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TREATMENT OF METASTATIC MELANOMA WITH AUTOLOGOUS, HAPTEN-MODIFIED MELANOMA VACCINE: REGRESSION OF PULMONARY METASTASES

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A human cancer vaccine composed of autologous tumor cells modified with the hapten dinitrofluorobenzene (DNP) induces cell-mediated immunity to the tumor cells and the development of inflammatory responses within metastatic sites. In this study we determined whether DNP vaccine could induce regression of established metastases. Ninety-seven patients (83 evaluable) with surgically incurable metastatic melanoma were treated with DNP vaccine preceded by low-dose cyclophosphamide. Tumor regression was assessed by standard criteria. The development of cell-mediated immunity to melanoma-associated antigens was measured by delayed-type hypersensitivity (DTH) testing before and after DNP vaccine treatment. Survival analysis was performed by the Kaplan-Meier method. There were 11 antitumor responses: 2 complete, 4 partial and 5 mixed. Both complete responses and 2 of the 4 partial responses occurred in patients with lung metastases. Response durations were as follows: partial responses—5, 6, 8 and 47+ months; and complete responses—12 and 29 months. Tumor regression required at least 4 months to become evident and in 2 cases maximum regression was not observed until 1 year after beginning treatment. Patients who exhibited tumor regression survived longer than those who did not (median survival times: responders, 21.4 months; non-responders, 8.7 months; $p = 0.010$). DTH to DNP-modified and unmodified autologous melanoma cells was induced in 87% and 42% of patients, respectively. The DTH response to unmodified cells was significantly associated with prolonged survival. Autologous DNP-modified melanoma vaccine can induce clinically meaningful regression of metastases and small lung metastases appear to be unusually sensitive. The development of DTH to unmodified, autologous tumor cells may be an important indicator of the vaccine's efficacy.

Key words: *melanoma; vaccine; immunotherapy; autologous; cyclophosphamide*

Studies performed and published over the past 30 years have made it clear that immunizing a tumor-bearing patient to his or her own tumor cells is exceedingly difficult. An appealing explanation for this difficulty is that tumor antigens are weakly immunogenic and often induce tolerance, *i.e.*, paralysis of the immune response, rather than effective immunity.[1]

Fortunately, advances in basic immunology have disclosed a number of ways of breaking immunologic tolerance. One way is the judicious use of cytotoxic drugs. In 1967, Maguire and Ettore[2] made the surprising discovery that administration of cyclophosphamide to animals prior to an immunization caused the cell-mediated immune response to be augmented rather than suppressed. We have shown that this phenomenon applies to cancer-bearing humans as well. Thus, the administration of low-dose (300 mg/m^2) cyclophosphamide prior to an immunization with autologous tumor cells greatly increased the development of cell-mediated immunity to those cells.[3]

A second approach to circumventing tolerance is the use of haptens—small molecules that are incapable of inducing an immune response unless they are coupled to a larger molecule. Coupling of a hapten to a protein, *i.e.*, hapten modification, often results in the induction of an immune response to the protein that would otherwise not be possible. For example, Weigle,[4] in a series of now classic experiments, immunized mice with hapten-modified thyroglobulin. This resulted in the induction of an immune response not only to the hapten-modified thyroglobulin, but also to unmodified, *i.e.*, native, thyroglobulin and produced autoimmune thyroiditis. A similar phenomenon was observed more recently by Neurath *et al.*[5] They found that application of the hapten dinitrophenyl to the colonic mucosa of mice resulted in the development of an autoimmune colitis, similar to human ulcerative colitis. Initially, these mice developed cellular immunity to the hapten-modified mucosa. However, as the chemically modified mucosa cells were shed, a potent immune response to the normal, unmodified mucosa developed, which led to the autoimmune pathology.

The immunologic basis of these interesting phenomena is now clear: there are T lymphocytes generated by immunization with hapten-modified cells that are reactive to both modified and unmodified cells. For example, class I MHC-restricted T-cell clones generated from mice immunized with TNP-modified syngeneic lymphocytes respond to MHC-associated, TNP-modified "self" peptides. Furthermore, some TNP-reactive clones respond to certain MHC-binding, unmodified peptides as well.[6]

These observations in animal models provide a strong rationale for a human cancer vaccine consisting of autologous tumor cells modified with a hapten, DNP, preceded by the administration of low-dose cyclophosphamide. We have observed that administration of DNP vaccine to patients with metastatic melanoma induces a unique reaction— the development of inflammation in metastatic masses.[7] Histologically, the response consists of infiltration of T lymphocytes, most of which are CD8+.[8] These T cells usually produce interferon-γ in situ.[9] Moreover, they represent expansion of T-cell clones with novel T-cell receptor structures.[10]

Previously we have reported that administration of DNP vaccine to melanoma patients with bulky, resectable nodal metastases produced relapse-free and overall survivals that appeared to be as

Abbreviations: BCG, bacille Calmette-Guérin; cfu, colony-forming unit; CR, complete response; DNFB, dinitrofluorobenzene; DNP, dinitrophenyl; DTH, delayed-type hypersensitivity; GM-CSF, granulocyte-macrophage colony-stimulating factor; LDH, lactate dehydrogenase; MHC, major histocompatibility complex; MR, mixed response; PBC, peripheral blood lymphocyte; PPD, purified protein derivative; PR, partial response; TNP, trinitrophenyl.

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good as or better than those reported with the standard postsurgical therapy, high-dose interferon-α.[11] In that study, the induction of DTH to autologous, unmodified melanoma cells was a significant predictor of clinical outcome. Here we extend the investigations to patients with surgically incurable melanoma metastases. We show that clinically significant regression of metastases, particularly small pulmonary metastases, can be achieved and we confirm that the induction of DTH to unmodified melanoma cells correlates with survival.

MATERIAL AND METHODS

Patients

The study population consisted of 97 patients with metastatic melanoma. Their clinical characteristics are summarized in Table I. The criteria for inclusion in the study were as follows: (*i*) metastatic melanoma that was not surgically curable (the 6 patients with stage III disease had had regional metastases that could not be resected—3 with nodal metastases and 3 with extensive regional skin metastases); (*ii*) at least 1 large (≥2.5 cm diameter) metastasis that could be excised and used for vaccine preparation; (*iii*) Karnovsky performance status of ≥ 70; and (*iv*) life expectancy ≥ 6 months. The major criteria for exclusion were as follows: (*i*) brain metastases; (*ii*) need for corticosteroids; (*iii*) radiation therapy within the preceding 6 months; and (*iv*) cytotoxic chemotherapy within the preceding 2 months.

Patients were considered evaluable only if they completed at least 2 months of the DNP vaccine program with pre- and post-treatment skin testing. By this definition, 14 patients were considered unevaluable. Four of the unevaluable patients received no vaccine injections and 6 others received only 2 or 3 of the intended 6 weekly treatments. Thus, 83 patients were evaluable for clinical and immunologic results.

Tumor processing and vaccine preparation

Metastatic tumor was excised, maintained at 4°C and delivered to the laboratory within 48 hours of excision. The tumors were processed as previously described.[3] In brief, cells were extracted by enzymatic dissociation with collagenase and DNAse, aliquoted, frozen in a controlled rate freezer and stored in liquid nitrogen in a medium containing human albumin and 10% dimethylsulfoxide until needed. On the day that a patient was to be treated, an aliquot of cells was thawed, washed and irradiated to 2500 cGy. Then they were washed again and modified with DNP by the method of Miller and Claman.[12] This involves a 30-minute incubation of tumor cells with DNFB, followed by washing with saline.

Each vaccine consisted of 2.5–25 × 10^6 intact (trypan blue excluding) tumor cells suspended in 0.2 ml Hanks' solution. There were variable numbers of lymphocytes and dead cells in all specimens and the mean cell composition was as follows: intact tumor cells, 31 ±2%; lymphocytes, 27 ± 2%; and dead cells, 42± 2%. After mixing with BCG (see below), the suspension was injected intradermally into 3 adjacent sites, usually on the upper dorsal arm, excluding sites ipsilateral to a lymph node dissection.

TABLE I – CLINICAL CHARACTERISTICS OF STUDY POPULATION

	All	Evaluable
Total	97	83
Sex		
Men	53	43
Women	44	40
Age [median (range)]	55 (28–81)	56 (28–81)
Metastatic site		
Skin	24	23
Nodes, other soft tissue	18	15
Lung only	23	18
Lung + soft tissue	12	12
Other visceral	20	15
LDH		
Normal	65	59
High	25	17
Prior treatment		
Chemotherapy	41	33
Dartmouth regimen	35	27
Radiation therapy	10	9
Interferon-α	13	9
Other vaccines	5	5
None	51	47
Karnovsky performance status	90 (70–100)	90 (70–100)
Stage		
III	6	6
IV	91	77

Vaccine administration

The studies were approved by the Institutional Review Board of Thomas Jefferson University and informed consent was obtained from all patients. Four vaccine dosage schedules were tested sequentially, as follows: schedule A, October, 1988 to March, 1993; schedule B, April, 1993 to March, 1994; schedule C, April, 1994 to August, 1995; and schedule D, September, 1995 to March, 1999. The dosage schedules are summarized in Table II.

For schedules A, B and C, patients were initially 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 IV rapid infusion was given 3 days prior to DNFB application. For schedule A, DNP vaccine mixed with BCG (Tice, Organon Teknika, Durham, NC) was administered every 4 weeks for a total of 8 doses; cyclophosphamide 300 mg/m^2 was administered 3 days before the first and second doses. All vaccine injections were given in the same site on a limb (usually the upper dorsal arm) that had not been subjected to a lymph node dissection. For schedule B, vaccine was administered weekly for 6 weeks; after a 4-week reevaluation period, vaccine was again administered weekly for 6 weeks. The first 3 vaccines of each course were DNP-modified and the last 3 were unmodified. BCG was admixed only with the 1st and 4th vaccine of each course. All the DNP vaccine injections were given into 1 area and all the unmodified vaccine injections were given into a second area. Cyclophosphamide 300 mg/m^2 was administered 3 days prior to 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 1 series of 6 weekly DNP-modified vaccines was administered; we have reported that this dosage schedule is particularly efficient at inducing DTH to autologous melanoma cells.[13]

For all dosage schedules the dose of BCG was progressively attenuated to produce a local reaction consisting of an inflammatory papule without ulceration. The attenuation schedule was as follows:

#1 0.1 ml of a 1:10 dilution (1–8 × 10^6 cfu)

#2 0.1 ml of a 1:100 dilution (1–8 × 10^5 cfu)

#3 0.1 ml of a 1:1000 dilution (1–8 × 10^4 cfu)

Because of the progressive development of cell-mediated immunity, most patients were receiving the lowest BCG dose by the 5th vaccine injection.

Clinical response criteria

Standard criteria were used, as follows: complete response, complete disappearance of all metastases for at least 3 months without the growth of other lesions or the development of new metastases; partial response, ≥50% reduction in the mean diameter of a measurable metastasis for at least 3 months without the growth of other lesions or the development of new metastases; mixed response, ≥50% reduction in the mean diameter of a measurable metastasis with concomitant growth of another metastasis; and stable, <25% increase in the mean diameter of any measurable metastasis. All patients were followed in the Melanoma Clinic of Thomas Jefferson University.

TABLE II – SUMMARY OF VACCINE DOSAGE-SCHEDULES

Schedule[1]	No. of patients	No. of doses cyclophosphamide	DNFB sensitization	Vaccine schedule	Vaccine dosage range	BCG mixed with:
A	20	3	+	q 28 days × 8	$5.0–25.0 \times 10^6$	Every dose
B	13	3	+	weekly × 12	$5.0–25.0 \times 10^6$	1/3 of doses
C	8	3	+	weekly × 12	$5.0–25.0 \times 10^6$	Every dose
D	42	1	−	weekly × 6	$2.5–7.5 \times 10^6$	Every dose

[1]*Schedule A:* Cyclophosphamide 300 mg/m^2 iv 3 days before DNFB and 3 days before first two dose of vaccine.–*Schedule B:* Cyclophosphamide 300 mg/m^2 iv 3 days before DNFB and 3 days before each 6-week series of vaccine; half of the vaccines were DNP-modified and half unmodified.–*Schedule C:* Cyclophosphamide 300 mg/m^2 iv 3 days before DNFB and 3 days before each 6-week series of vaccine; all vaccines were DNP-modified.–*Schedule D:* Cyclophosphamide 300 mg/m^2 iv 3 days before first vaccine only; booster injections of vaccine were given at 6 and 12 months.

TABLE III – ANTITUMOR RESPONSES

Response	Metastatic site				Total
	Skin	Other soft tissue	Lung	Other viscera	
Complete	0	0	2	0	2
Partial	2	0	2	0	4
Mixed	3	1	1	0	5
Stable	0	0	2	0	2
None	18	14	23	15	70
Total	23	15	30	15	83

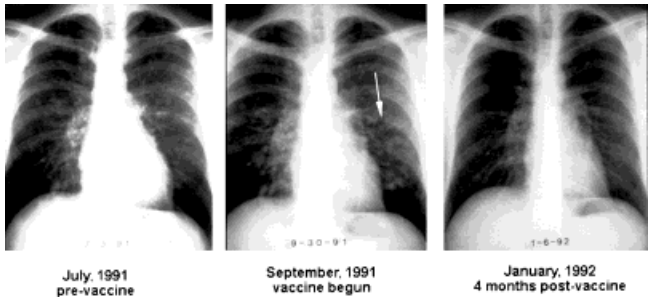

FIGURE 1 – Regression of lung metastases in patient #20063. Multiple lung metastases are seen in July, 1991, most prominently in the left lower lobe (arrow), which had increased in size and number in the September, 1991 x-ray. Four months after beginning DNP vaccine treatment, the metastases have completely regressed.

DTH testing

Patients were tested for DTH by a standard method that we have previously described.[3] Cryopreserved, mechanically dissociated melanoma cell suspensions and PBLs were thawed, washed and irradiated (2500 cGy). DNP modification of melanoma cells and PBLs was performed as described above. Melanoma cells (1×10^6) and PBLs (3×10^6), each either DNP-modified or unmodified, were suspended in Hanks' balanced salt solution without serum, phenol red or antibiotics and injected intradermally into the ventral forearm. The mean diameter of induration was measured after 48 hr. A positive response was defined as maximum diameter of induration $\geq$ 5 mm. Patients were also skin-tested with intermediate strength PPD (5 TU). DTH testing was performed before the treatment program was initiated and at various times post treatment (schedule A, 2 weeks after second monthly vaccine; schedules B, C and D, 2½ weeks after 6th weekly vaccine). Only 1 patient exhibited a small DTH response to autologous, unmodified PBLs after treatment, which excludes the possibility of spurious responses to the cryopreservation medium or its components.

Statistics

Survival was measured from the date that patients began the vaccine program (date of initial skin testing). 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

Antitumor responses

Among the 83 evaluable patients there were 11 responses—2 complete, 4 partial and 5 mixed; 2 patients were judged to have stable disease. All responders had stage IV disease. As shown in Table III, both complete responses and 2 of the 4 partial responses occurred in patients with lung metastases. Response durations were as follows: partial responses, 5, 6, 8 and 47+ months; complete responses, 12 and 29 months.

Summary of patients with clinical responses

Patient #20063 (CR), a 28-year-old man, developed multiple bilateral lung metastases shortly after tumor tissue had been obtained from a regional lymph node metastasis (Fig 1, first panel). These metastases increased in size and number just prior to starting DNP vaccine treatment (Fig.1, second panel). After a course of DNP vaccine administration (schedule B), the appearance of the metastatic nodules was unchanged (not shown). However, 2 months later, the metastases had completely regressed (Fig. 1, third panel),. The patient remained tumor-free until 1 year later when mediastinal and hilar recurrence was noted. His survival from the beginning of DNP vaccine treatment was 34.5 months.

Patient #20254 (CR), a 77-year-old man, presented simultaneously with a regional lymph node metastasis in the neck and 2 cm diameter mass in the lung adjacent to the cardiac border that increased in size over 2 months of observation (Fig. 2*a*). At 5 months after beginning DNP vaccine treatment (schedule D), the same mass was thought to be slightly smaller (Fig. 2*b*). The mass continued to regress showly and by the 2-year point it had regressed completely (Fig.2*c*). After a remission of 29 months, the patient developed recurrent melanoma in the anterior mediastinum, which was incompletely resected. His overall survival was 48.4 months.

Patient #20294 (PR), a 77-year-old woman, underwent resection of what appeared to be a solitary lung metastasis from a primary melanoma in the paranasal sinus. However, postoperative evaluation showed several new lung metastases (Fig. 3*a*). Following DNP vaccine administration (schedule D), the nodules gradually regressed completely (Fig. 3*b*,*c*). However, before regression was complete, the patient developed a solitary brain metastasis, which was resected. The patient remains alive with disease at 47 months after beginning DNP vaccine treatment.

Patient #20295 (PR), a 64-year-old man, developed multiple lung metastases from a primary head and neck melanoma. These partially regressed after combination chemotherapy with the Dartmouth regimen,[14] and a residual tumor mass was resected. The patient then developed a large recurrent pulmonary metastasis, which was resected and used for vaccine preparation. Postoperative evaluation showed 2 new lung nodules (Fig. 4*a*). Following DNP vaccine treatment (schedule D), 1 nodule regressed completely (not shown) and the second, larger, nodule regressed by about 75% (Fig. 4*b*). The patient remains alive and well at 47

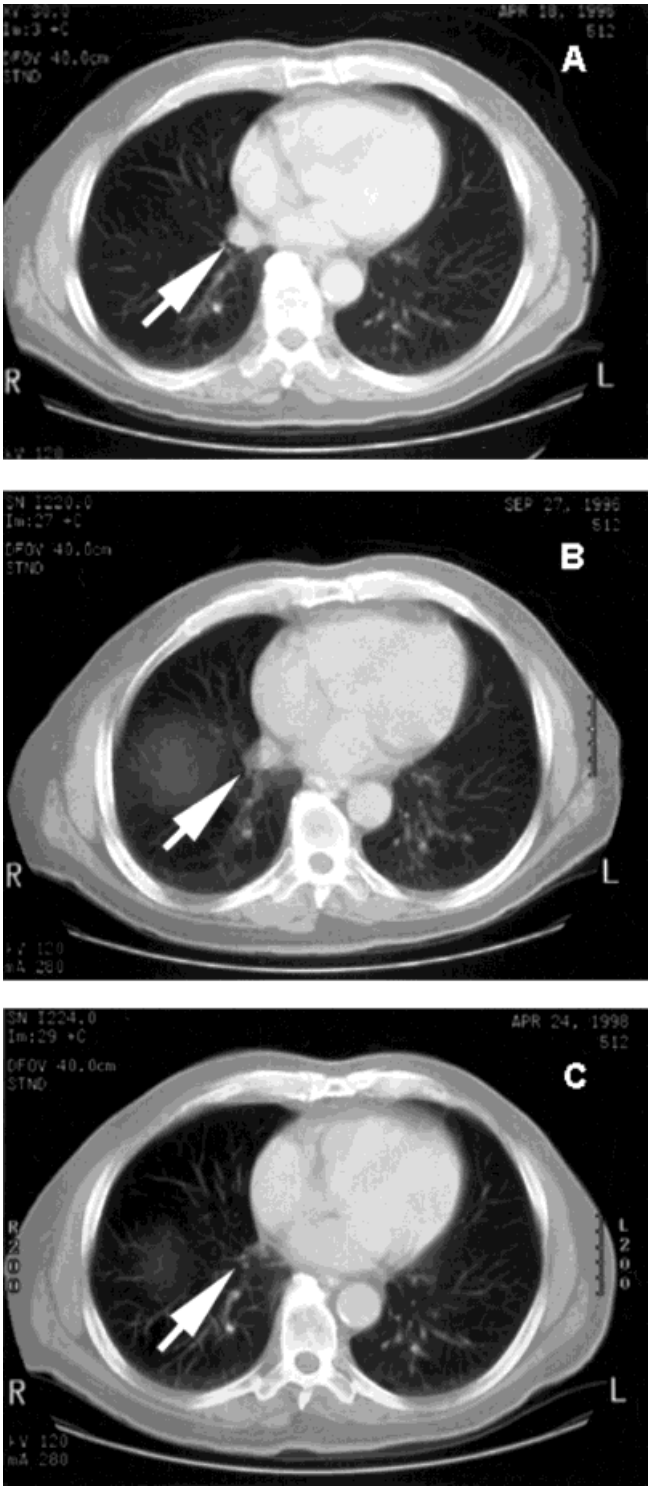

FIGURE 2 – Regression of lung metastasis in patient #20254. (*a*) April, 1996, prevaccine. A 2 cm diameter mass is seen adjacent to the cardiac boarder (arrow). (*b*) September, 1996, 5 months after beginning DNP vaccine treatment. The mass is slightly smaller. (*c*) April, 1998, 2 years after beginning DNP vaccine treatment. The mass has completely regressed.

months after beginning treatment. Subsequent computed tomography scans up to the present continue to show only a very small nodule at the known tumor site (Fig. 4*c*); it is not clear whether or not this represents residual tumor.

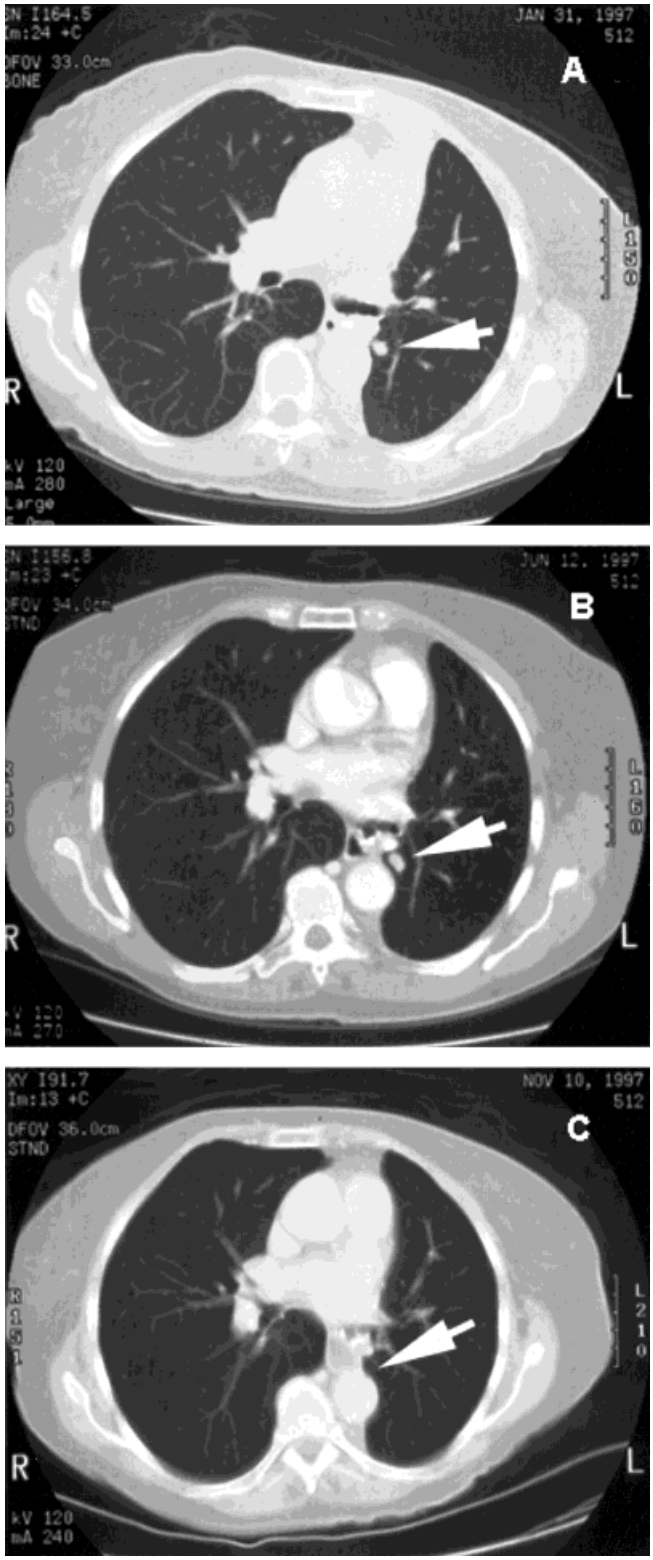

FIGURE 3 – Regression of lung metastasis in patient #20294. (*a*) January, 1997, prevaccine. A 1 cm diameter metastasis is seen in the left upper lobe adjacent to the descending aorta (arrow). (*b*) June, 1997, 5 months after beginning DNP vaccine treatment – The mass is unchanged. (*c*) November, 1997, 10 months after beginning DNP vaccine treatment. The mass has regressed. A second metastasis regressed as well (not shown).

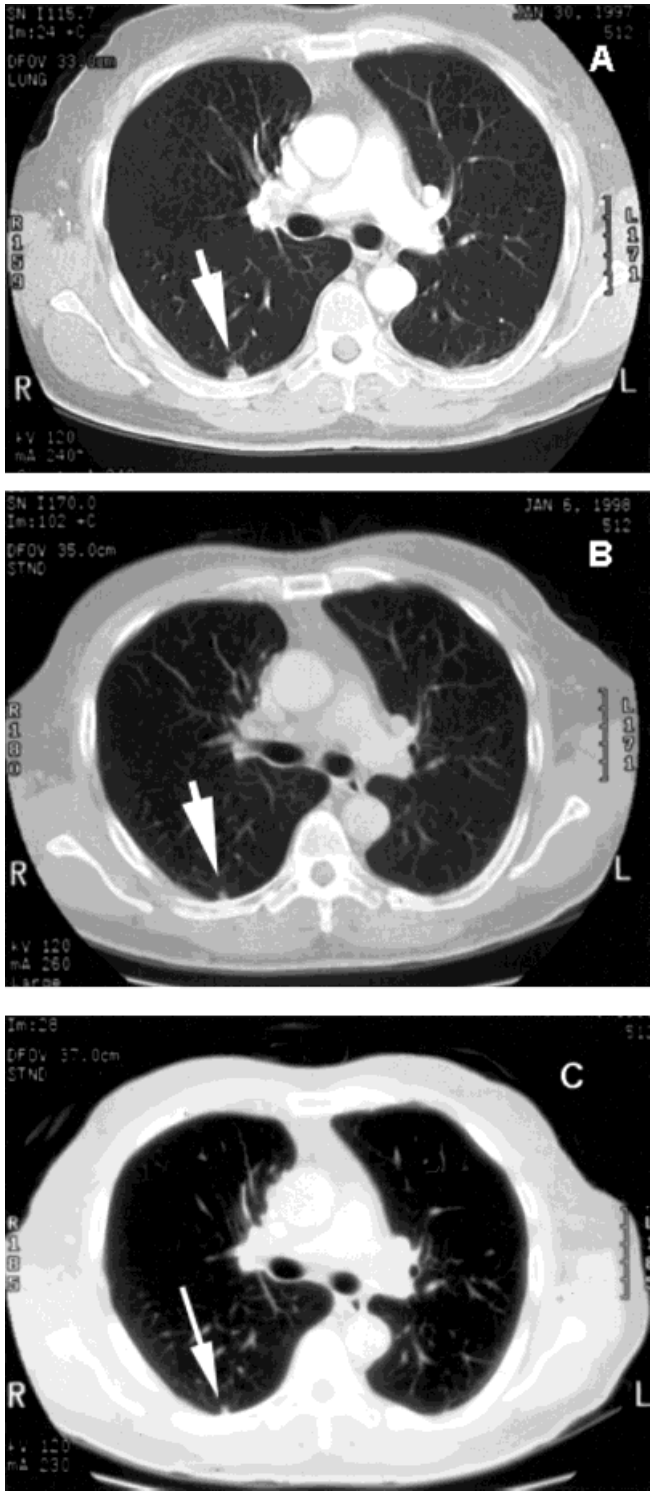

FIGURE 4 – Regression of lung metastasis in patient #20295. (*a*) January, 1997, prevaccine. A 1.2 cm metastasis is seen in the periphery of the right lower lobe. (*b*) January, 1998, 1 year post vaccine. The mass has decreased in size by about 75%. (*c*) January, 1999, 2 years post vaccine. The appearance of the mass remains stable.

These examples of regression of lung metastases are noteworthy because the regressions developed slowly and only after a latent period. Evidence of response required at least 4–5 months and in 2 of these cases maximum regression required at least 1 year.

Tumor inflammatory responses

We have reported that patients treated with DNP vaccine develop inflammatory responses in superficial metastatic sites.[7] This finding was confirmed in this expanded study: 27/83 patients (33%) were observed to have tumor inflammation. In most cases, inflamed tumors were subcutaneous metastases. An example was patient #20297, who developed clinically evident inflammation in 2 subcutaneous metastases on his abdomen and inguinal area 4–5 months after starting treatment with DNP vaccine. These tumors were excised and the histopathology is illustrated in Figure 5. The tumor tissue is densely infiltrated with lymphocytes that were shown to be CD3+ by immunohistochemistry (not shown).

Delayed-type hypersensitivity responses

The results are summarized in Table IV.These data represent the maximum DTH responses to each test material. The median time to development of a maximum DTH response was 8 weeks, *i.e.*, after 2 monthly vaccine administrations for schedule A or after 1 course of 6 weekly vaccines for schedules B, C and D. Only 7 patients exhibited small DTH responses (5–6 mm) to autologous melanoma cells prior to vaccine treatment.

Almost all patients developed a strong PPD response, which indicates that they were sufficiently immunocompetent to respond to a strong antigen. Most patients also exhibited a large DTH response to DNP-modified autologous melanoma cells. As expected, a lower proportion of patients developed DTH to unmodified autologous melanoma cells. The proportions of positive responses and their magnitude are similar to those we have reported for patients with micrometastatic disease treated with DNP vaccine.[11]

The high frequency of DTH to DNP-modified autologous peripheral blood lymphocytes has been previously reported as well;[11] it indicates that patients have been immunized to 1 or more DNP-modified normal proteins. However, only 1 patient developed a small (6 mm diameter) DTH to unmodified autologous lymphocytes.

Survival analysis

As shown in Table V, univariate analysis of patient-related variables showed reduced survival for patients who had been previously treated with interferon-α. There was a trend to reduced survival for patients who had previously received cytotoxic chemotherapy. As noted by other studies,[15] patients with elevated LDH (≥10% increase over normal value) at the time of beginning vaccine had significantly shorter survival than those with normal levels.

Table VI shows the results of univariate analysis of the impact of treatment-related variables. Patients who had an antitumor response (complete, partial or mixed) had a significantly longer survival time, about 2.5 times that of nonresponders (Fig. 6). The development of larger DTH responses to autologous melanoma cells or to PPD was associated with longer survival. However, only the impact of the DTH response to *unmodified* autologous melanoma cells was statistically significant ($p = 0.023$). Neither mean vaccine dose nor dosage schedule determined survival. The importance of dosage schedule to induction of immunologic and clinical effects has been elucidated in a larger series of studies of patients treated with DNP vaccine following complete resection of bulky regional lymph node metastases.[11,16]

The induction of a tumor inflammatory response is characteristic of the autologous DNP-modified melanoma vaccine.[7] In this study, patients whose metastases became inflamed did survive longer than those without tumor inflammation, but the difference was of borderline significance.

Multivariate analysis was performed by adding each of the treatment-related variables to a basic model consisting of all of the patient-related variables (sex, age, metastatic site, LDH, prior chemotherapy, prior interferon, prior radiation, prior vaccine therapy). Antitumor response (complete, partial or mixed versus stable

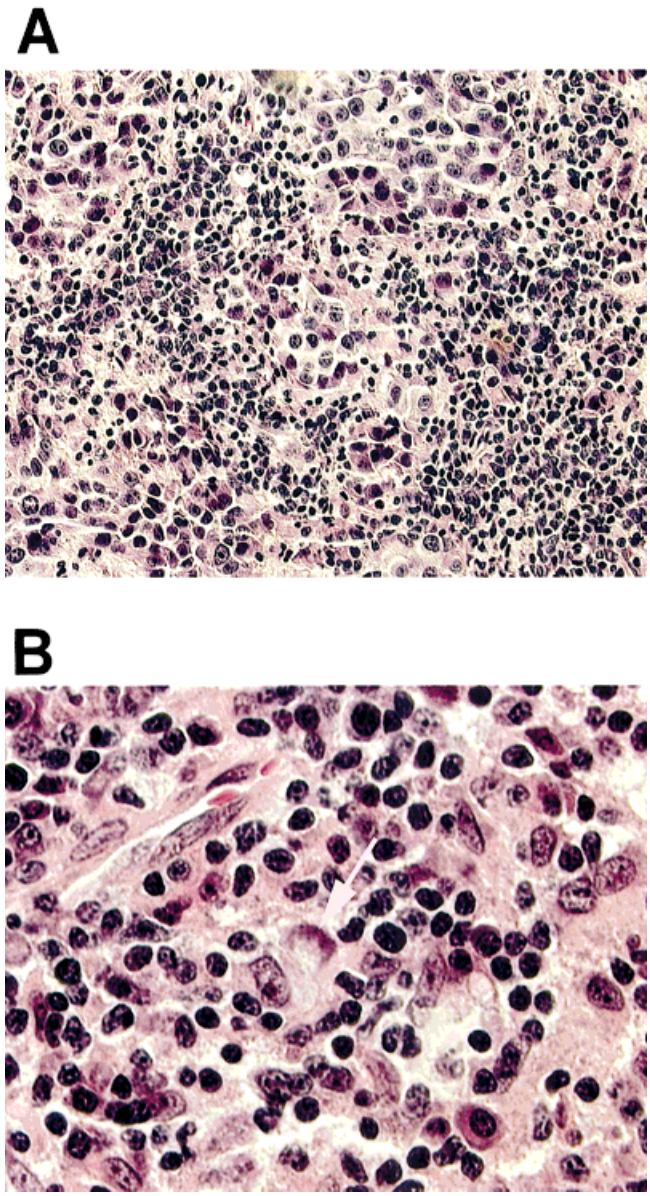

FIGURE 5 – Photomicrograph showing inflammatory response in subcutaneous metastasis excised from patient #20297. The clinically inflamed tumor was excised 5 months after beginning treatment with DNP vaccine. (*a*) The tumor tissue is intensely infiltrated with lymphocytes. (*b*) Necrosis of individual tumor cells is apparent (arrow). Original magnification × 200 (*a*); ×600 (*b*).

TABLE IV – DTH RESPONSES

Response	No. or %
Peak DTH to unmodified melanoma cells (mechanically dissociated)	
Median (range)	4 (0–15)
% ≥ 5 mm	42
% ≥ 10 mm	8
Peak DTH to DNP-modified melanoma cells	
Median (range)	15 (0–80)
% ≥ 5 mm	87
% ≥ 10 mm	74
Peak DTH to DNP-modified lymphocytes	
Median (range)	8 (0–120)
% ≥ 5 mm	68
% ≥ 10 mm	42
Peak DTH to unmodified lymphocytes	
Median (range)	0 (0–6)
% - 5 mm	1
% ≥ 10 mm	0
Peak DTH to PPD	
Median (range)	23 (0–60)
% ≥ 10 mm	94
% ≥ 20 mm	68

TABLE V – UNIVARIATE ANALYSIS OF PATIENT-RELATED PROGNOSTIC VARIABLES

Factor	No. of patients	Median survival (mo)	*p*-value*
Age (yr)			
≤ 50	32	8.7	
> 50	51	12.1	0.394
Sex			
M	43	8.7	
F	40	11.3	0.869
Prior vaccine			
No	78	9.5	
Yes	5	11.8	0.295
Prior interferon			
No	74	11.4	
Yes	9	4.5	**0.047**
Prior chemotherapy			
No	50	11.9	
Yes	33	8.6	**0.081**
Prior radiation			
No	74	11.4	
Yes	9	8.5	0.146
Metastatic site			
Skin	23	12.9	
Nodes	15	8.9	
Lung	30	9.9	
Other visceral	15	6.9	0.154
LDH			
Normal	59	12.2	
Increased	17	6.5	**0.001**

*p values (log rank test) ≤ 0.100 are in bold face.

or none) remained a highly significant predictor of survival (hazard ratio, 0.242; 95% confidence interval, 0.098–0.600, $p = 0.002$). The effect of DTH to unmodified autologous melanoma cells (≥5 vs. <5 mm) was of borderline significance (hazard ratio, 0.562; 95% confidence interval, 0.294–1.070, $p = 0.080$).

Toxicity

Toxicity was similar to what we have previously reported.[11] There were no serious medical events. A local inflammatory response consisting of papules or pustules with small ulcerations developed at the vaccine injection sites, which were primarily due to BCG. These sites healed in about 3 months, leaving small scars. Less than 5% of patients complained of fever or malaise lasting for 24 hr following individual vaccine injections. About 25% of the patients experienced nausea, sometimes with grade I vomiting following administration of cyclophosphamide; this was easily controlled by use of prochlorperazine. There were no significant changes in blood counts or routine serum chemistries. There was neither clinical nor laboratory evidence of autoimmunity; specifically. Vitiligo was never observed.

DISCUSSION

The clinical application of active immunotherapy to human cancer is now about 30 years old,[17] an awkward age for a man or for an idea. Immunotherapy no longer generates the enthusiasm and optimism of its youth, but it has not matured sufficiently to takes its place in the conceptual framework of human tumor biology. Depending on whether one is a proponent or a skeptic,

TABLE VI – UNIVARIATE ANALYSIS OF TREATMENT-RELATED VARIABLES

Factor	No. of patients	Median survival (mo)	*p*-value[1]
Dosage schedule			
1	20	12.1	
2	13	8.6	
3	8	11.9	
4	42	8.0	0.758
Mean vaccine dose			
$\leq 7.5 \times 10^6$	50	9.5	
$> 7.5 \times 10^6$	33	11.3	0.875
Tumor response			
C-P-M[2]	11	21.4	
None	72	8.7	**0.010**
Tumor inflammation			
Yes	27	14.5	
No	56	8.4	**0.080**
DTH to unmodified tumor cells			
<5 mm	30	8.4	
≥5 mm	22	16.5	
nd	31	8.6	**0.023**
DTH to DNP-modified tumor cells[3]			
<20 mm	24	7.9	
≥20 mm	30	14.5	
nd	29	8.4	**0.074**
DTH to DNP-modified lymphocytes[3]			
<10 mm	42	8.6	
≥10 mm	30	12.1	
nd	11	6.9	0.446
DTH to PPD[3]			
<20 mm	28	6.8	
≥20 mm	40	12.1	
nd	15	9.5	**0.055**

[1]*p*-values (log rank test) ≤ 0.100 are in bold face.–[2]C, complete response; P, partial response; M, mixed response.–[3]These breakpoints were chosen because they approximated the median values for each parameter.

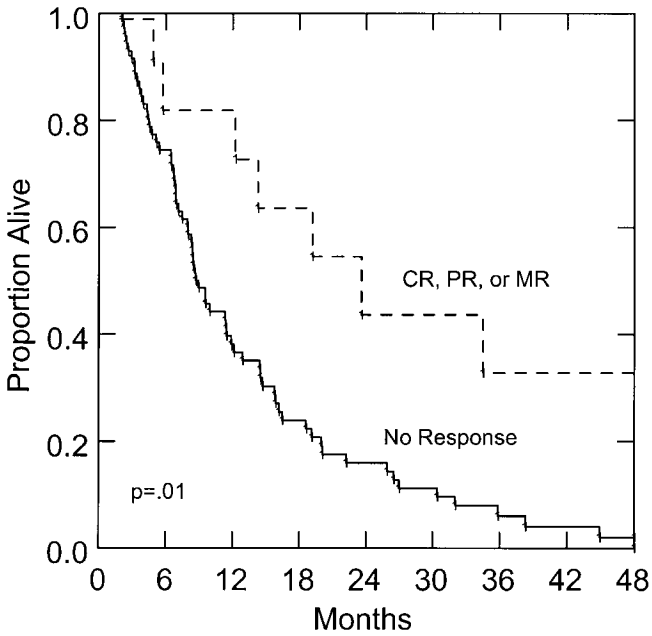

FIGURE 6 – Effect of antitumor response on survival. The Kaplan-Meier plot compares the survival of patients who had an antitumor response (CR, PR, MR; $n = 11$) versus those who did not respond ($n = 72$). The curves were analyzed by a log-rank test. Survival was measured from the date that patients began the vaccine program (date of first skin test application).

human tumor immunology could be said to be in development or in limbo.

Conventional teaching dictates that active immunotherapy is most suitable for patients with micrometastases. Proof of efficacy in the postsurgical adjuvant setting requires large, randomized clinical trials and few of the currently available cancer vaccine technologies have been subjected to such trials. However, vaccines have been studied in a more difficult clinical setting—patients with surgically incurable metastases. Despite the animal data suggesting that tumor vaccines shouldn't work well against established tumors, there are a number of reports of "positive" clinical trials in patients with metastatic solid tumors, mainly melanoma.

In 1 of the earliest papers, Laucius *et al.*[18] observed tumor regression in 4/18 patients after treatment with an autologous, whole-cell vaccine mixed with BCG. Our group continued this work by adding low-dose cyclophosphamide as a pretreatment 3 days before the vaccine injections.[19] Again, we observed a low frequency of tumor regressions (5/40). McCune *et al.*[20] applied the autologous cell approach to adenocarcinoma of the kidney; 4/14 patients with measurable metastases responded. Allogeneic vaccines can apparently induce tumor regression as well: in a recent report of the vaccine developed by Morton, Hsueh *et al.*[21] described regression of cutaneous in-transit metastases in 9/54 patients following administration of a vaccine comprising a pool of irradiated, melanoma cell lines. Mitchell *et al.*[22] observed tumor regression in 4/25 patients treated with Melacine®, a preparation of lysates derived from melanoma cell cultures mixed with the adjuvant DETOX.

Schirrmacher *et al.*[23] have reported promising results using autologous tumor cells modified with a nonlytic strain of Newcastle disease virus. This virus renders tumor cells more immunogenic through a variety of mechanisms, including induction of adhesion molecules and production of chemokines

Several investigators have been trying to increase the immunogenicity of autologous tumor cell vaccines by genetic modification of the cells. For example, Simons *et al.*[24] reported a case of regression of multiple lung metastases from metastatic adenocarcinoma of the kidney among 18 patients entered onto a phase I trial. Their vaccine consisted of autologous tumor cells transfected with the gene for GM-CSF. However, the need for genetic modification of each and every vaccine preparation was questioned by remarkable results reported by Leong *et al.*[25] They reported 4/20 partial or complete responses, with regression of bulky metastases in multiple visceral sites, following treatment of melanoma patients with a simple vaccine, consisting of irradiated, autologous tumor cells followed by injections of GM-CSF.

The identification of proteins selectively expressed on melanoma cells and the synthesis of small peptides derived from those proteins has produced a new generation of tumor vaccines. Rosenberg *et al.*[26] reported 14 cases of tumor regression (1 CR, 13 PR) following treatment with a synthetic peptide or a mutant peptide with a single amino acid substitution derived from the melanoma-associated antigen MART-1. Interpretation of the results was complicated by the fact that 13/14 responders also received high doses of interleukin-2, a drug known to have antitumor effects.[27] Marchand *et al.*[28] observed regression of metastases in 7/25 patients treated with subcutaneous injections of an aqueous solution of a synthetic peptide derived from a protein encoded by the melanoma-associated gene *MAGE-3* and presented in the context of HLA-A1. Nestle *et al.*[29] treated 16 metastatic melanoma patients with a novel immunotherapy: autologous dendritic cells pulsed with either synthetic peptides or lysates of autologous melanoma tissue and then injected directly into the regional lymph node. There were 5 responses (2 CR, 3 PR).

We believe that our study makes several important contributions to the human cancer vaccine literature: (*i*) It is the largest reported phase II study of measurable metastases treated with an autologous tumor vaccine. (*ii*) We have identified a set of patients who seem

most likely to respond (those with small volume lung metastases) and have provided documentation of the tumor regressions that should allow oncologists to determine their significance. (*iii*) The antitumor responses that occurred were clinically meaningful in that they were associated with prolonged survival. (*iv*) We have identified an immunologic parameter—delayed-type hypersensitivity to autologous tumor cells—that appears to be a significant predictor of prolonged survival.

The particular sensitivity of small lung metastases to autologous, DNP vaccine was an unexpected finding, since most antitumor responses reported with other vaccines have occurred in skin metastases.[18,21,22,28] Even more remarkable was the kinetics of the tumor regression. Significant tumor shrinkage required at least 4–5 months and in 2 of these cases maximum regression was not observed for 1 year. A similar pattern of tumor regression was noted by Marchand *et al.*[28] in patients treated with the MAGE-3 peptide vaccine and by Leong *et al.*[25] in patients given autologous vaccine with GM-CSF. However, in most other published vaccine studies, responses occurred shortly after the inception of therapy. For example, after administration of the MART-1 peptide with interleukin-2, it appears that the time to tumor regression was short: in 1 illustrated case it was only 8 days. In the study of Nestle *et al.*,[29] all the responses were detected between weeks 6 and 10, *i.e.*, immediately after completion of the initial round of treatment. Responses to Melacine occurred within a few weeks of beginning treatment.[22]

On reflection, it seems more reasonable that tumor regression following administration of a cancer vaccine would require months, rather than days or weeks. After all, before a metastasis can begin to regress, 2 rate-limiting events must occur: (*i*) the development of a cell-mediated immune response to weakly immunogenic tumor antigens; and (*ii*) infiltration of tumor antigen-reactive T cells into the tumor tissue. In contrast, a therapeutic response to a cytokine, such as interleukin-2, might occur rapidly if it depends on a nonimmunologic mechanism, such as increased permeability of tumor vasculature.[30]

The kinetics of tumor regression effectively rule out the possibility that the responses were caused by a direct cytotoxic effect of cyclophosphamide, a drug that is inactive in metastatic melanoma, even at full oncostatic doses.[31] Furthermore, it seems certain that the antitumor effects were not attributable to BCG. Although capable of causing regression of directly injected skin metastases, BCG by itself does not affect distant metastases.[32] Moreover, a number of randomized trials have indicated that BCG is not effective as a postsurgical adjuvant treatment of melanoma.[33] Similarly, the topical application of DNFB that we employed cannot be considered therapeutic. Neither DNFB nor the related compound, DNCB, has systemic antitumor effects;[34] furthermore, 4 of the 6 patients who exhibited partial or complete tumor regression in our study did not receive DNFB before sensitization.

The duration of the antitumor responses is generally given little emphasis in cancer vaccine studies. However, it is apparent that responses induced by the various vaccines commonly last for 6 months and several of the studies report at least 1 or 2 long-term survivors.[21,28,29] In our study, the median response duration was 10 months and the most durable response continues for more than 47 months. The effect of vaccine-induced tumor regression on survival is generally ignored, perhaps because the small sample sizes render difficult the demonstration of statistically significant differences between responders and nonresponders. In 1 study that included survival analysis,[21] clinical response to vaccine produced no survival benefit: nonresponders who underwent excision of skin metastases lived as long as patients whose metastases regressed after vaccine administration. Our study, then, appears to be unique in its demonstration of prolonged survival of vaccine responders, even in a multivariate analysis

Progress in human cancer immunology has been limited by the absence of a test that reliably determines whether or not a patient has developed a cell-mediated immune response to tumor-associated antigens. Two types of tests have been evaluated, DTH and *in vitro* assays of T-lymphocyte reactivity (most commonly, generation of cytotoxic T cells). No studies, including our own, have been able to demonstrate that tumor regression depends on the development of an antitumor immune response. This may be a statistical, rather than a biologic, problem, *i.e.*, small sample sizes and low frequency of tumor regression. Some studies[11,23] have reported a correlation between the development of cell-mediated immunity and clinical outcome when tumor vaccines were used as postsurgical adjuvant therapy . What we have been able to show here is that the development of a positive DTH response to unmodified autologous melanoma cells is associated with longer survival in patients with measurable metastases.

Autologous DNP vaccine induces a unique immunologic effect—inflammatory responses in metastatic tumors. Although 33% of patients developed tumor inflammation, confirming our original observation,[7] the impact of this response on survival was of borderline statistical significance. Although tumor infiltration with T lymphocytes is necessary for tumor regression, it is evidently not sufficient.

The autologous DNP-modified cancer vaccine has several theoretical and practical advantages over other approaches: *(i)* the immunologic rationale is well established;[6]. *(ii)* it does not depend on a patient's tumor expressing a single antigen or on the patient's HLA type; *(iii)* there is minimal biohazard, since each patient is injected with his or her own tumor tissue and genetic modification is not required; *(iv)* the need to generate autologous dendritic cells for each patient is avoided and *(v)* the technology can be extended from melanoma to other human cancers without the need for extensive research and development; we have already reported preliminary results of the application of DNP vaccine to ovarian carcinoma.[35]

Of course, the major impediment to the routine use of autologous DNP vaccine is the need to obtain and process an adequate amount of tumor tissue from each patient. The establishment of a manufacturing facility[#] several thousand patients yearly has addressed this problem. This facility is supporting the ongoing multisite, randomized trial of DNP vaccine as adjuvant therapy in patients with bulky, resectable regional lymph node metastases. The results reported here support the conduct of a second multisite trial in patients with metastatic melanoma limited to small lung metastases.

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[#]Avax Technologies, Inc. opened the facility in Philadelphia in April, 1999.

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