapten-modified normal tissue antigens.

### *DTH Testing*

Patients were tested for DTH to the following materials: autologous melanoma cells, DNP-modified and unmodified; autologous lymphocytes, DNP-modified and unmodified, separated from blood by density-gradient centrifugation and cryopreserved; purified protein derivative (PPD) intermediate (5 tuberculin units); and diluent. For each cellular skin test material, 0.15 mL was drawn into a 0.5-mL Lo-Dose insulin syringe (Becton Dickinson, Franklin Lakes, NJ) and injected intradermally into the ventral forearm, making sure that a wheal was raised by the injection. After 48 $\pm$ 4 hours, each reaction was palpated to determine the borders of the area of induration by running the index finger along the skin toward the skin test reaction until an edge was felt, and each edge was marked by a pen line. The distances between each of the opposite sets of two pen lines were measured. A positive

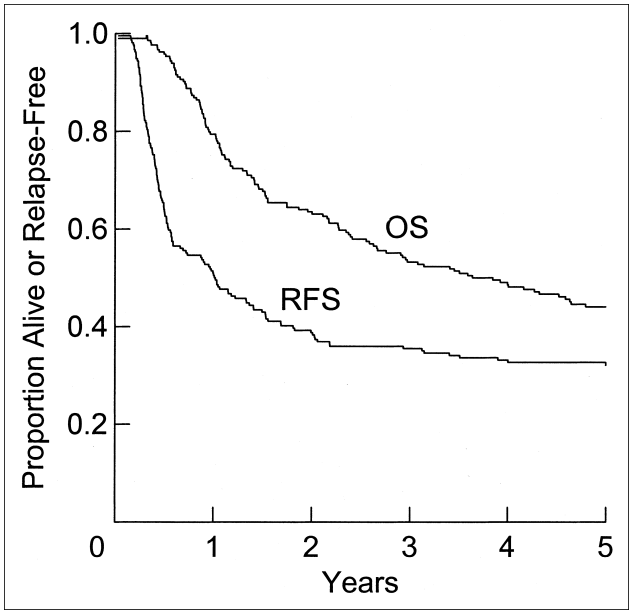


**Fig 1.** Overall survival (OS) and relapse-free survival (RFS) of 214 patients with clinical stage III melanoma treated postoperatively with autologous, dinitrophenyl-modified vaccine.

response was defined as a maximum diameter of induration ≥ 5 mm. Patients were also skin-tested with intermediate strength PPD (5 tuberculin units).

DTH testing was performed before the treatment program was initiated and posttreatment (see Results). Analyses were performed by determining the maximum DTH response exhibited by each patient to each of the test reagents.

### Statistics

Survival was plotted by the Kaplan-Meier method, and the difference between survival curves was determined by the log-rank test of Mantel. The effect of prognostic variables on survival was determined by proportional hazards regression (Cox).

## RESULTS

### Effect of Patient-Related Variables on Survival

Figure 1 shows the RFS and OS of all 214 patients treated postlymphadenectomy with autologous, DNP vaccine. The data are essentially complete, because all but four surviving patients have completed the 5-year follow-up. The 5-year RFS and OS rates are 33% and 44%, respectively. Univariate analysis of known prognostic variables (Tables 1 and 3) indicated that the major determinant of survival was the number of positive nodes; eg, the 5-year OS rates of patients with a palpable mass only versus those with a mass plus three or more microscopically positive nodes were 61.9% and 21.4%, respectively. However, the presence of metastatic disease in two nodal sites and extranodal extension were significant independent predictors of OS as confirmed by the multivariate analysis (Table 4). It should be noted that only grossly evident extranodal extension was recorded, because processing of the mass precluded a routine examination for microscopic invasion of the entire lymph node capsule. Finally, neither the presence of intransit metastases nor ulceration of the primary tumor alone were significant determinants of survival in DNP vaccine–treated patients, although both were previously shown to be important variables in large series of patients treated with surgery alone [21].

### Effect of Vaccine Dosage Schedule on Survival

Table 5 lists the range of vaccine doses administered. The dose of vaccine was defined by the number of live tumor cells, ie, tumor cells that excluded the supravital dye, trypan blue. These live cells were proliferation-incompetent as shown by failure to incorporate a radioactive DNA precursor after in vitro incubation (data not shown). Subsequently we found that dead tumor cells also may have contributed to the immunogenicity of the vaccine [18], so we summarized the number of dead and total (live plus dead) cells administered as well. Univariate analysis (Table 6) indicated that none of these cell dosage parameters significantly affected RFS or OS. However, schedule C seemed to be associated with lower 5-year RFS and OS, although the difference was of borderline statistical significance only for RFS.

### Effect of Vaccine-Induced DTH Responses on Survival

Table 5 provides a summary of the DTH responses induced by administration of DNP vaccine. For each patient, the maximum DTH response to each material is tabulated. For most patients, DTH to unmodified autologous melanoma cells was tested at only one posttreatment time point (week 10 for study A, week 11 for studies B and C, week 9 for study D). Sixty-eight patients had a second DTH test to unmodified tumor cells. In studies A, B, and C ($n = 34$), the follow-up DTH test was performed after a median interval of 1.9 months; in study D ($n = 34$), the follow-up DTH test was performed after a median interval of 9.5 months. For each patient, there was a tight correlation between DTH responses elicited at the two time points; eg, for DTH to mechanically dissociated tumor cells, the squared multiple $r = 0.690$ ($P < .001$). These data suggest that the DTH responses shown in Table 5 were durable as well as reproducible. Pretreatment positive DTH responses to autologous melanoma cells, either DNP-modified or unmodified, were observed in only 23 patients (8%) and were generally small (median, 6 mm).

We tested for DTH to both autologous melanoma cells that had been dissociated with enzymes (collagenase and DNAse) and to melanoma cells that had been mechanically dissociated only. The two preparations usually elicited similar DTH responses, as indicated by regression analysis (Fig 2). However, 30 patients (14%) developed an apparent DTH response to the enzymes themselves, as measured by skin testing with enzyme-treated autologous peripheral-

**Table 3.** Univariate Analysis of Effect of Patient-Related Variables on RFS and OS

| Parameter | No. of Patients | 5-Year RFS (%) | P* | 5-Year OS (%) | P |
|---|---|---|---|---|---|
| Sex | | | .259 | | **.035** |
| Male | 122 | 28.7 | | 36.4 | |
| Female | 92 | 38.0 | | 54.2 | |
| Age, years | | | .486 | | .711 |
| ≤ 50 | 97 | 29.9 | | 44.1 | |
| ≥ 50 | 117 | 34.9 | | 43.9 | |
| Site of primary melanoma | | | .393 | | **.022** |
| Extremity | 74 | 33.8 | | 53.9 | |
| Trunk, head/neck, AL | 129 | 31.0 | | 37.6 | |
| Ulceration of primary melanoma | | | .793 | | .813 |
| Positive | 52 | 38.3 | | 48.0 | |
| Negative | 128 | 34.4 | | 44.2 | |
| No data or no primary | 34 | | | | |
| Time from primary to nodal metastasis, months | | | .712 | | .632 |
| < 12 | 104 | 31.3 | | 43.7 | |
| ≥ 12 | 99 | 33.7 | | 43.3 | |
| No. of nodal sites involved† | | | **.024** | | **.064** |
| One | 154 | 37.0 | | 46.9 | |
| Two | 40 | 20.0 | | 32.5 | |
| No. of positive nodes | | | **< .001** | | **< .001** |
| Mass only | 74 | 50.0 | | 61.9 | |
| Mass and 1-2 microscopic, + | 62 | 29.0 | | 43.2 | |
| Mass and ≥ 3 microscopic, + | 68 | 14.6 | | 21.4 | |
| Gross extranodal extension | | | **.096** | | **.036** |
| Positive | 21 | 23.8 | | 22.2 | |
| Negative | 189 | 33.3 | | 46.3 | |
| No data | 4 | | | | |
| In-transit metastases‡ | | | .235 | | .505 |
| Present | 20 | 30.0 | | 45.0 | |
| Absent | 154 | 37.0 | | 46.9 | |

Abbreviations: RFS, relapse-free survival; OS, overall survival; AL, acrolentiginous.
*Log-rank test; *P* values < .10 are bolded.
†In-transit metastases excluded.
‡Two nodal sites excluded.

blood mononuclear cells, so we analyzed only their DTH response to mechanically dissociated melanoma cells. Thirty percent of these enzyme-reactive patients also exhibited positive DTH to mechanically dissociated cells. It is noteworthy that DTH to the enzymes was induced in 60% of patients treated with dosage schedule B, which included immunization with unmodified as well as DNP-modified melanoma cells, but in only 7% of patients treated by dosage schedules A, C, and D, in which all vaccine cells were hapten-modified.

**Table 4.** Multivariate Analysis of Patient-Related Variables

| Factor | Relapse-Free Survival | | | Overall Survival | | |
|---|---|---|---|---|---|---|
| | Hazard Ratio* | 95% CI | P | Hazard Ratio | 95% CI | P |
| Sex, female | 0.75 | 0.49 to 1.14 | .174 | 0.61 | 0.40 to 0.95 | **.028** |
| Age > 50 years | 0.92 | 0.62 to 1.35 | .654 | 0.92 | 0.62 to 1.36 | .670 |
| Time to nodal metastases ≥ 12 months | 1.00 | 0.67 to 1.51 | .983 | 1.01 | 0.67 to 1.52 | .979 |
| No. of nodal sites, 2 | 1.44 | 0.87 to 2.38 | .157 | 1.70 | 1.02 to 2.83 | **.045** |
| Extranodal extension, present | 1.97 | 1.04 to 3.74 | **.038** | 2.52 | 1.31 to 4.85 | **.006** |
| No. of positive nodes, mass + ≥ 1 microscopic | 1.70 | 1.09 to 2.65 | **.020** | 1.52 | 0.97 to 2.37 | **.067** |
| Primary site, not extremity | 0.95 | 0.62 to 1.44 | .801 | 1.05 | 0.69 to 1.60 | .821 |
| Ulceration of primary, present | 0.96 | 0.61 to 1.51 | .867 | 0.90 | 90.57 to 1.42 | .645 |

*Hazard ratios with *P* < .100 are bolded.

**Table 5.** Treatment-Related Variables

| Parameter | All | Dosage Schedule | | | |
|---|---|---|---|---|---|
| | | A | B | C | D |
| No. of patients | 214 | 47 | 30 | 50 | 87 |
| Vaccine dose, live cells | | | | | |
| Median | 7.5* | 11.9 | 10.0 | 10.8 | 7.0 |
| Range | 1.5-25.0 | 2.7-25.0 | 2.0-25.0 | 2.8-23.9 | 1.5-7.5 |
| Vaccine dose dead cells | | | | | |
| Median | 11.8 | 12.2 | 5.5 | 13.3 | 13.6 |
| Range | 1.3-49.4 | 2.2-40.2 | 1.5-34.8 | 2.1-44.8 | 1.3-49.4 |
| Vaccine dose, total cells | | | | | |
| Median | 22.3 | 26.8 | 16.1 | 25.6 | 19.4 |
| Range | 3.4-56.9 | 5.7-53.1 | 3.8-43.8 | 6.6-53.6 | 3.4-56.9 |
| Peak DTH to DNP-modified tumor cells | | | | | |
| Median | 20 | 22 | 22 | 25 | 16 |
| Range | 4-70 | 7-55 | 9-70 | 7-55 | 4-70 |
| ≥ 5mm, % | 99 | 100 | 100 | 100 | 99 |
| ≥ 10mm, % | 87 | 86 | 96 | 85 | 86 |
| Peak DTH to unmodified tumor cells | | | | | |
| Median | 4 | 4 | 2 | 1 | 6 |
| Range | 0-22 | 0-22 | 0-6 | 0-8 | 0-13 |
| ≥ 5mm, % | 47 | 47 | 11 | 18 | 68 |
| ≥ 10mm, % | 6 | 9 | 0 | 0 | 9 |
| Peak DTH to DNP-modified lymphocytes | | | | | |
| Median | 10 | 16 | 15 | 15 | 7 |
| Range | 0-50 | 4-45 | 5-35 | 4-45 | 0-50 |
| ≥ 5mm, % | 87 | 98 | 100 | 98 | 70 |
| ≥ 10mm, % | 52 | 70 | 70 | 64 | 28 |
| Peak DTH to enzyme-coated lymphocytes | | | | | |
| Median | 0 | 0 | 5 | 0 | 0 |
| Range | 0-40 | 0-20 | 0-40 | 0-15 | 0-22 |
| ≥ 5mm, % | 15 | 11 | 60 | 8 | 5 |
| ≥ 10mm, % | 9 | 5 | 27 | 5 | 4 |
| Peak DTH to PPD | | | | | |
| Median | 25 | 23 | 23 | 25 | 25 |
| Range | 8-85 | 12-60 | 10-35 | 8-50 | 9-85 |
| ≥ 5mm, % | 100 | 100 | 100 | 100 | 100 |
| ≥ 10mm, % | 99 | 100 | 100 | 98 | 99 |
| ≥ 20mm, % | 75 | 65 | 80 | 82 | 74 |

Abbreviations: DTH, delayed-type hypersensitivity; DNP, dinitrophenyl; PPD, purified protein derivative.
*$\times 10^6$.

Virtually all patients developed positive DTH to DNP-modified autologous melanoma cells and to PPD after DNP vaccine treatment, and the intensity of these response was similar among the four dosage schedules ($P = .507$ and $P = .103$, respectively, Kruskal-Wallis test). In contrast, the DTH response to DNP-modified autologous lymphocytes was significantly lower in patients treated with schedule D as compared with A, B, and C ($P < .001$). This difference was likely due to the omission of DNFB contact sensitization in schedule D, which might have primed patients for a T-cell response to DNP-modified normal tissue antigens.

DTH responses to unmodified autologous melanoma were induced less frequently and were smaller. Schedules A and D generated significantly larger responses to unmodified tumor cells than schedules B and C ($P < .001$). Only three patients exhibited positive DTH to unmodified, autologous peripheral-blood mononuclear cells; these responses could have represented the induction of autoimmunity, but because no patients developed changes in the number of circulating leukocytes or clinical signs of autoimmunity, they were more likely artifactual.

Univariate analyses of the effect of the DTH responses on survival are shown in Table 6. The group of patients who developed positive DTH to unmodified, autologous melanoma cells had a doubling of both RFS and OS, which was highly significant ($P < .001$; Fig 3). In contrast, the magnitudes of the DTH response to DNP-modified melanoma cells and PPD were not predictive of longer survival (Fig 4).

Finally, as shown in Table 7, a positive DTH response to unmodified tumor cells remained statistically significant

**Table 6.** Univariate Analysis of Effect of Treatment-Related Variables on RFS and OS

| Parameter | No. of Patients | 5-Year RFS (%) | P* | 5-Year OS (%) | P |
|---|---|---|---|---|---|
| Dosage schedule | | | **.055** | | .570 |
| A | 47 | 42.6 | | 53.2 | |
| B | 30 | 32.3 | | 43.3 | |
| C | 50 | 20.0 | | 34.0 | |
| D | 87 | 34.5 | | 44.9 | |
| Vaccine dose, live cells† | | | .986 | | .926 |
| $< 7.0 \times 10^6$ | 68 | 33.8 | | 43.8 | |
| $7.0\text{-}9.9 \times 10^6$ | 73 | 31.5 | | 40.6 | |
| $\geq 10.0 \times 10^6$ | 72 | 33.3 | | 48.6 | |
| Vaccine dose, total cells† | | | .647 | | .675 |
| $< 20.0 \times 10^6$ | 92 | 35.7 | | 42.9 | |
| $\geq 20.0 \times 10^6$ | 121 | 30.6 | | 45.2 | |
| DTH to unmodified TC, mm | | | **< .001** | | **< .001** |
| < 5 | 78 | 21.6 | | 29.3 | |
| ≥ 5 | 99 | 43.4 | | 59.3 | |
| ND | 37 | | | | |
| DTH to DNP-modified TC, mm | | | .517 | | .967 |
| ≤ 20 | 92 | 31.4 | | 40.8 | |
| > 20 | 83 | 25.3 | | 42.0 | |
| ND | 39 | | | | |
| DTH to PPD, mm | | | .562 | | .419 |
| ≤ 20 | 68 | 33.8 | | 52.4 | |
| 21-30 | 91 | 39.6 | | 45.9 | |
| > 30 | 38 | 21.1 | | 36.3 | |
| ND | 17 | | | | |

Abbreviations: RFS, relapse-free survival; OS, overall survival; DTH, delayed-type hypersensitivity; TC, tumor cells; ND, not done; DNP, dinitrophenyl; PPD, purified protein derivative.
*Log-rank test; *P* values < .10 are bolded.
†Vaccine dose information not available for one patient.

for both RFS and OS in multivariate analyses that included the previously determined important patient-related variables: sex, number of positive lymph nodes, and presence or absence of extranodal extension. None of the other treatment-related parameters tested in this model (DTH responses to DNP-modified tumor cells, DTH to PPD, or number of tumor cells per vaccine dose) had significant effects on survival.

### *Timing of the Vaccine Induction Dose*

Because the development of positive DTH to unmodified melanoma cells was so important to clinical outcome, we performed retrospective analyses to determine why dosage schedules B and C were inferior to A and D in regard to stimulation of that immune response. All four dosage schedules included the following procedures: baseline skin testing performed by intradermal injection of $1 \times 10^6$ irradiated, autologous, DNP-modified tumor cells without BCG into the ventral forearm; administration of low-dose cyclophosphamide; and multiple intradermal injections of DNP-modified melanoma cells mixed with BCG beginning 3 days after cyclophosphamide administration. One difference between schedules was the timing of the baseline skin tests. A majority of patients (n = 122) received baseline skin testing 3 to 8 days before cyclophosphamide administration (day −3 to −8), whereas 78 patients received baseline skin testing on the day of cyclophosphamide administration (day 0) or one day after (day +1). The remaining 14 patients received baseline skin testing 12 to 14 days before cyclophosphamide administration or not at all.

DTH responses to autologous, unmodified melanoma cells that developed after a course of DNP vaccine were significantly larger in patients who had received baseline skin testing on day −3 to −8 than in those who had been skin-tested on day 0 or day +1 (Fig 5; $P < .001$). The percentage of patients who developed a positive DTH (≥ 5 mm in diameter) was as follows: day −3 to −8, 73%; day 0 or +1, 20% ($P < .01$; Fisher's exact test). The difference in timing of the baseline skin tests explains the differential effect of the four dosage schedules on induction of DTH to unmodified melanoma cells: 91% of patients treated with schedules B and C (lower DTH responses) received baseline skin testing at the suboptimal time, compared with 14% of patients treated with schedules A and D (higher DTH responses). The timing of baseline skin testing had no significant effects on the development of DTH to DNP-modified melanoma cells or to PPD (data not shown).

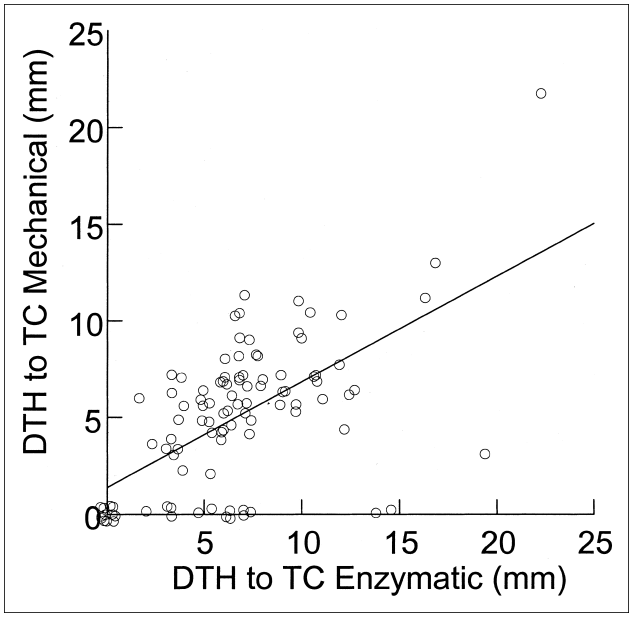


**Fig 2.** Scatter plot comparing the delayed-type hypersensitivity (DTH) responses elicited by autologous, mechanically dissociated tumor cells (TC Mechanical) versus autologous, enzymatically dissociated tumor cells (TC Enzymatic) in 100 patients who were tested with both preparations. Squared multiple $r = 0.751$; $P < .001$.

Surprisingly, this variable influenced survival as well. Five-year OS was 51% in the group that had baseline skin testing on day −3 to −8 versus 33% in the group with baseline skin testing on days 0 or +1 ($P = .007$; Fig 6). A similar dichotomy was observed for 5-year RFS (40% *v* 22%, respectively; $P = .005$). This effect remained significant in a multivariate analysis that included the known prognostic variables: number of positive nodes, presence of extranodal extension, and sex (hazard ratio for skin test on day 0 or +1 *v* days −3 to −8 = 1.618; range, 1.117 to 2.343; $P = .011$).

These data indicate that what we have considered merely a baseline skin test may serve as an induction dose of vaccine. The timing of the induction dose relative to administration of cyclophosphamide apparently determines whether the subsequent course of DNP vaccine results in tumor immunity or unresponsiveness.

### *Relapse Sites and Postrelapse Survival*

Of the 148 patients who experienced relapse, 83 patients developed their first metastatic site in soft tissues (subcutaneous or lymph nodes) and 65 patients in viscera (lung, n = 20; liver, n = 14; brain, n = 17; bone, n = 8; other abdominal sites, n = 6). As we have noted previously [16], the brain was not a common site for first recurrence, an observation that fails to support the prevalent belief that the CNS is an immunologically privileged site that would not be protected by the development of active tumor immunity [22].

The 5-year postrelapse survival rate was 16.1%. We performed an analysis of the patient-related and treatment-related variables that might determine postrelapse survival in these patients. As expected, postrelapse survival was significantly higher in patients who experienced relapse late ( > 6 months postlymphadenectomy) versus early (≤ 6 months), who experienced relapse in soft tissue versus viscera, and whose first relapse was resectable versus nonre-

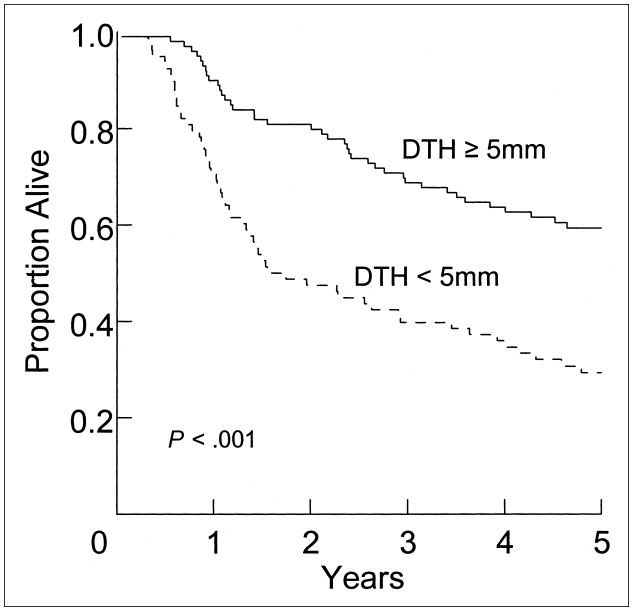


**Fig 3.** Overall survival of patients with stage III melanoma treated with autologous, dinitrophenyl-modified vaccine stratified by peak delayed-type hypersensitivity (DTH) response to unmodified autologous melanoma cells.

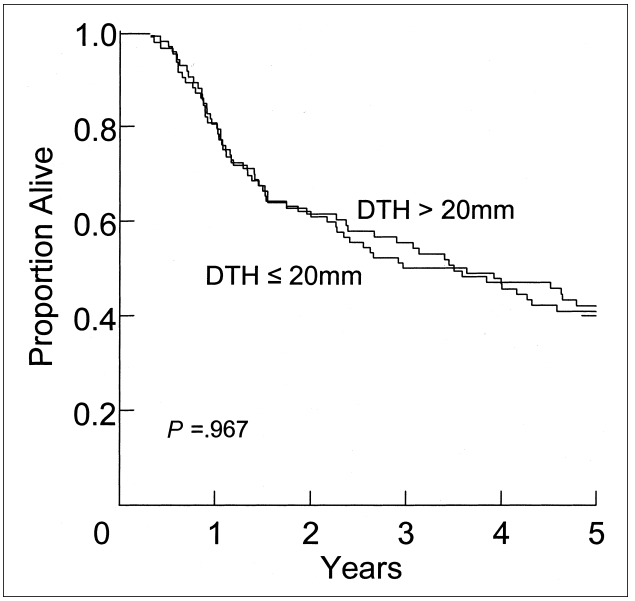


**Fig 4.** Overall survival of patients with stage III melanoma treated with autologous, dinitrophenyl (DNP)-modified vaccine stratified by peak delayed-type hypersensitivity (DTH) response to DNP-modified autologous melanoma cells.

**Table 7.** Multivariate Analysis of Treatment-Related Variables

| Factor* | Relapse-Free Survival | | | Overall Survival | | |
|---|---|---|---|---|---|---|
| | Hazard Ratio | 95% CI | P† | Hazard Ratio | 95% CI | P† |
| Vaccine dose, total cells > $20.0 \times 10^6$ | 0.93 | 0.61 to 1.42 | .752 | 0.90 | 0.57 to 1.42 | .648 |
| DTH to unmodified TC ≥ 5 mm | 0.59 | 0.39 to 0.90 | **.015** | 0.53 | 0.33 to 0.85 | **.009** |
| DTH to DNP-modified TC > 20 mm | 0.93 | 0.62 to 1.41 | .747 | 0.93 | 0.59 to 1.46 | .745 |
| DTH to PPD > 20 mm | 0.96 | 0.63 to 1.46 | .831 | 1.26 | 0.78 to 2.04 | .341 |

Abbreviations: DTH, delayed-type hypersensitivity; TC, tumor cells; DNP; dinitrophenyl; PPD, purified protein derivative.
*Adjusted for patient-related variables: sex, No. of positive nodes, extranodal extension.
†Hazard ratios with $P < .100$ are bolded.

sectable (Table 8). Other patient-related prognostic variables, such as number of positive lymph nodes obtained from the original lymphadenectomy, age (not shown), and sex (not shown), were not significantly predictive of postrelapse survival.

Of 85 patients who received combination chemotherapy (73 with the Dartmouth regimen [23]) for postrelapse metastases, the responses of 80 were documented: 18 patients (23%) had complete or partial responses, and these responders lived longer. For some patients, it was possible to resect the metastases and produce a new DNP-modified vaccine. The 29 patients who received DNP vaccine retreatment lived significantly longer after experiencing relapse than those who did not, but this effect was impossible to separate from the beneficial effect of having a resectable metastasis to begin with.

Unexpectedly, 5-year postrelapse survival was significantly greater in patients who had developed a positive DTH response (≥ 5 mm induration) to autologous, unmodified melanoma cells after the original vaccine treatment (positive DTH, 25.2%; negative DTH, 12.3%; $P < .001$, log-rank test; Fig 7). This effect remained significant in a multivariate analysis that included the other significant variables (hazard ratio for positive *v* negative DTH = 0.488; range, 0.274 to 0.869; $P = .015$). This finding can be at least partially explained by the observation that patients with positive DTH to unmodified melanoma cells had a significantly higher likelihood of experiencing relapse with tumors that were resectable (positive DTH, 62% resectable; negative DTH, 41% resectable; $P = .022$, Fisher's exact test). Postrelapse survival was not affected by the magnitude of the DTH response to DNP-modified autologous tumor cells.

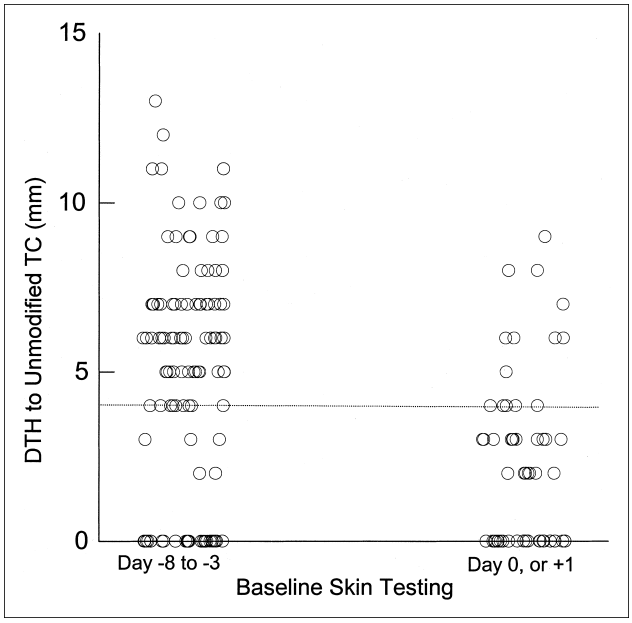


**Fig 5.** Delayed-type hypersensitivity (DTH) responses to unmodified autologous melanoma tumor cells (TC) stratified by timing of the baseline skin test (induction dose). Each marker represents an individual patient.

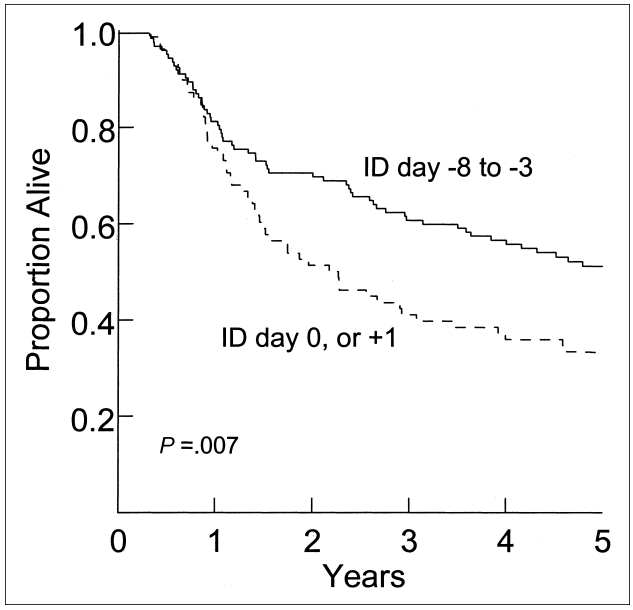


**Fig 6.** Overall survival of patients with stage III melanoma treated with autologous, dinitrophenyl-modified vaccine stratified by timing of the baseline skin test. ID, induction dose.

**Table 8.** Effect of Patient-Related and Treatment-Related Variables on Postrelapse Survival

| Parameter | No. of Patients | 5-Year Postrelapse Survival (%) | P* |
|---|---|---|---|
| Time to relapse, months | | | **< .001** |
| < 6 | 77 | 11.7 | |
| ≥ 6 | 71 | 20.5 | |
| Site of relapse | | | **.001** |
| Subcutaneous or lymph node | 83 | 24.9 | |
| Visceral | 65 | 4.8 | |
| Relapse resectable | | | **< .001** |
| Yes | 71 | 29.8 | |
| No | 77 | 3.9 | |
| Response to chemotherapy | | | **.001** |
| CR or PR | 18 | 11.1 | |
| None | 62 | 3.5 | |
| Vaccine re-treatment | | | **< .001** |
| Yes | 29 | 37.4 | |
| No | 119 | 10.5 | |
| DTH to unmodified TC, mm | | | **< .001** |
| < 5 | 63 | 12.3 | |
| ≥ 5 | 58 | 25.2 | |
| DTH to DNP-modified TC, mm | | | .636 |
| ≤ 20 | 65 | 18.2 | |
| > 20 | 63 | 20.2 | |
| No. of positive nodes | | | .621 |
| Mass only | 40 | 18.0 | |
| Mass + ≥ 1 microscopic | 108 | 15.2 | |

Abbreviations: CR, complete response; PR, partial response; DTH, delayed-type hypersensitivity; TC, tumor cells; DNP, dinitrophenyl.
*Log-rank test; *P* values < .10 are bolded.

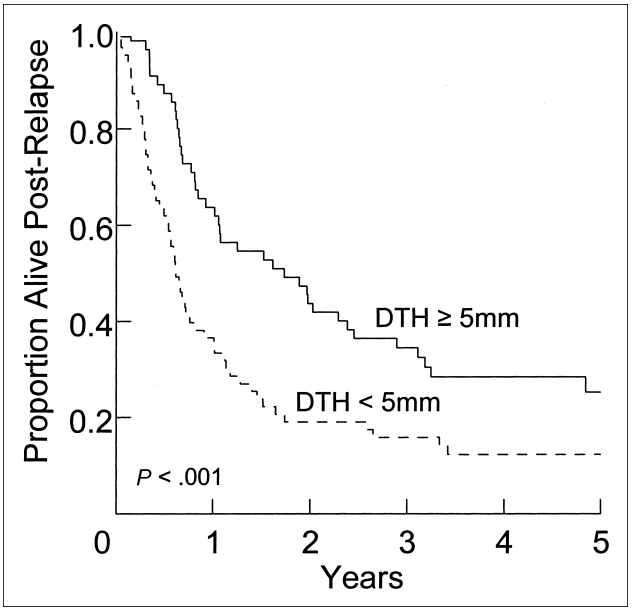


**Fig 7.** Survival of patients who experienced relapse (from date of relapse to date of death or date of last follow-up) stratified by peak delayed-type hypersensitivity (DTH) response to unmodified autologous melanoma cells.

### *Toxicity*

All patients developed reactions at the vaccine injection sites consisting of pruritic papules that progressed to pustules, sometimes with small ulcerations. The intensity of the reactions was ameliorated by reducing the dose of BCG. Less than 5% of patients noted fever or chills after vaccine administration, and no patient experienced a decrease in performance status attributable to vaccine administration. We observed no clinical evidence of autoimmunity. No patients developed depigmentation after treatment.

## DISCUSSION

Our approach to active immunotherapy of cancer is based on two principles: first, autologous tumor cells are the optimal source of tumor antigens; second, hapten modification is an effective way to increase their immunogenicity. The first premise is strongly supported by the classic literature of tumor immunology [10] as well as by more recent work suggesting that antigens shared by tumors of the same histology may not make effective vaccines [11].

Hapten modification is a venerable immunologic trick that allows the generation of an immune response against proteins that are otherwise not immunogenic [24]. Several animal models confirm that immunization with hapten-modified tumor cells confers protection against challenge with unmodified tumor cells [25]. Most recently, Sojka et al [13], using the highly metastatic 410.4 murine mammary carcinoma, reported that administration of a vaccine consisting of irradiated tumor cells haptenized with DNP caused significant prolongation of RFS, whereas an unmodified vaccine was ineffective. The molecular basis of the hapten effect has been elucidated by Martin et al [20]. They have demonstrated that immunization of mice with trinitrophenyl peptides induced cytotoxic T cells that recognized unmodified peptides. They hypothesized that haptenization increases the binding of autoreactive T cells to self peptides, and, once activated, these T cells react with unmodified peptides as well.

Previously, we reported the immunologic and clinical results of 77 patients with clinical stage III melanoma treated with autologous, DNP-modified vaccine after lymphadenectomy [16]. This article represents a final update of the initial studies, the sample size having been increased to 214 patients and almost all surviving patients having completed the planned 5-year follow-up. There are two major findings, each of which provides insight into the immunopharmacology of the vaccine: first, the development of a positive DTH response to unmodified, autologous melanoma cells after vaccine administration is a significant determinant of clinical outcome; second, the development of a positive DTH response is, to a great extent, determined by schedule of vaccine administration.

Patients who developed a positive DTH to unmodified melanoma cells had 5-year RFS and OS that were double those of patients with negative DTH responses (59% *v* 29%, respectively). This effect size is actually larger than what we originally reported, and we interpret it as indicating that the vaccine works by inducing T-cell–mediated immunity to as yet unidentified melanoma antigens. However, we have performed additional analyses to be certain that interpretation of these data was not obscured by technical or statistical artifacts.

The major technical artifact in the use of autologous tumor cells for DTH testing is the presence of immunogenic contaminants, especially heterologous serum and the enzymes used for tumor dissociation. We did not use serum in the preparation of the vaccines, but the cells were exposed to collagenase (bacterial origin) and DNAse (bovine origin). In a previous study in which patients were administered an autologous melanoma vaccine that was not hapten-modified, we observed large DTH responses to both collagenase and DNAse in almost all patients after treatment [26], apparently owing to trace amounts of enzyme remaining on the cells after the tumor dissociation process. To avoid that problem, the patients described in this article were skin-tested with autologous tumor cells obtained by mechanical dissociation (without enzymes) as well as with enzymatically dissociated cells, and the two preparations produced almost identical responses. Surprisingly, only 14% of patients who received DNP-modified vaccine developed DTH to the enzymes, as measured by skin testing with enzyme-coated autologous peripheral-blood mononuclear cells, and most of them had been treated by dosage schedule B, which included immunization with unmodified as well as DNP-modified melanoma cells. These data suggest that DNP modification of tumor cells either removes the contaminating enzymes or renders them nonimmunogenic.

Statistical artifacts interfering with interpretation of DTH results are more subtle. It is possible that patients who developed positive responses survive longer because they are healthier to begin with. We have addressed this issue by analyzing the effect of other immunologic parameters on clinical outcome and by performing multivariate analyses. In contrast to the significant effect of DTH to unmodified melanoma cells, survival was not affected by the responses to DNP-modified tumor cells or to PPD. Moreover, a positive DTH response to unmodified tumor cells remained a statistically significant determinant of both RFS and OS in multivariate analyses that included the important patient-related variables: sex, number of positive lymph nodes, and presence or absence of extranodal extension.

An unexpected finding was the significant effect of DTH to unmodified tumor cells on postrelapse survival. Patients who had developed positive DTH after their initial vaccine administration had a 5-year postrelapse survival rate that was double that of patients who had negative DTH responses (25% *v* 12%, respectively). This finding can be at least partially explained by the observation that patients with positive DTH to unmodified melanoma cells had a significantly higher likelihood of experiencing relapse with tumors that were resectable. Thus it is possible that the induction of antitumor immunity changes the biology of the disease so that the growth of metastases is modulated even when not prevented.

It is noteworthy that several other investigators have now reported that DTH is a meaningful measure of tumor immunity in cancer patients undergoing immunotherapy. Chang et al [27] found that eight of 10 and four of 10 patients developed $\geq$ 5 mm DTH reactions to keyhole limpet hemocyanin and autologous tumor cells, respectively, after administration of dendritic cells loaded with autologous lysates and the hemocyanin. Lee et al [28] showed that injection of a vaccine consisting of the melanoma-associated peptide gp100 induced a DTH response to this peptide in 85% of patients and that the response was augmented by coadministration of interleukin-12. Finally, Disis et al [29] reported that immunization with a HER-2/*neu* peptide vaccine induced DTH to the peptide that seemed to be correlated with in vitro T-cell proliferative responses.

The second major finding of this study was the importance of the vaccine dosage schedule on the immunologic response. Patients treated on schedules A and D developed significantly greater DTH responses to unmodified, autologous melanoma cells than those treated on schedules B and C. This difference was not due to variations in vaccine dose, but rather to the schedule of administration—apparently, to the timing of what we are calling an induction dose. This is defined as the intradermal injection of DNP-modified autologous melanoma cells without BCG before or simultaneously with cyclophosphamide. When these protocols were designed, the purpose of this injection was to serve as a baseline skin test, so therefore its timing was not considered critical. However, a retrospective analysis indicated that patients who received baseline skin testing 3 to 8 days before cyclophosphamide administration (day $-3$ to $-8$) subsequently developed significantly larger DTH responses than those who received baseline skin testing on the day of cyclophosphamide administration (day 0) or one day after (day $+1$). Moreover, the timing of the induction dose seemed to affect clinical outcome in that 5-year OS was significantly greater in the day $-3$ to $-8$ group, even after a multivariate analysis.

Given the number of treatment variables that differentiated the four dosage schedules, the induction dose phenomenon should be viewed as a hypothesis until more definitive clinical data are obtained. However, it is of interest that it has been reproduced in an animal model: in the 410.4 murine mammary carcinoma model described above, the administration of an induction dose 7 days before cy-

clophosphamide further increased RFS (M.B. Mokyr, personal communication, 2002). Moreover, there is a plausible immunologic explanation for this phenomenon based on melanoma-specific regulatory T cells (Tr) [8]. Administration of the induction dose (autologous melanoma cells without adjuvant) could induce the expansion of Tr cells, which are killed or functionally inhibited by the administration of cyclophosphamide 3 to 8 days later. Then, administration of vaccine 3 days after cyclophosphamide would allow the presentation of tumor antigens with an adjuvant in the absence of specific Tr. These events could increase the probability of generating an effective cell-mediated immune response.

An assessment of the clinical effectiveness of the autologous, DNP-modified melanoma vaccine requires a randomized trial. However, as phase II data, the results (44% 5-year OS) seem promising when compared with the outcomes of most published surgical series of patients with clinically detectable nodal metastases, which report 5-year survivals of 20% to 25% [30-32]. Even in the more recent large data analysis that formed the basis of the newest American Joint Committee on Cancer staging system, patients with stage IIIC disease, who comprised the majority of our patients, were reported to have 5-year OS of only approximately 25% [17]. Additional evidence that the DNP vaccine can induce clinically meaningful antitumor immunity is provided by our study of patients with measurable metastases, in which six of 83 patients had documented clinical responses [6].

The findings resulting from this work on an autologous, hapten-modified vaccine may be applicable to other human cancer vaccines. Whether vaccines are composed of whole cells, cellular extracts, purified proteins, peptides, or DNA, an understanding of their immunopharmacology is necessary for their clinical development. After all, vaccines are drugs, thus the way they are administered (eg, dose, schedule, and route of administration) is likely to be as important to their efficacy as their composition. These variables are best analyzed by using an immunologic end point that makes biologic sense and that is strongly predictive of clinical outcome.


## *Acknowledgment*

We thank Carmella Clark and Jason Kuchar for technical assistance and Ellen Bloome, research nurse.


## *Authors' Disclosures of Potential Conflicts of Interest*

The following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. Owns stock (not including shares held through a public mutual fund: David Berd, AVAX Technologies Inc. Acted as a consultant within the last 2 years: David Berd, AVAX Technologies Inc. Received more than $2,000 a year from a company for either of the last 2 years: David Berd, AVAX Technologies Inc.

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