Cancer Immunol Immunother (2002) 51: 320–326
DOI 10.1007/s00262-002-0285-3

ORIGINAL ARTICLE

David Berd · Takami Sato · Michael J. Mastrangelo

Effect of the dose and composition of an autologous hapten-modified melanoma vaccine on the development of delayed-type hypersensitivity responses

Received: 10 January 2002 / Accepted: 22 February 2002 / Published online: 6 June 2002

Abstract We have reported that treatment of melanoma patients with a vaccine consisting of autologous tumor cells modified with the hapten, dinitrophenyl (DNP) and preceded by low-dose cyclophosphamide induces delayed-type hypersensitivity (DTH) to autologous, unmodified tumor cells and that this response is a significant predictor of survival. We analyzed the vaccines prepared for 284 patients who were treated following resection of regional or distant metastases to find out whether the dose and composition determined the immunological response. A positive DTH response (≥5 mm induration) to unmodified autologous tumor cells was induced in 57% of the patients (median: 5 mm; range: 0–22 mm). Regression analysis showed no significant association between the magnitude of DTH and the number of live (trypan blue exclusion) melanoma cells per dose over a dosage range of 0.5–25.0×10^6. Surprisingly, there was a small but significant positive relationship between the mean number of dead cells in the vaccines of a given patient and that patient's maximum DTH to unmodified melanoma cells. Only 37% of patients whose vaccines contained >50% live cells developed DTH, as compared with 69% and 65% of patients whose vaccines contained 26% to 50% or ≤25% live cells, respectively. Thus, it appears that dead tumor cells contribute to the immunogenicity of the DNP vaccine, but other factors such as the administration schedule may be more important determinants of immunological and clinical outcome.

Keywords Autologous · Cyclophosphamide · Immunotherapy · Melanoma · Vaccine

D. Berd (✉) · T. Sato · M.J. Mastrangelo
Department of Medicine,
Kimmel Cancer Center,
Thomas Jefferson University,
1015 Walnut St., Suite 1024
Philadelphia 19107, PA, USA
E-mail: d_berd@mail.jci.tju.edu
Tel.: +1-215-9558875
Fax: +1-215-9230797

Abbreviations *BCG* bacille Calmette–Guérin · *DNFB* dinitrofluorobenzene · *DNP* dinitrophenyl · *DTH* delayed-type hypersensitivity

Introduction

We have developed a human cancer vaccine consisting of autologous tumor cells modified with the hapten, dinitrophenyl (DNP). The rationale, supported by studies in preclinical models, is that coupling of a hapten to a protein often results in the induction of an immune response to the protein that would otherwise not be possible [14, 20]. Administration of the vaccine is preceded by an intravenous injection of low-dose cyclophosphamide, because of multiple reports that this drug, properly timed, can augment the development of T cell mediated immune responses [3].

We have observed that administration of DNP vaccine to patients with metastatic melanoma induces a unique reaction – the development of inflammation in metastatic masses [6]. Histologically, the response consists of infiltration of T lymphocytes, most of which are $CD8^+$ [4]. These T cells usually produce gamma interferon in situ [9]. Moreover, they represent the expansion of T cell clones with novel T cell receptor structures [18].

Previously we have reported that administration of DNP vaccine to melanoma patients with bulky resectable nodal metastases produced both relapse-free and overall survival that appeared to be better than those reported with surgery alone [5]. In that study, the induction of delayed-type hypersensitivity (DTH) to autologous unmodified melanoma cells was a significant independent predictor of clinical outcome: Patients who developed a positive (≥5 mm induration) DTH response to unmodified autologous melanoma cells had significantly higher 5-year survival (71% versus 49%) than those who did not. In contrast, the other DTH responses that developed following administration of DNP vaccine – to DNP-modified autologous melanoma cells or to PPD – were not indicators of prolonged survival.

In that initial report, we tested two dosage schedules of DNP vaccine and could detect no difference between them in regard to immunological or clinical outcomes. In this report, we have expanded the studies to include 284 patients tested at five dosage-schedules. We observed no significant relationship between the development of DTH to unmodified autologous melanoma cells and vaccine dose, as defined by the number of live tumor cells. However, the vaccine composition, e.g. relative numbers of live and dead tumor cells, did have a small but significant effect on DTH responses. Unexpectedly, we found that dead tumor cells appeared to have made a contribution to the vaccine's immunogenicity.

Materials and methods

Patients

The study population consisted of 284 patients with metastatic melanoma that was completely resectable. Their clinical characteristics are summarized in Table 1. Most of the patients had clinical stage III disease with large (≥2.5 cm diameter) regional lymph node metastases. Some of these patients had in-transit metastases as well, but all were excised prior to entry into the protocols. The remainder of the patients had stage IV melanoma and had undergone resection of one or more distant metastases, the most common site being the lung.

All protocols were approved by the institutional review board of Thomas Jefferson University. The major entry criteria were: (1) at least one resectable metastasis that was processed in the laboratory to yield a sufficient number of melanoma cells for vaccine preparation; (2) no evidence of metastatic disease post-operatively as determined by physical examination, laboratory tests, and computed tomography (CT) or magnetic resonance imaging (MRI); (3) no chemotherapy within eight weeks of entry and no radiation therapy within six months of entry; (4) no concomitant anti-melanoma therapy.

Table 1. Patient characteristics

Total	284
Men	161
Women	123
Age (yr): median (range)	53 (16–83)
Stage	
III: One nodal site	179
III: In-transit metastases	29
III: Two nodal sites	53
IV: Disseminated	23

Tumor processing and vaccine preparation

Metastatic tumor was excised, maintained at 4°C and delivered to the laboratory within 48 h of excision. The tumors were processed as previously described [3]. In brief, cells were extracted by enzymatic dissociation with collagenase and DNAse, aliquotted, frozen in a controlled rate freezer, and stored in liquid nitrogen in a medium containing human albumin and 10% dimethylsulfoxide until needed. In most cases, 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 2,500 cGy. Then they were washed again and modified with DNP by the method of Miller and Claman [12]. This involved a 30-min incubation of tumor cells with dinitrofluorobenzene (DNFB), followed by washing with saline.

Each vaccine fell within a specified dose range of live (trypan blue exclusion) tumor cells suspended in 0.2 ml of Hank's solution; in addition, there were variable numbers of lymphocytes and dead cells in all specimens. After mixing with bacille Calmette–Guérin (BCG), the suspension was injected intradermally into three adjacent sites, usually on the upper dorsal arm, excluding sites ipsilateral to a lymph node dissection.

Vaccine administration

Five 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; schedule E: October 1998 to March 2001. These are summarized in Table 2. 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 at the same site on the ventral upper arm; cyclophosphamide 300 mg/M^2 i.v. rapid infusion was given 3 days prior to DNFB application. For schedule A, DNP vaccine mixed with BCG (Tice; produced by Organon Teknika, Durham, N.C.) 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 dose. All vaccine injections were given in the same site on a limb (usually the upper dorsal arm) that had not been subjected to lymph node dissection. For schedule B, the vaccine was administered weekly for 6 weeks; after a 4-week reevaluation period, the 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 in one area, and all of the unmodified vaccine injections were given in a second area. Cyclophosphamide (300 mg/M^2) was administered 3 days prior to the

Table 2. Summary of vaccine schedules

Schedule	No. patients	No. doses cyclophosphamide	DNFB sensitization	Vaccine schedule	Vaccine dosage range	BCG mixed with
A	47	3	+	28 days ×8	5.0–25.0×10^6	Every dose
B	30	3	+	Weekly ×12	5.0–25.0×10^6	One-third of doses
C	50	3	+	Weekly ×12	5.0–25.0×10^6	Every dose
D	87	1	–	Weekly ×6	2.5–7.5×10^6	Every dose
E	70	1	–	Weekly ×6	0.5–2.0×10^6	Every dose

Schedule A: cyclophosphamide 300 mg/M^2 i.v. three days before DNFB and three days before first two dose of vaccine
Schedule B: cyclophosphamide 300 mg/M^2 i.v. three days before DNFB and three days before each six-week series of vaccine; half of the vaccines were DNP-modified and the other half unmodified
Schedule C: cyclophosphamide 300 mg/M^2 i.v. three days before DNFB and three days before each six-week series of vaccine; all vaccines were DNP-modified
Schedules D, E: cyclophosphamide 300 mg/M^2 i.v. three days before the first vaccine only; booster injection of vaccine were given at six and 12 months

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. Schedules D and E were simplified regimens in which DNFB presensitization was omitted, and only one series of six weekly DNP-modified vaccines was administered. These schedules differed only by dose ranges: D = 2.5–7.5×10^6 versus E = 0.5–2.0×10^6 tumor cells per dose.

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: no. 1: 0.1 ml of a 1:10 dilution (1–8×10^6 CFU); no. 2: 0.1 ml of a 1:100 dilution (1–8×10^5 CFU); no. 3: 0.1 ml of a 1:1,000 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 fifth vaccine injection.

DTH testing

Patients were tested for DTH by a standard method that we have previously described [3]. Cryopreserved melanoma cell suspensions and peripheral blood lymphocytes (PBL) were thawed, washed and irradiated (2,500 cGy). DNP modification of melanoma cells and PBL was performed as described above. Melanoma cells (1×10^6) and PBL (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 the induration was measured after 48 h. A positive response was defined as: maximum diameter of induration ≥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 the second monthly vaccine; schedules B, C, D, E: 2.5 weeks after the sixth weekly vaccine). Analyses were performed by determining the maximum DTH response exhibited by each patient to each of the test reagents.

Pretreatment positive DTH responses to autologous melanoma cells, either DNP-modified or unmodified, were observed in 23 patients (8%) and were generally small (median: 6 mm; range: 5–18 mm).

Most patients were tested for DTH to autologous melanoma cells that had been dissociated with enzymes (collagenase and DNAse) and to melanoma cells that had been mechanically dissociated only. There was a strong correlation between DTH responses to the two preparations (adjusted squared multiple $R = 0.752$). Twenty-six patients (9%) developed an apparent DTH response to the enzymes, as measured by skin testing with enzyme-coated autologous PBL. Of these, 16 had been treated on schedule B, which included immunization with unmodified as well as DNP-modified melanoma cells. If a patient developed DTH to enzyme-coated PBL, we analyzed only their DTH response to mechanically-dissociated melanoma cells.

Statistics

All statistical analyses were performed using Systat software (SPSS, Chicago, Ill.). Scatter plots were fitted with a LOWESS smoother which runs along the x values and finds predicted values from a weighted average of nearby y values. This allows the surface to flex locally to better fit the data.

Results

DTH responses

A summary of the DTH responses observed following administration of the DNP vaccine is shown in Table 3. Almost all patients developed positive responses to DNP-modified autologous melanoma cells and to PPD; these responses were usually at least 10 mm in diameter. Responses to unmodified autologous tumor cells were induced only in a subset of these patients and were smaller (usually 5–10 mm in diameter).

Following vaccine treatment, five patients (2%) exhibited a small (5–6 mm) DTH response to autologous unmodified PBL after treatment. This could have represented a T cell response against normal tissue antigens, but is more likely to be artifactual, since no other manifestations of autoimmunity were observed.

The magnitude of the DTH response to unmodified melanoma cells was not related to the clinical stage. There was no significant difference between the responses of the 179 patients with metastases to one nodal site compared to the 105 patients with more advanced disease, i.e. nodal disease with in-transit metastases, two nodal sites involved, or distant metastases (for both groups, median DTH response: 5 mm, $P = 0.880$; Kruskal–Wallis test).

Effect of vaccine dose and composition on DTH

Table 4 presents a summary of the composition of the vaccines administered. All vaccines contained live (trypan blue exclusion) tumor cells, dead (trypan blue positive) tumor cells and lymphocytes. As can be seen from

Table 4. Composition of vaccines

Dose parameter	Median (range)
No. live tumor cells (×10^6)	6.8 (0.5–25.0)
No. dead tumor cells (×10^6)	8.0 (0.1–71.2)
No. live + dead tumor cells (×10^6)	16.6 (0.5–73.0)
% Live tumor cells	44 (3–88)
% Lymphocytes	34 (0–86)

Table 3. DTH responses

Test reagent	DTH response (mm) median (range)	% Patients with response ≥5 mm	% Patients with response ≥10 mm
DNP-modified autologous melanoma cells	17 (0–70)	95	77
Unmodified autologous melanoma cells	5 (0–22)	57	13
PPD	24 (0–85)	99	98

the table, there was considerable variation in vaccine composition among patients. However, for a given patient the composition of multiple vaccines manufactured over a period time was similar (data not shown). Therefore, for all analyses we used the mean value for each patient.

Fig. 1 is a scatter plot with a regression line of the dose of live tumor cells versus the maximum DTH response to autologous unmodified melanoma cells. No significant relationship was observed (adjusted squared multiple $R < 0.01$; $P = 0.512$).

Since all of the vaccines contained dead cells (i.e. staining with trypan blue) and lymphocytes, we analyzed the effects of these components on the development of DTH. Surprisingly, as shown in Fig. 2, there was a small but significant positive relationship between the mean number of dead cells in the vaccines of a given patient and that patient's maximum DTH to unmodified melanoma (adjusted squared multiple $R = 0.060$; $P < 0.001$). There was a direct relationship of similar degree between DTH and the total vaccine dose, defined as the mean number of live tumor cells added to the mean number of dead tumor cells, as shown in Fig. 3 (adjusted squared multiple $R = 0.039$; $P = 0.001$).

We observed a significant inverse relationship between the magnitude of DTH and the proportion of live tumor cells per dose (calculated as the number of live tumor cells divided by the total number of tumor cells), as shown in Fig. 4 (adjusted squared multiple $R = 0.063$; $P < 0.001$). Moreover, patients whose vaccines contained $> 50\%$ live cells developed significantly smaller DTH responses than those whose vaccines contained 26% to 50% or $\leq 25\%$ live cells (Fig. 5; $P < 0.001$). Only 37% of patients whose vaccines contained $> 50\%$ live cells developed DTH to unmodified melanoma, as compared with 69% and 65% of patients whose vaccines contained $\leq 25\%$ or 26% to 50% live cells, respectively.

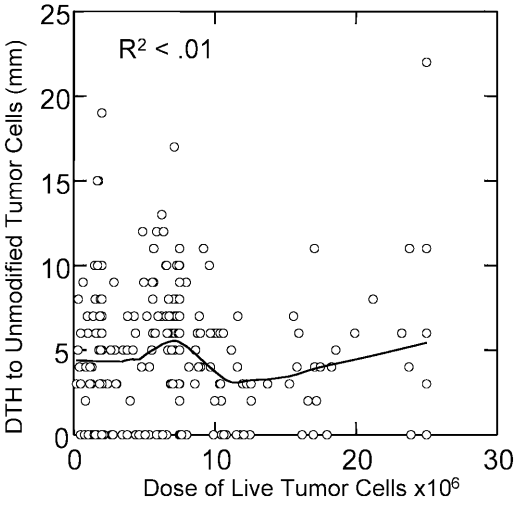

Fig. 1. Scatter plot showing the effect of the mean dose of live (trypan blue exclusion) tumor cells per vaccine on the development of DTH to autologous unmodified melanoma cells. Each *dot* represents a single patient. A LOWESS smoother was applied. Regression analysis: adjusted squared multiple $R < 0.01$; $P = 0.512$

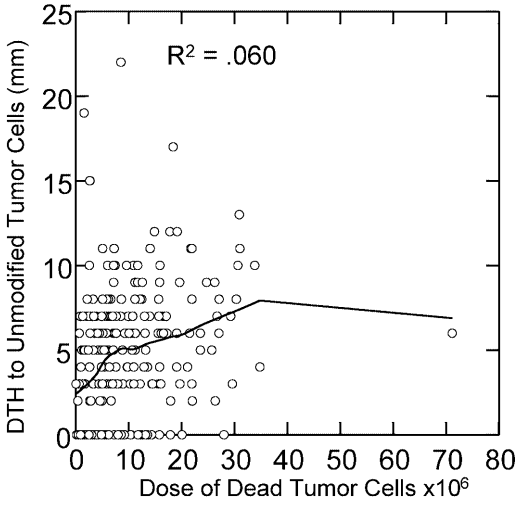

Fig. 2. Scatter plot showing the effect of the mean dose of dead (trypan blue-positive) tumor cells per vaccine on the development of DTH to autologous unmodified melanoma cells. Each *dot* represents a single patient. A LOWESS smoother was applied. Regression analysis: adjusted squared multiple $R = 0.060$; $P < 0.001$

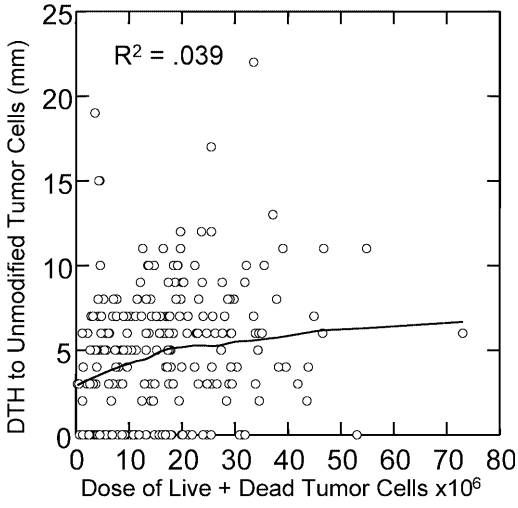

Fig. 3. Scatter plot showing the effect of the mean dose of live + dead tumor cells per vaccine on the development of DTH to autologous, unmodified melanoma cells. Each *dot* represents a single patient. A LOWESS smoother was applied. Regression analysis: adjusted squared multiple $R = 0.039$; $P = 0.001$

Almost all of the vaccines contained some lymphocytes. Since most of the tumors used for vaccine preparation were lymph node metastases, the lymphocyte content of some vaccines exceeded 50% (see Table 4). However, as expected, the proportion of contaminating lymphocytes in a vaccine had no effect on the development of DTH to unmodified melanoma cells (Fig. 6) (adjusted squared multiple $R < 0.01$; $P = 0.355$).

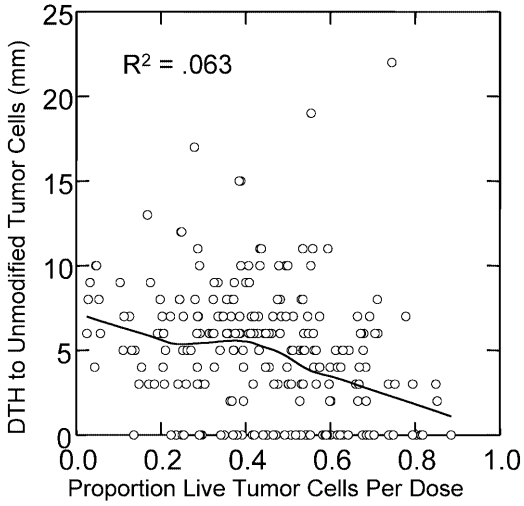

Fig. 4. Scatter plot showing the effect of the proportion of live tumor cells per vaccine (calculated as the number of live tumor cells divided by the total number of tumor cells) on the development of DTH to autologous unmodified melanoma cells. Each *dot* represents a single patient. A LOWESS smoother was applied. Regression analysis: adjusted squared multiple $R = 0.063$; $P < 0.001$

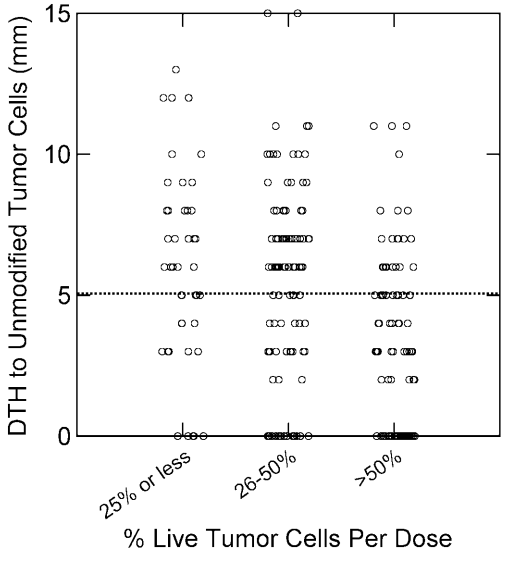

Fig. 5. Peak DTH responses to autologous unmodified melanoma cells stratified by proportion of live tumor cells per vaccine. Each *dot* represents a single patient. The patients whose vaccines contained > 50% live cells had significantly lower DTH responses than those whose vaccine contained 26% to 50% or ≤ 25% live cells (Kruskal–Wallis test; $P < 0.001$). The *dashed line* indicates the delineation between positive and negative responses

Clinical outcomes

The clinical results of these studies, measured as relapse-free and overall survival times, have been reported previously [2, 5] and will be the subject of future reports. However, we did analyze survival using the Kaplan–Meier method in which patients were stratified by each of the vaccine composition parameters. None of these

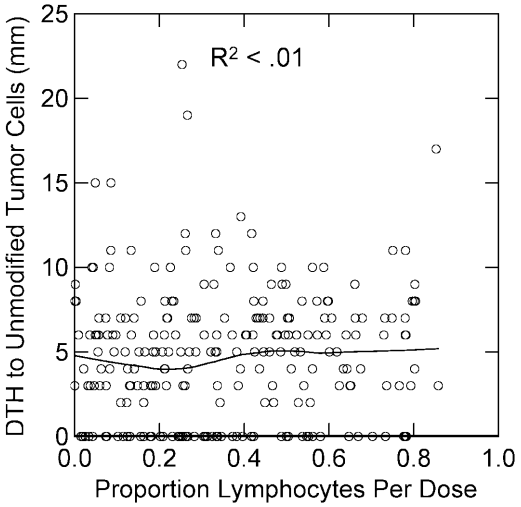

Fig. 6. Scatter plot showing the effect of the mean proportion of lymphocytes per vaccine on the development of DTH to autologous unmodified melanoma cells. Each *dot* represents a single patient. A LOWESS smoother was applied. Regression analysis: adjusted squared multiple $R < 0.01$; $P = 0.355$

parameters had any significant effect on relapse-free or overall survival (data not shown).

The DTH responses induced by DNP vaccine were never associated with clinical evidence of autoimmunity. Three patients developed erythema around their lymphadenectomy sites following vaccine administration. This was asymptomatic and spontaneously abated. Skin biopsy of one of these patients showed a non-specific vasculitis. No patients developed vitiligo following treatment.

Discussion

Our previous studies have demonstrated that the efficacy of autologous DNP-modified melanoma vaccine is dependent on the induction of DTH to autologous unmodified melanoma cells. In patients who received DNP vaccine following resection of bulky resectable nodal metastases, the induction of this response was a highly significant predictor of relapse-free and overall survival. Moreover, it was independent of important prognostic variables, such as the number of positive lymph nodes [5]. Even in patients with measurable metastases who had much larger tumor burdens, a positive DTH following vaccine treatment was associated with longer survival [7].

Here we show that the intensity of the DTH response to autologous, unmodified melanoma cells was not primarily determined by the doses of vaccine administered, at least over the dosage range ($0.5–25.0 \times 10^6$) that we tested. We defined the dose by the number of melanoma cells that were live, i.e. excluding the supravital dye, trypan blue, although proliferation was rendered incompetent by irradiation and DNP modification. Live tumor cells had been assumed to be the active compo-

nent of the vaccine because of reports in animal systems emphasizing the superior immunogenicity of live tumor cells over dead ones [15]. However, in our study, we could find no significant relationship between the number of live cells per dose and the DTH response to unmodified melanoma cells that was induced.

Surprisingly, there was a direct correlation between DTH and the number of dead cells per dose. This effect, although statistically significant, was small, the number of dead cells per dose accounting for only 6% of the variation in DTH responses. That it is biologically significant is reinforced by the observation that DTH responses were greater in patients whose vaccine had the lowest proportions of live cells. Therefore, the data suggest that dead tumor cells contribute to the immunogenicity of the DNP-modified vaccine.

On reflection, this result makes biological sense. There is evidence indicating that dead tumor cells, including necrotic cells, are effectively processed by antigen-presenting cells which can result in a T cell response to tumor-associated antigens [1]. Since DNP modification makes cells fragile, the uptake of trypan blue by a DNP-modified cell may mean only that its membrane has been disrupted. As long as the tumor antigens have not been destroyed, DNP-modified cells should be just as immunogenic when live as when dead.

There are a number of human cancer vaccine technologies in clinical trials, particularly for the treatment of melanoma [10, 11, 13, 16]. Many of these have been shown to have positive clinical effects, usually measured by regression of established metastases in a small proportion of patients. However, progress in this field 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. Furthermore, no systematic dose-response analyses have been published. Simons et al. [19] tested three dosage levels of an autologous renal cell carcinoma vaccine transfected with the gene for GM–CSF, but the number of patients at each dosage level (seven, ten, and two, respectively) was too small to differentiate their immunological effects. Schirrmacher et al. [17], in their studies on an autologous vaccine infected with Newcastle disease virus, have claimed that vaccines with a higher proportion of live tumor cells and a lower proportion of lymphocytes produced better clinical outcomes. They did not analyze the effect of vaccine composition on immunological results.

A most provocative observation was made by Chandawarkar et al. [8] in a murine tumor model. They reported that mice immunized with optimal doses of autologous tumor-derived gp96 heat-shock protein resisted a challenge with the tumor that was the source of gp96. However, immunization with quantities of gp96 five to ten times larger than the optimal dose did not elicit tumor immunity and actually appeared to induce tolerance. We were not able to detect a supraoptimal dose of vaccine in our studies, even after testing doses of tumor cells that varied over a 50-fold range.

In retrospect, immunotherapy researchers have placed insufficient emphasis on the pharmacological aspects of cancer vaccines. These vaccines are, after all, drugs. Thus, their efficacy is likely to depend not only on the quality and quantity of tumor antigens that they express and present, but also on parameters of much less fascination to immunologists, such as dose, administration schedule, and route of administration. More studies on these mundane but critical issues should be welcomed.

Acknowledgements This work was funded by PHS grant CA-39248 and by a research grant from AVAX Technologies. The authors wish to acknowledge the technical assistance of C. Clark and J. Kuchar and also the assistance provided by E. Bloome.

References

1. Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK (2000) Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappaB pathway. Int Immunol 12:1539
2. Berd D, Maguire HC, Kairys JC, Sato T, Nathan FE, Bloome E, Mastrangelo MJ (2000) Post-surgical treatment of clinical stage III melanoma with autologous, hapten-modified vaccine: expanded sample size and long-term follow-up [abstract 2254]. Proc Am Soc Clin Oncol 19:572a
3. Berd D, Maguire HC Jr, Mastrangelo MJ (1986) Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. Cancer Res 46:2572
4. Berd D, Maguire HC Jr, Mastrangelo MJ, Murphy GF (1994) Activation markers on T cells infiltrating melanoma metastases after therapy with dinitrophenyl-conjugated vaccine. Cancer Immunol Immunother 39:141
5. Berd D, Maguire HC Jr, Schuchter LM, Hamilton T, Hauck WW, Sato T, Mastrangelo MJ (1997) Autologous, hapten-modified melanoma vaccine as post-surgical adjuvant treatment after resection of nodal metastases. J Clin Oncol 15:2359
6. Berd D, Murphy G, Maguire HC Jr, Mastrangelo MJ (1991) Immunization with haptenized, autologous tumor cells induces inflammation of human melanoma metastases. Cancer Res 51:2731
7. Berd D, Sato T, Cohn H, Maguire HC Jr, Mastrangelo MJ (2001) Treatment of metastatic melanoma with autologous, hapten-modified melanoma vaccine: regression of pulmonary metastases. Int J Cancer 94:531
8. Chandawarkar RY, Wagh MS, Srivastava PK (1999) The dual nature of specific immunological activity of tumor-derived gp96 preparations. J Exp Med 189:1437
9. Lattime EC, Mastrangelo MJ, Bagasra O, Li W, Berd D (1995) Expression of cytokine mRNA in human melanoma tissues. Cancer Immunol Immunother 41:151
10. Leong SPL, Enders-Zohr P, Zhou Y-M, Stuntebeck S, Habib FA, Allen RE, Sagebiel RW, Glassberg AB, Lowenberg DW, Hayes A (1998) Recombinant human granulocyte–macrophage-colony stimulating factor and autologous melanoma vaccine mediate tumor regression in patients with metastatic melanoma. J Immunother 22:166
11. Marchand M, Van Baren N, Weynants P, Brichard V, Dréno B, Tessier MH, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, Vanwijck R, Liénard D, Beauduin M, Dietrich PY, Russo V, Kerger J, Masucci G, Jäger E, De Greve J, Atzpodien J, Brasseur F, Coulie PG, Van der Bruggen P (1999) Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene *MAGE-3* and presented by HLA-A1. Int J Cancer 80:219

12. Miller SD, Claman HN (1976) The induction of hapten-specific T cell tolerance by using hapten-modified lymphoid cells. I. Characteristics of tolerance induction. J Immunol 117:1519
13. Nestle FO, Alijacic S, Gilliet M, Sun Y, Grabbe S, Dummer R, Schadendorf D (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nature Med 4:328
14. Neurath MF, Fuss I, Kelsall BL, Stuber E, Strober W (1995) Antibodies to interleukin 12 abrogate established experimental colitis in mice. J Exp Med 182:1281
15. Peters LC, Brandhorst JS, Hanna MG Jr (1979) Preparation of immunotherapeutic autologous tumor cell vaccines from solid tumors. Cancer Res 39:1353
16. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dudley ME, Schwarz SL, Spiess PJ, Wunderlich JR, Parkhurst MR, Kawakami Y, Seipp CA, Einhorn JH, White DE (1998) Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma [see comments]. Nature Med 4:321
17. Schirrmacher V, Ahlert T, Pröbstle T, Steiner H-H, Herold-Mende C, Gerhards R, Hagmüller E (1998) Immunization with virus-modified tumor cells. Semin Oncol 25:677
18. Sensi ML, Farina C, Maccalli C, Lupetti R, Nicolini G, Anichini A, Parmiani G, Berd D (1997) Clonal expansion of T lymphocytes in human melanoma metastases after treatment with a hapten-modified autologous tumor vaccine. J Clin Invest 99:710
19. Simons JW, Jaffee EM, Weber CE, Levitsky HI, Nelson WG, Carducci MA, Lazenby AJ, Cohen LK, Finn CC, Clift SM, Hauda KM, Beck LA, Leiferman KM, Owens AH Jr, Piantadosi S, Dranoff G, Mulligan RC, Pardoll DM, Marshall FF (1997) Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by ex vivo granulocyte–macrophage colony-stimulating factor gene transfer. Cancer Res 57:1537
20. Weigle WO (1965) The production of thyroiditis and antibody following injection of unaltered thyroglobulin without adjuvant into rabbits previously stimulated with altered thyroglobulin. J Exp Med 122:1049