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Chemoimmunotherapy Increases the Lymphocyte Reactivity of Melanoma Patients*

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Abstract—*Eight surgically incurable melanoma patients receiving methyl-CCNU +vincristine +BCG +allogeneic melanoma cells, 8 receiving methyl-CCNU +vincristine, and 8 receiving only methyl-CCNU were measured for* in vitro *lymphocyte reactivity against an allogeneic melanoma cell line before and after treatment. In the presence of fetal calf serum (FCS), the lymphocytes of 5 of 8 patients receiving chemoimmunotherapy and 0 of 16 patients receiving chemotherapy alone showed a significant increase in reactivity. In the presence of patient's autologous serum, 4 of 8 receiving chemoimmunotherapy and 2 of 16 receiving chemotherapy alone increased significantly. The differences between pre- and post-treatment reactivity were significantly greater in the group receiving chemoimmunotherapy than in the other two groups. The increase in lymphocyte reactivity demonstrated by the patients receiving chemoimmunotherapy did not correlate with tumor regression and was not reflected in an improved survival.*

INTRODUCTION

WE HAVE demonstrated in previous studies [1] that surgically incurable melanoma patients undergoing immunotherapy consisting of intradermal bacillus Calmette-Guêrin (BCG) +autologous irradiated melanoma cells showed increased peripheral blood lymphocyte reactivity (LR)** against melanoma cells *in vitro* after treatment. We further demonstrated [2] that melanoma patients receiving chemotherapy with dimethyl triazeno imidazole carboxamide or BCNU (1, 3-bis (2-chloroethyl)-l-nitrosourea) plus vincristine exhibited no significant decrease in LR even after prolonged administration of these drugs. Combination chemoimmunotherapy is currently under intense investigation in melanoma patients. The purposes of this study were to (a) determine if methyl-CCNU with or without vincristine reduced LR to allogeneic melanoma target cells, and (b) determine if immunization with allogeneic melanoma cells +BCG enhanced LR in the face of simultaneous chemotherapy. To achieve these goals, the reactivity of lymphocyte samples from each patient was tested before and 4 and/or 8 weeks after administration of chemotherapy or chemoimmunotherapy. The changes noted in each treatment group were compared with those in the other two groups.

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**Used to include both cytotoxic and cytostatic effects observed with mononuclear cells obtained by Ficoll–Hypaque separation of heparinized blood.

MATERIALS AND METHODS

Lymphocyte donors

Melanoma patients entered into the study had histologically documented, surgically incurable malignant melanoma and estimated life expectancies of at least 3 months. Patients with brain metastases and those receiving steroid therapy were excluded. The clinical profile of the study patients is presented in Table 1.

Patients were randomly allocated to one of the following treatment regimens: (1) methyl-CCNU (NSC 95441; 1-(2-chloroethyl)-3-(4-

Table 1. Clinical profile of study patients

	Therapy		
	MeCCNU	MeCCNU + VCR	MeCCNU + VCR + TC + BCG
Total patients	8	8	8
Male/female	3/5	4/4	5/3
Age (yr): Mean	56	50	44
Median	57	52	49
Range	22–82	22–71	26–68
Metastatic sites			
Subcutaneous	4/8	4/8	4/8
Lymph node	2/8	5/8	2/8
Lung	5/8	4/8	5/8
Bone	2/8	2/8	2/8
DNCB reactivity	6/7*	6/8	8/8
Survival (MOS)			
Mean	9.6+	9	9.7
Median	7+	8	10
Range	5–24+	3–18	5–16
Prior chemotherapy	1/8	1/8	2/8
Prior immunotherapy	1/8	3/8	0/8
Objective responders	4/8	3/8	3/8

MeCCNU = methyl-CCNU; VCR = vincristine; TC = allogeneic tumor cells; BCG = Bacillus Calmette-Guerin.
* = One patient not tested.

methyl-cyclohexyl)-1-nitrosourea) alone at a dose of 200 mg/m^2 orally every 8 weeks; (2) methyl-CCNU as above plus vincristine at a fixed dose of 2 mg intravenously, every 4 weeks, or (3) methyl-CCNU plus vincristine as above plus 1–2 × 10^8 allogeneic irradiated (15,000 rad) tumor cells admixed with BCG (Glaxo, 2–4.5 × 10^6 organisms) intradermally (at 5 sites) every 2 weeks. An adequate trial was considered to be completion of one 8 week cycle. The first 8 patients entered in each arm constitute the study population.

Preparation of vaccine

A single melanoma patient with a large tumor burden provided the allogeneic melanoma cells used for all patients receiving chemoimmunotherapy. The fresh specimen was minced finely into a cell suspension and frozen in 1–2 × 10^8 viable cells/ml aliquots as described previously [1].

Microcytotoxicity assay

Lymphocytes were separated from heparinized blood on a Ficoll–Hypaque gradient and stored in liquid nitrogen as described previously [1]. Blood from melanoma patients was allowed to clot at room temperature, the sera separated by centrifugation and stored at −4°C. Immediately prior to use, the sera were heat inactivated at 56°C for 30 min. A single melanoma cell line (M14), pooled and stored in liquid nitrogen as previously described [1], was used throughout the study.

Our modification of the Takasugi and Klein [3] technique has been described in detail elsewhere [1]. Prior to addition of lymphocytes either heat inactivated fetal calf serum (FCS) diluted 1/6 with medium containing no serum or patients autologous serum (PS) also diluted 1/6 with medium was added to the target cells in 0.01 ml amounts. Patients' lymphocytes in medium containing 20% FCS were then added to the target cells and the mixture incubated for 48 hr, stained and counted as previously described [1]. Pretreatment and post-treatment lymphocyte samples from a single patient were on the same plate. In addition on each plate, there were 8 wells containing target cells plus FCS diluted 1/6 with medium without lymphocytes.

Criteria for analysis

The survival fraction for each lymphocyte sample was calculated by dividing the mean number of tumor cells remaining in 8 wells containing tumor cells + lymphocytes by the

mean number remaining in wells containing tumor cells alone. The difference between the survival fraction before treatment and the survival fraction 4 or 8 weeks after the beginning of treatment was calculated for each patient and the statistical significance determined by comparing the 8 pretreatment wells with the 8 post-treatment wells using the two-tailed Student's *t*-test. The differences in the survival fractions for all patients in each treatment group by the Student's *t*-test to determine any significant differences in changes in reacctivity among the various treatment regimens.

RESULTS

To determine the changes in reactivity after treatment, lymphocyte samples from each of the 24 melanoma patients studied were retrieved from liquid nitrogen and tested simultaneously against allogeneic melanoma cells (M14) as described in Materials and Methods. The effect of chemotherapy on LR was determined by comparing the results obtained with pretreatment and 8 week post-treatment samples from 8 patients treated with methyl-CCNU (group 3) and 8 treated with methyl-CCNU +vincristine (group 2). As shown in Table 2, none of the patients in groups 2 and 3 exhibited a significant increase in LR in the presence of FCS and 1 patient in each group (M271,310) increased in LR in the presence of autologous PS. No patient in either of these two groups decreased significantly in LR in the presence of FCS. One patient (M279) in group 3 decreased significantly in reactivity in the presence of autologous PS. A subset of patients was tested at more frequent intervals, to determine if the chemotherapy suppressed lymphocyte reactivity 4 or 6 weeks after treatment, a time of significant hematologic suppression. These serial data are presented in Fig. 1. Of 3 patients receiving methyl-CCNU +vincristine and 4 patients receiving methyl-CCNU alone, one (M333) experienced a significant decrease in LR that was evident at 6 weeks and not at 8 weeks; this was noted only in the presence of FCS and not in autologous PS.

The pretreatment and 8 week post-treatment survival fractions for the eight chemoimmunotherapy patients (group 1) are shown in Table 2. Statistical analysis of each patient revealed that 5 of 8 patients (M336, 300, 290, 275, 316) showed increased lymphocyte reactivity (decreased tumor cell survival) in the presence of FCS. In the presence of autologous PS, the lymphocyte reactivity of 4 of 8 patients (M336, 300, 290, 316) increased significantly. No patient receiving chemoimmunotherapy showed significant decrease in reactivity in the presence of FCS or PS.

The eight patients receiving chemoimmunotherapy were sampled serially to determine at which point in time the increases in LR first became manifest (Fig. 2). At 2 weeks, 2 patients (M290, 336) had significant increases in LR in both FCS and PS; by 4 weeks, 5 patients (M283, 316, 300, 290, 336) had increased LR in the presence of PS and 3 (M275, 300, 336) showed increases in the presence of FCS.

A quantitative analysis of the change in lymphocyte reactivity after treatment is presented in Table 3. The numbers represent the survival fraction of melanoma cells exposed to each patient's pretreatment lymphocytes minus the survival fraction of cells exposed to his post-treatment lymphocytes. In the chemoimmunotherapy group the survival fraction decreased by mean values of 0.23 and 0.26 in the presence of FCS and PS respectively. In the two chemotherapy groups the mean decrease in survival fraction was 0.06 or less. By Student's *t*-test, the mean decrease in group 1 was significantly greater than the decreases in groups 2 and 3 when the assays were performed in FCS. In the presence of PS only the difference between groups 1 and 3 was of statistical significance ($P=0.05$).

DISCUSSION

There has been much debate over the significance of *in vitro* assays of lymphocyte-mediated cytotoxicity in patients with cancer. The controversy has centered on the specificity of the reaction and on its relevance to the clinical course of the tumor. In studying these 24 patients with metastatic malignant melanoma we have not addressed ourselves to these questions. Instead we sought to determine whether the therapy had any effect on the degree of cytotoxicity. More specifically, the study was designed to determine if the cytotoxic drugs methyl-CCNU and vincristine suppressed lymphocyte-mediated cytotoxicity and if immunotherapy with an allogeneic tumor cell vaccine and BCG as an adjuvant increased cytotoxicity.

Lymphocyte-mediated cytotoxicity did not show any consistent changes after chemotherapy with methyl-CCNU or vincristine. Only 1 of 16 patients demonstrated decreased

Table 2. Lymphocyte reactivity before and after treatment of melanoma patients with chemotherapy or chemoimmunotherapy

Group 1 MeCCNU + VCR + TC + BCG			Group 2 MeCCNU + VCR			Group 3 MECCNU		
Patient No.	SF L + FCS	SF L + PS	Melanoma No.	SF L + FCS	SF L + PS	Melanoma No.	SF L + FCS	SF L + PS
131	(a) 0.64	(a) 0.68	271	(a) 0.47	(a) 0.58	339*	(a) 0.80	(a) 0.84
	(b) 0.59	(b) 0.62		(b) 0.41	(b) 0.41†		(b) 0.76	(b) 0.86
192*	(a) 0.63	(a) 0.53	243*	(a) 0.70	(a) 0.72	325*	(a) 0.68	(a) 0.54
	(b) 0.50	(b) 0.55		(b) 0.58	(b) 0.69		(b) 0.58	(b) 0.48
336	(a) 0.83‡	(a) 0.93‡	241	(a) 0.63	(a) 0.72	333*	(a) 0.40	(a) 0.64
	(b) 0.40†	(b) 0.25†		(b) 0.46	(b) 0.60		(b) 0.50	(b) 0.55
283	(a) 0.52	(a) 0.71	231*	(a) 0.57	(a) 0.66	18	(a) 0.54	(a) 0.59
	(b) 0.52	(b) 0.70		(b) 0.52	(b) 0.57		(b) 0.47	(b) 0.58
300	(a) 0.61‡	(a) 0.58‡	282*	(a) 0.56	(a) 0.61	201	(a) 0.25	(a) 0.16
	(b) 0.32†	(b) 0.26†		(b) 0.48	(b) 0.61		(b) 0.19	(b) 0.13
290*	(a) 0.60‡	(a) 0.69‡	179	(a) 0.30	(a) 0.27	310	(a) 0.66	(a) 0.79
	(b) 0.22†	(b) 0.22†		(b) 0.37	(b) 0.26		(b) 0.60	(b) 0.63†
275*	(a) 0.57‡	(a) 0.55	196	(a) 0.52	(a) 0.52	279	(a) 0.46	(a) 0.56
	(b) 0.30†	(b) 0.57		(b) 0.51	(b) 0.47		(b) 0.54	(b) 0.71
316	(a) 0.61	(a) 0.68‡	269	(a) 0.59	(a) 0.57	351*	(a) 0.62	(a) 0.48
	(b) 0.33	(b) 0.09†		(b) 0.52	(b) 0.56		(b) 0.56	(b) 0.45

SF = survival fraction calculated by dividing the mean number of cells in 8 wells containg tumor cells + lymphocytes by the mean number of cells in 8 wells containing tumor cells alone: L = lymphocytes; FCS = fetal calf serum; PS = patient's serum: a = pre-treatment; b = after 8 weeks of treatment; † = a statistically significant ($P \leqq 0.05$) increase in lymphocyte cytotoxicity; ‡ = a statistically significant ($P \leqq 0.05$) decrease in lymphocyte cytotoxicity; * = objective tumor regression; MeCCNU = methyl-CCNU; VCR = vincristine; TC = allogeneic tumor cells.

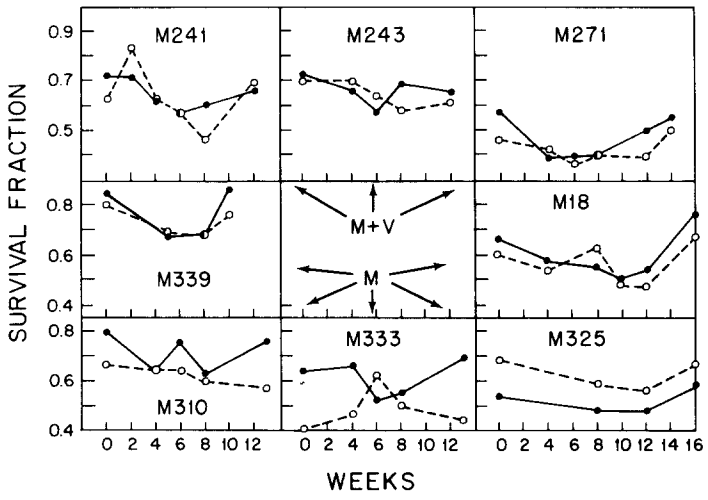

Fig. 1. Sequential lymphocyte reactivity in the presence of FCS (—○—○—) or autologous serum (—●—) of three melanoma patients (M241, M243, M271) receiving methyl-CCNU plus vincristine, and 5 melanoma patients (M339, M18, M310, M333, M325) receiving methyl-CCNU therapy.

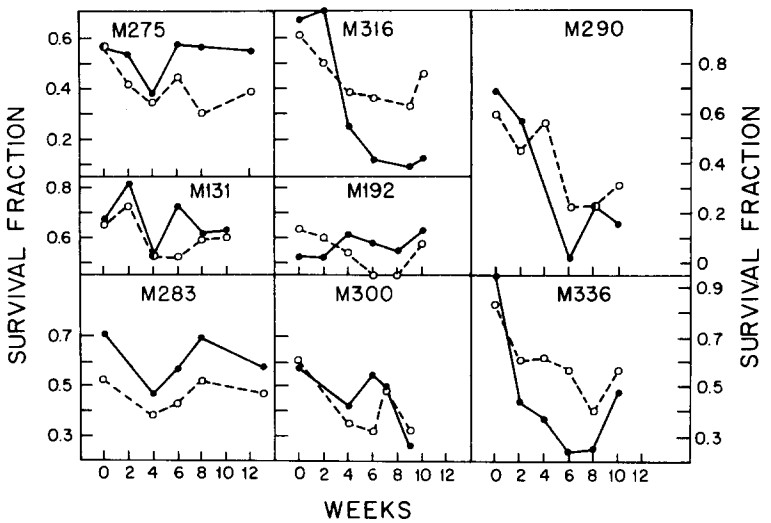

Fig. 2. Sequential lymphocyte reactivity in the presence of FCS (—○—○—) or autologous serum (—●—) of 8 melanoma patients receiving methyl-CCNU plus vincristine plus BCG plus allogeneic melanoma cells.

cytotoxicity 8 weeks after administration of methyl-CCNU. The myelosuppressive effects of this drug are maximal about 4 weeks after administration, and it could be argued that maximal immunosuppressive effects would also be observed at this time point. However, of 7 patients whose lymphocytes were also tested 4 weeks after methyl-CCNU, only 1 showed suppression of cytotoxicity.

Both methyl-CCNU and vincristine are immunosuppressive in experimental animals [4], but have not been studied in this regard in humans except by Mitchell *et al.* [6]. They administered methyl-CCNU or the related drugs, CCNU and BCNU, to patients with melanoma and measured lymphocyte cytotoxicity weekly. They found that cytotoxicity was markedly reduced in 8 of 9 patients 3 weeks after administration but returned to baseline levels by 4 weeks. These data are consistent with our observations. There is evidence that established or secondary immunologic responses are much more resistant to immunosuppressive drugs than are primary responses [7]. The reactivity of peripheral blood lymphocytes to cultured melanoma

*Table 3. Decrease in the survival fraction of melanoma cells exposed to post-treatment lymphocytes as compared to pre-treatment lymphocytes**

Group 1 MeCCNU + VCR + TC + BCG†				Group 2 MeCCNU + VCR			Group 3 MECCNU Alone	
Patient No.	L + FCS	L + PS	M No.	L + FCS	L + PS	M No.	L + FCS	L + PS
131	0.05	0.06	271	0.06	0.17	339	0.04	−0.02
192	0.13	−0.02	243	0.12	0.03	325	0.10	0.06
336	0.43	0.68	241	0.17	0.12	333	−0.10	0.09
283	0.00	0.01	231	0.05	0.09	18	0.07	0.01
300	0.29	0.32	282	0.08	0.00	201	0.06	0.03
290	0.38	0.47	179	−0.07	0.01	310	0.06	0.16
275	0.27	−0.07	196	0.01	0.05	279	−0.08	−0.15
316	0.28	0.59	269	0.07	0.01	351	0.06	0.03
Mean ±	0.23 ±	0.26 ±		0.06 ±	0.06 ±		0.03 ±	0.03 ±
Standard error‡	0.05	0.11		0.03	0.02		0.03	0.03

*Numbers represent the survival fraction of melanoma cells incubated with pre-treatment lymphocytes minus the survival fraction of melanoma cells incubated with lymphocytes obtained 8 weeks after the initiation of chemotherapy or chemoimmunotherapy.

†Abbreviations: Me-CCNU = methyl-CCNU; VCR = vincristine; TC = allogeneic tumor cells; L = lymphocytes; FCS = fetal calf serum; PS = patient's serum.

‡2-tailed Student's *t*-test between groups +

(a) FCS: 1 vs 2, $P < 0.020$.
1 vs 3, $P < 0.005$.

(b) PS: 1 vs 2, $P < 0.100$.
1 vs 3, $P < 0.050$.

cells, whether tumor is specific or not, is clearly an established function at the time the cytotoxic agents are administered.

In contrast we did demonstrate significant changes in lymphocyte-mediated cytotoxicity in patients given immunotherapy along with chemotherapy. Five of eight patients showed increased cytotoxicity over the 8 week period of testing, while only 1 of 8 patients in each of the chemotherapy alone groups manifested increases. Augmentation of *in vitro* cytotoxicity was not associated with tumor regression. Of the 10 patients whose tumors partially or completely regressed as a result of therapy, only 2 developed increased lymphocyte cytotoxicity. Of the 7 patients who developed increased cytotoxicity, only 2 manifested tumor regression.

The augmentation of cytotoxicity in *in vitro* assays by immunotherapy and its lack of correlation with antitumor responses has been noted by other investigators. Ikonopisov *et al.* [8] treated 13 patients with irradiated autochthonous melanoma cells, injected subcutaneously at 6 sites. The therapy was repeated at varying intervals. A transient increase in tumor-specific antibodies was observed, lasting 10–14 days, but no regression of nodules. Krementz *et al.* [9] treated 19 patients with intradermal injections of autochthonous cells at multiple sites. Treatments were repeated 6 times over a 2-week period and then once a week for 6 weeks. One patient with dermal and subcutaneous metastases had a complete remission and remained free of disease for over 3 yr, when he died of unrelated causes. The other 18 patients showed no clinical improvement. However, the patients developed increases in tumor-specific antibodies. Currie *et al.* [10] treated 22 patients with autochthonous melanoma cells injected subcutaneously. Five of nineteen patients showed an enhanced specific lymphocyte cytotoxicity to their own cultured melanoma cells, but none demonstrated nodule regression. The lymphocyte cytotoxicity was transient, lasting only 7–14 days.

We previously treated 18 patients with metastatic melanoma with a mixture of irradiated autochthonous tumor cells and BCG intradermally once every 2 weeks for 5 treatments [11]. Four patients experienced tumor regressions, and 2 of these had complete remissions, although the responses were of short duration (mean = 3 months). The lymphocytes of 9 of these patients were tested for reactivity against cultured allogeneic melanoma cells, and this was found to be increased after immunotherapy. Patterns of reactivity against melanoma cells and against bladder carcinoma cells were similar, indicating a lack of specificity for melanoma antigens.

The lack of correlation between *in vitro* cytotoxicity and *in vivo* tumor growth may be explained by the work of Mitchell *et al.* [6]. They noted that after administration of BCG, lymphocyte cytotoxicity against melanoma cells increased but only transiently and within 8 weeks returned to baseline levels even though BCG therapy was continued. Perhaps a more sustained high level of cytotoxicity is necessary to promote immunologically-mediated tumor regression. It is equally likely that the *in vitro* interactions between lymphocytes and tumor cells do not at all reflect events *in vivo*. Potentially cytotoxic lymphocytes might, for example, be inhibited *in vivo* by humoral or cellular suppressive factors. Our data do not provide evidence for a circulating humoral suppressive "blocking" factor, since, in most cases, cytotoxicity in the presence of patients' serum was the same as in FCS.

The allogeneic melanoma line used as the target cell in the present study was the same cell line as a target in our prior report utilizing autologous cells for immunization [1]. The allogeneic cells used for immunization in this study significantly enhanced lymphocyte microcytoxicity against this target cell as did the autologous vaccine in the prior report. Thus, allogeneic melanoma cells were as effective as autologous melanoma cells for immunization, when measured against an allogeneic target.

Although it is encouraging that concomitant chemotherapy did not abrogate the development of tumor associated immunity as reflected by this assay, the failure of test results to reflect clinical outcome makes it unlikely that this assay will be useful in the design of clinical trials.

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