Clin. Exp. Metastasis, 1995, **13,** 97–104

Effects of prolonged treatment with decarbazine on tumour metastatic potential in mice bearing Lewis lung carcinoma

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(*Received 15 July 1994; accepted in revised form 28 November 1994*)

The effects of decarbazine on tumour growth and metastatic dissemination upon treatment protracted for 10 tumour transplant generations were examined in mice bearing Lewis lung carcinoma. Primary tumour growth is unaffected by the drug, independently from the duration of the treatment. In contrast, dacarbazine significantly inhibits the formation of lung metastasis. The proportion of mice with metastasis decreases for an increasing number of transplant generations of treatment, and after 10 transplant generations of treatment metastatic capacity is completely lost in immunocompetent mice. The reduction in metastatic potential is relatively stable, being retained for three successive transplant generations without treatment. The metastatic potential of treated tumours in immunosuppressed mice is substantially similar to that in immunocompetent hosts, indicating that chemical xenogenization of tumour cells does not occur as reported for transplantable mouse leukaemias. The results obtained using clonally selected tumour lines with different metastatic potential rule out the selection by dacarbazine of tumour cell sublines with reduced metastatic potential as the mechanism of the drug's action. Upon prolonged treatment, dacarbazine appears to cause a rather stable and dramatic loss in metastatic potential, not accompanied by resistance, which might be attributed to genotypic alteration(s) of tumour cells, and which might participate into the clinical effects of the drug.

Keywords: dacarbazine, DTIC, metastatic potential, prolonged treatment

Introduction

Dacarbazine (DTIC, 5-3,3-(dimethyl-l-triazeno)imidazole-4-carboxamide is a drug with established clinical anti-tumour activity. As a single agent, dacarbazine has significant activity against Hodgkin's disease, sarcomas and melanoma [1–3]; certain amine precursor uptake and dacarboxylation (APUD) tumours also appear to be responsive to the drug. Dacarbazine in combinations with other anti-tumour drugs has been also shown to be clinically effective [5–7]. In animal tumour systems dacarbazine displays a broad spectrum of anti-tumour action, which is evident either against solid tumours, or on ascitic ones as well as on transplantable leukaemias [8, 9].

The mechanism of the anti-tumour action of dacarbazine has been shown to involve metabolic activation to chemically reactive species [10, 11]. The most likely cytotoxic lesion caused by the drug's metabolite(s) seems to be the methylation of DNA guanine residues in the O^6-position [12].

Besides these anti-tumour properties ascribed to a cytotoxic antiproliferative action, dacarbazine has further interesting properties in animal models. In mice bearing solid malignant transplantable tumours, the formation of systemic spontaneous metastasis is

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inhibited [13] via a mechanism which is not related to the cytotoxic properties of the drug [14, 15]. Moreover, when the treatment of transplantable leukaemias is prolonged for several successive transplant generations, the drug induces the appearance of stable tumour-associated transplantation antigens that cause the rejection of the drug-modified tumour cells by immunocompetent hosts (chemical xenogenization) [16–18].

To our knowledge, the effects of the treatment with dacarbazine prolonged for more than one tumour transplant generation have not been examined in mice bearing solid malignant tumours. The aim of the present investigation was to determine the effects of the treatment with dacarbazine, serially performed for a maximum of 10 successive transplant generations, in mice bearing Lewis lung carcinoma. The capacity of such treated tumour cells to grow locally and metastasize spontaneously to the lungs has been evaluated in immunocompetent and immunodepressed recipients at the end of each transplant generation. The stability of the effects of dacarbazine after treatment for one tumour transplant generation has been determined in three subsequent transplant generations. Finally, in order to study the possible drugs-induced clonal selection of tumour cell subpopulations, experiments were performed using clonally selected tumour cell lines endowed with different metastatic potential.

Materials and methods

Tumour transplantation and evaluation

The Lewis lung carcinoma parental line used was originally obtained from the National Cancer Institute (Bethesda, MD); the clonally selected Lewis lung carcinoma lines endowed with high (M1087) or low (BM21548) metastatic potential [19, 20], were originally provided by Dr G. Zupi (Istituto Regina Elena, Rome, Italy). The tumour lines are maintained in C57BL/6 mice by s.c. injection in the axillary region of 50 mm^3 of minced tumour tissue, aseptically prepared from donors similarly inoculated 2 weeks previously [21]. The implantation of tumour for experimental purposes was performed intramuscularly in the calf of the left hind leg of BD2F1 mice. Female mice weighing 18–20 g (purchased from Charles River Laboratories, Calco, Como, Italy) were used. All animals received humane care, in accordance with EC and Italian regulations.

Tumour implantation was performed either in normal immunocompetent mice, or in mice which were immunosuppressed by i.p. treatment with cyclophosphamide, 180 mg/kg body weight, 24 h before tumour inoculation.

Primary tumour weight was determined on day 15 after tumour inoculation by calliper measurements of short (a) and long (b) axes, taking tumour density equal to 1:

$$\text{Tumour weight} = 6 \times a^2 \times b$$

The number of metastases was determined at killing on day 21 from tumour inoculation by examining the surface of the lungs by a low-power stereomicroscope. The weight of metastases was determined as the sum of their individual weights calculated according to the above equation after determination of their dimension by an ocular micrometer [22].

Drug treatment

Dacarbazine was kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD); cyclophosphamide was a generous gift of Shering SpA (Milan, Italy). Dacarbazine (60 mg/kg per day) was administered intraperitoneally daily for 14 consecutive days starting 24 h after tumour implantation, in volumes of 0.1 ml/10 g of animal weight, as a freshly prepared suspension in isotonic saline containing 1% Na-carboxymethylcellulose. Cyclophosphamide (180 mg/kg) was administered as a solution in isotonic saline (0.1 ml/10 g of animal weight), as a single dose 24 h before tumour implantation.

The experimental protocols concerning drug treatment, and evaluation of metastatic potential of drug-treated tumour cells in immunocompetent and immunodepressed host mice, are further detailed in Figure 1.

Results

The dosage employed for dacarbazine is the maximum tolerated one for the treatment schedule used (daily administration for 14 days after tumour implantation), as already determined in mice bearing Lewis lung carcinoma [23]. The treatment of mice bearing intramuscular implants of the parental line of Lewis lung carcinoma with this dosage and schedule of dacarbazine does not cause any significant alteration in the growth of the primary intramuscular tumour as measured on days 11, 13, 15 and 21, independently from the number of tumour transplant generations (TG1–TG10, Figure 1).

However, when the effects on metastasis are expressed in terms of metastasis number or weight, they are similarly pronounced and significantly

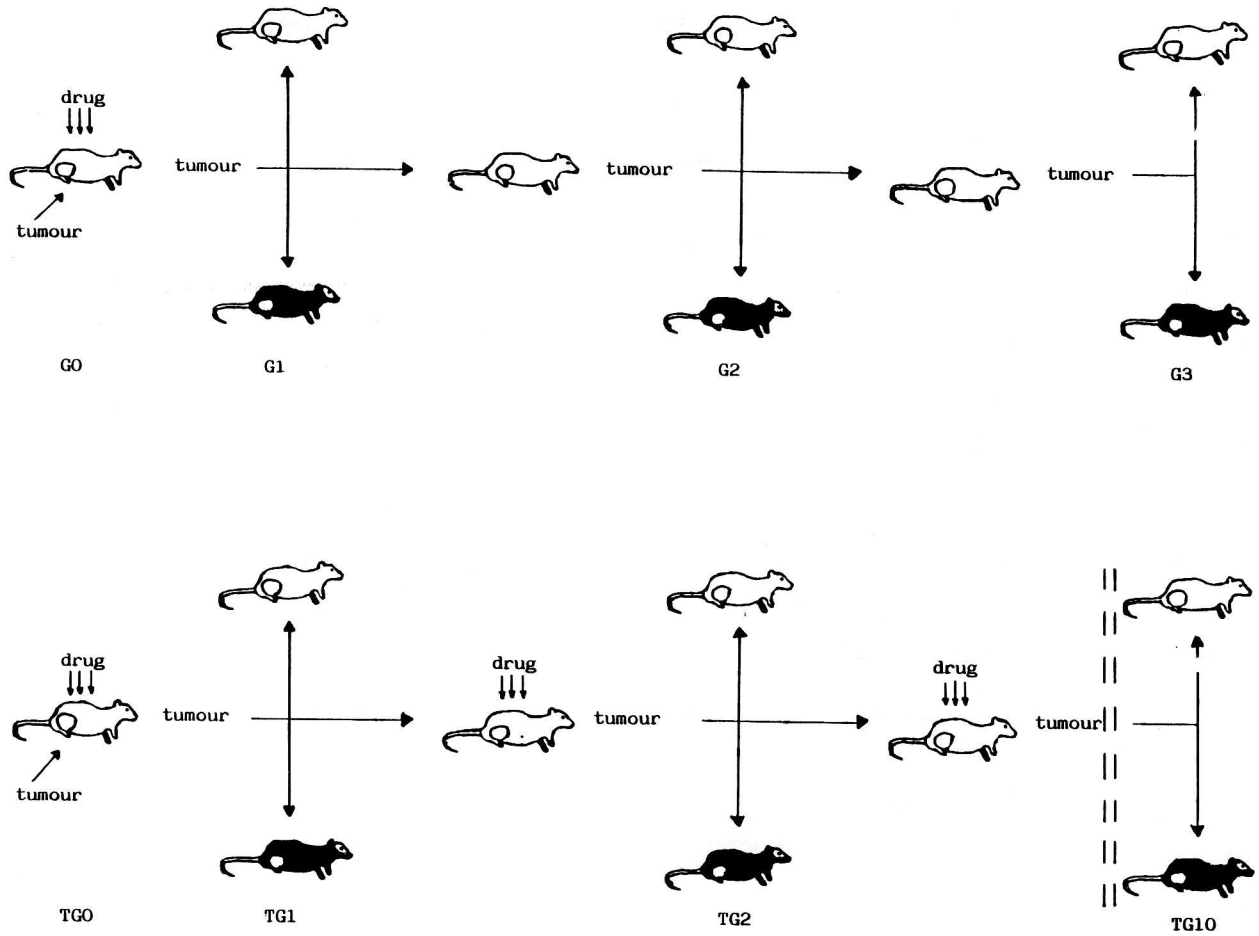

Figure 1. Experimental protocols. Groups of four tumour-bearing mice were treated with DTIC daily on days 1–14 from tumour inoculation. The mice were killed 24 h after the last drug administration; then the pooled i.m. tumours obtained from these treated animals were serially transplanted intramuscularly into non-treated recipient mice for three successive transplant generations (G1, G2 and G3). Alternatively, the pooled i.m. tumours obtained from DTIC-treated animals were serially transplanted and treated with DTIC into recipient mice for 10 successive transplant generations (TG1–TG10). For each transplant generation, the tumour was also implanted into groups of at least seven normal immunocompetent (□) mice or cyclophosphamide-immunodepressed mice (■), for measurement of primary tumour growth and metastasis formation.

correlate (linear regression analysis for the total population of mice used, $r = 0.922$, $P < 0.0001$). For these reasons, the results reported below for the parental line of Lewis lung carcinoma illustrate the effects of treatment on metastasis weight only. Moreover, the observations obtained with the parental line of Lewis lung carcinoma have been duplicated in a separate series of experiments, providing strictly similar results (not reported).

Treatment for one transplant generation

Treatment with dacarbazine of mice bearing the parental line of Lewis lung carcinoma produces a significant decrease in the weight of spontaneous lung metastasis, which persists for three transplant generations following that of treatment (G1–G3, Figure 1). The weight of lung metastasis is significantly smaller in immunocompetent mice, compared with immunodepressed ones in the first transplant generation following that of treatment (G1) (Table 1).

Treatment for 10 transplant generations

The effects of treatment with dacarbazine of mice bearing the parental line of Lewis lung carcinoma for a maximum of 10 transplant generations (TG1–TG10) are reported in Table 2. The formation of spontaneous lung metastasis is significantly attenuated by the drug treatment (TG1–TG10). At the same time, the proportion of animals with metastases significantly decreases for an increasing number of transplant generations of treatment (TG6–TG10). Metastasis weight is significantly smaller in immunocompetent

Table 1. Metastatic potential of Lewis lung carcinoma in three transplant generations following that of *in vivo* treatment with DTIC

Transplant generation	Immunocompetent hosts		Immunodepressed hosts	
	Lung metastasis		Lung metastasis	
	Weight (T/C%)	n^a	Weight (T/C%)	n^a
G1	23.1 ± 4.7*[b]	7/7	55.9 ± 8.1*[b]	7/7
G2	54.2 ± 10.0*	7/7	61.9 ± 7.4*	7/7
G3	47.0 ± 7.1*	7/7	63.3 ± 8.6*	7/7

[a] Animals with lung metastasis at the time of killing.
The weight of spontaneous lung metastasis for three transplant generations following that of treatment (G1–G3) has been determined in immunocompetent or immunodepressed hosts (see Figure 1). Each reported value is the mean percent ratio (±S.E.M.) obtained in these recipient mice in comparison with the relevant controls (recipient mice receiving tumour implants from drug untreated donor mice). The actual mean value ± S.E.M. of metastasis weight was 151.5 ± 21.3 for immunocompetent drug untreated control groups, and 182.4 ± 18.9 for immunodepressed drug untreated control groups (difference not statistically significant).
* Means statistically different from controls ($P < 0.01$). Means marked with the same letter are statistically different ($P < 0.05$); Student–Newmann Keuls test [35].

Table 2. Metastatic potential of Lewis lung carcinoma following *in vivo* treatment with DTIC for 10 transplant generations

Transplant generation	Immunocompetent hosts		Immunodepressed hosts	
	Lung metastasis weight (T/C%)	n^a	Lung metastasis weight (T/C%)	n^a
TG1	23.1 ± 4.7*[b]	7/7[defgh]	55.9 ± 8.1*[b]	7/7[ijk]
TG2	9.1 ± 2.6*[c]	10/12	28.8 ± 4.7*[c]	8/8
TG3	16.4 ± 3.5*	7/8	25.0 ± 4.1*	8/8
TG4	8.9 ± 2.3*	6/9	15.3 ± 2.4*	8/8
TG5	8.6 ± 1.7*	5/7	19.8 ± 2.5*	7/7
TG6	9.1 ± 3.8*	3/7[d]	15.9 ± 2.0*	5.6
TG7	3.6	1/8[e]	10.7 ± 2.5*	3/7[i]
TG8	2.4 ± 0.0*	2/8[f]	5.8 ± 3.2*	3.6
TG9	9.5 ± 3.0*	2/8[g]	7.0 ± 1.3*	4/12[j]
TG10	0.0	0/8[g]	4.2 ± 0.6*	3/7[k]

[a] Animals with lung metastasis at the time of killing.
The weight of spontaneous lung metastasis after the indicated number of transplant generation of treatment (TG1–TG10) has been determined in immunocompetent or immunodepressed hosts (see Figure 1). Each reported value is the mean percent ratio (±S.E.M.) obtained in these recipient mice in comparison with the relevant controls (recipient mice receiving tumour implants from drug untreated donor mice): the mean has been calculated excluding mice without metastasis. The actual mean value ± S.E.M. of metastasis weight was 199.0 ± 31.6 for immunocompetent drug-untreated control groups, and 252.6 ± 49.1 for immunodepressed drug-untreated control groups (difference not statistically significant).
* Means statistically different from controls ($P < 0.01$). Means marked with the same letter are statistically different ($P < 0.05$); [b,c] Student–Newmann Keuls test [35], or [d–k] Fisher's exact test [37].

recipient mice in comparison with immunodepressed ones (TG1–TG2). After 10 transplant generations of treatment (TG10), metastasis occurred in three out of seven immunodepressed recipients and in none of immunocompetent ones (difference marginally significant; Fisher's exact test $P = 0.07$); the primary tumours retained the capacity to locally grow at a rate identical to that of the original drug untreated line (TG0). The loss of metastatic potential in immunocompetent recipients is retained for at least four successive transplant generations of observation (not shown).

Treatment of clonally selected tumour lines with different metastatic potential

The treatment for one transplant generation of mice bearing the Lewis lung carcinoma line M1087 (high metastatic potential) causes in three transplant generations following that of treatment (G1–G3) a reduction in the weight of lung metastasis accompanied by a lack of effects on intramuscular primary tumour growth, which are identical to those observed using the parental line and which are unreported here.

Using the tumour line with low metastatic potential BM21548, the weight of spontaneous lung metastasis in the transplant generation following that of treatment (G1) is reduced to the same degree observed using the parental and M1087 lines of Lewis lung carcinoma. Primary tumour growth is significantly inhibited in the transplant generation of treatment (G0); this reduction is more evident in the following transplant generation (G1), and prevents further tumour transplantation and subsequent observations (Table 3). Differences between immunocompetent and immunodepressed recipient hosts are not statistically significant.

Metastatic potential of treated tumour cells in hosts pretreated with the same drugs

When the parental line of Lewis lung carcinoma is treated for one transplant generation (G1), no difference in the reduction of metastatic potential is observed in normal untreated recipient mice in comparison with mice treated before tumour implantation with dacarbazine (Table 4).

Table 4. Metastatic potential of Lewis lung carcinoma treated *in vivo* with DTIC for one transplant generation and further transplanted in normal hosts, or in hosts pretreated with the same drug

Pretreatment of tumour	Pretreatment of hosts	Lung metastasis	
		Weight (mg)	n^a
−	−	125.2 ± 17.9^{bc}	7/7
+	−	18.9 ± 5.7^{bd}	6/7
−	+	109.4 ± 42.7^{de}	6/6
+	+	11.5 ± 6.0^{ce}	3/6

[a] Fraction of animals with lung metastasis at the time of killing.

Groups of four immunocompetent mice were treated daily on days 1–14 after tumour inoculation. The pooled tumours obtained from these treated animals 24 h after the last drug administration (pretreatment of tumour, +), or tumours obtained from drug untreated donor mice (pretreatment of tumour, −), were implanted into groups of six recipient mice which were drug treated for the previous 14 days (pretreatment of hosts, +) or were untreated (pretreatment of hosts, −), as indicated. Each reported value is the mean (±S.E.M.) obtained in these recipient mice; the mean has been calculated excluding mice without metastasis.

Means marked with the same letter are statistically different ($P < 0.01$), Mann–Whitney test [36].

Table 3. Primary tumour growth and metastatic potential of BM21548 line of Lewis lung carcinoma in the transplant generation following that of *in vivo* treatment with DTIC

Group (G1)	Immunocompetent hosts			Immunodepressed hosts		
	Primary tumour weight (g)	Lung metastasis weight (mg)	n^a	Primary tumour weight (g)	Lung metastasis weight (mg)	n^a
Controls	2.3 ± 1.2^b	58.6 ± 10.6^c	13/15	2.4 ± 1.8^d	86.3 ± 11.3^e	14/15
Treated	1.2 ± 1.5^b	29.1 ± 5.2^c	10/16	0.8 ± 1.5^d	28.5 ± 7.8^e	13/18

[a] Fraction of animals with lung metastasis at the time of killing.

The weight of primary tumour and spontaneous lung metastasis after implantation from untreated donors (controls) or donors treated with DTOC (treated), has been determined in immunocompetent or immunodepressed hosts in the transplant generation following that of treatment (G1) (see Figure 1). Each reported value is the mean ± S.E.M. obtained in these recipient mice: for the weight of lung metastasis the mean has been calculated excluding mice without metastasis.

Means marked with the same letter are statistically different ($P < 0.01$), Student–Newmann Keuls test [35].

Discussion

The data illustrated so far confirm and extend reported findings on the anti-tumour and anti-metastatic action of dacarbazine. Indeed, in mice bearing Lewis lung carcinoma the formation of spontaneous lung metastasis is markedly reduced by dacarbazine, by its imidazole derivative BRL51308 and by structurally related p-substituted aryl-dimethyltriazenes, at maximum-tolerated dosages and with treatment schedules which are devoid of significant effects on primary tumour growth [13, 24]. The mechanism of the anti-metastatic action of dacarbazine might consist in the inhibition of tumour cell intravasation, since after treatment with this drug a marked reduction in the number of circulating tumour cells has been observed in mice bearing Lewis lung carcinoma [14]. The magnitude of tumour response to the cytotoxic effects of the drug was found to be increased, and the anti-metastatic response was conversely reduced, for a Lewis lung carcinoma line with low metastatic potential; an inverse pattern of cytotoxic and anti-metastatic response was displayed by a line with high metastatic potential [25].

When tumour local growth and metastatic spread are examined for three further transplant generations without treatment, the decrease in metastatic potential caused by dacarbazine appears to be stable. Upon transplantation in immunosuppressed recipient hosts, the decrease in metastatic potential of dacarbazine-treated tumours is significantly attenuated only in the first post-treatment transplant generation.

The treatment of the tumour was also prolonged for a maximum of 10 transplant generations. The purpose of this prolongation was to examine the same prolonged treatment conditions required to induce chemical xenogenization in mouse-transplantable leukaemias [26–29]. Moreover, the repeated prolonged contact between dacarbazine and tumour more closely reproduces the clinical use of the drug. Using these conditions, the potential of the tumour to proliferate locally is not affected by the treatment. In contrast, metastatic potential is progressively decreased with the prolongation of treatment, and is abolished in immunocompetent recipient mice after the tenth transplant generation of treatment. The metastatic potential is significantly higher in immunodepressed recipient hosts only after the first and second transplant generation of treatment. The loss of metastatic potential in immunocompetent recipients is retained for at least four successive transplant generations of observation.

Considering the immunosuppression of the hosts was induced by cyclophosphamide, it may be that the attenuation in the reduction of metastatic potential caused by the treatment which is observed in cyclophosphamide-pretreated animals is caused by pulmonary injury caused by cyclophosphamide, as reported by Steel and Adams [30] and by Carmel and Brown [31]. However, our data appear adequate to rule out the induction by dacarbazine of a significant chemical xenogenization of Lewis lung carcinoma as observed for transplantable leukaemias. Indeed, in mice bearing L1210 leukaemia the transplantability of the tumour which is completely lost after six transplant generations of treatment, is fully recovered in cyclophosphamide-pretreated hosts [32].

Considering that dacarbazine has immunodepressive properties [33], the pretreatment of recipient host mice with the drug has been examined. The reduction in metastatic potential caused by the drug is not different in mice pretreated with dacarbazine in comparison with untreated controls.

We also studied the possibility that treatment with dacarbazine induces a selection of clones of tumour cells endowed with reduced metastatic potential. For this purpose, Lewis lung carcinoma cell lines which were previously subjected to clonal selection for increased or reduced metastatic potential were used in place of the tumour parental line. The clonally selected line with high metastatic potential (M1087), similarly to the parental line, does not show a response in the primary tumour to the cytotoxic effects of dacarbazine. In contrast, primary tumours of the low metastatic line BM21548 display a high sensitivity to the cytotoxic effects of the drug. The possibility that the anti-metastatic effects of dacarbazine might be attributed to the clonal selection of tumour cell subpopulations with reduced metastatic potential can therefore be ruled out, since the clone with reduced metastatic potential is the most sensitive to the cytotoxic action of the drug.

Our results indicate that treatment with dacarbazine causes a reduction in metastatic potential of Lewis lung carcinoma which persists for several cell cycles after cessation of the contact with the drug. These effects could be attributed to genetic or epigenetic alterations produced by the drug, possibly caused by its capacity to alkylate DNA [34]. The nature of this alteration is not identified at present, and requires further elucidation; the possibility that dacarbazine may influence the expression and regulation of extracellular collagenolytic activity is currently being examined. It should be noted that prolonged treatment with dacarbazine causes a cumulative reduction in tumour metastatic potential, and does not appear to induce resistance to the anti-metastatic effects of the drug. Altogether, these findings support the view that

dacarbazine has, in addition to cytotoxic properties, interesting anti-metastatic effects in experimental animal tumour systems, which might participate in the clinical efficacy of the drug, and which deserve further consideration.

Acknowledgements

Supported by CNR Special Project ACRO contract no. 92.2192.PF39, and by MURST 40 and 60% (Prof. T. Giraldi) U.S.U. 1991.

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