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Priming Reduces the Bone Marrow Toxicity of Carboplatin

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Abstract—*A dose of* 170 mg/kg *of carboplatin is lethal in mice, death occurring through bone marrow failure. This lethality can be avoided by giving the animals* 200 mg/kg *cyclophosphamide* 1 *or* 2 *days before this dose of carboplatin. The improved normal tissue tolerance cannot be explained by altered pharmacokinetics of carboplatin. Increased survival appears to be associated with a more rapid regeneration of the haemopoietic stem cells. Tumour tissue is not protected in the same way and thus a therapeutic gain can be achieved using this protocol.*

INTRODUCTION

THE doses of anti-cancer drugs are limited by their toxic effects on normal tissues. Carboplatin, an analogue of cisplatin, has been shown to have activity against a variety of tumours including ovarian carcinoma [1] and small cell lung carcinoma (SCLC) [2]. Its dose limiting toxicity is myelosuppression [3].

We have previously shown in animal studies that pretreating or 'priming' recipients of chemotherapy or total body irradiation (TBI) with small doses of cytotoxic drug reduces both gastrointestinal and bone marrow toxicity [4–7]. The mechanism underlying this priming phenomenon is not understood but may involve the induction of DNA repair [8] or the production of a soluble factor which stimulates normal tissue cells to regenerate [9]. A precise understanding of the mechanism is not essential before priming is exploited clinically particularly since tumour tissue appears to remain unaffected (for a recent review; see [10]).

We have examined the effect of priming animals with cyclophosphamide prior to the administration of high dose carboplatin, in preparation for a phase I study of high-dose carboplatin in patients. The results of this study are presented here with particular reference to normal tissue tolerance and tumoricidal effect.

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MATERIALS AND METHODS

Animals

CBA mice aged between 10 and 12 weeks and weighing between 22 and 28 g were used throughout. In survival studies, mice were primed with 200 mg/kg cyclophosphamide on various days before or at the same time as 170 mg/kg carboplatin. The animals were checked on a daily basis and dead animals were removed promptly. Thirty-day survival was recorded.

Drugs

Carboplatin, supplied by the Bristol–Myers Co., was dissolved in saline to a concentration of 10 mg/ml just before being injected intraperitoneally into recipient mice at doses of 170 mg/kg except in the peripheral blood count experiment and in the tumour growth delay study where doses of 140 and 120 mg/kg were used respectively. Mice were weighed prior to injection and the volume of the carboplatin solution was adjusted to take account of the animal's weight and the concentration required. Cyclophosphamide (Koch–Light Labs. Ltd.) was dissolved in saline to a concentration of 20 mg/ml and 0.01 ml/g body wt injected intraperitoneally. The priming dose was always 200 mg/kg.

Tissue platinum levels

Tissues were removed, rinsed in saline and gently blotted dry. Tissue samples up to 250 mg were solubilized in 0.5 ml Hyamine hydroxide (1 M, Sigma Chemical Co.) at 50–60° C. The

solubilized samples were diluted in 0.1 N HCl and then analysed for platinum content using internal standards on an Instrumentation Laboratory atomic absorption spectrophotometer (model 457) in the flameless mode. Plasma and blood samples were diluted in 0.1 N HCl and analysed directly for platinum levels.

Blood urea nitrogen (BUN) levels

Urea levels in the plasma were determined calorimetrically by reaction with diacetyl monoxime [11].

Histology

Tissues were fixed in modified Methacarn (Methanol : Inhibisol : Acetic Acid, 6 : 3 : 1) and embedded in paraffin wax. Five micron thick sections were evaluated blind by one of us (JPS) who was unfamiliar with the numbering system on the slides.

Spleen colony forming cells (CFUs)

Pluripotent haemopoietic stem cells, CFUs, were measured using the method of Till and McCulloch [12]. Briefly, bone marrow was aspirated from femoral shafts either 1 day or 4 days after treatment with carboplatin. A single cell suspension was made and an appropriate cell inoculum injected intravenously into lethally irradiated (9 Gy) recipients. Seven days later the spleens of these recipeint mice were removed, fixed in Bouin's fixative and the surface colonies enumerated. With a knowledge of the dilution that took place to produce an appropriate cell inoculum, the number of CFUs per femur following carboplatin could be calculated. The primed group received in addition to the carboplatin 200 mg/kg cyclophosphamide 2 days prior to the carboplatin.

Peripheral blood count

Serial blood samples were withdrawn from each mouse by cutting a small piece off the end of the tail and sampling with a 20 μl haemoglobin pipette. After sampling a ligature was tied securely around the tail just proximal to the cut to minimize blood loss. The blood sample was placed into 130 μl of an 0.14% (w/v) EDTA solution in saline. The blood sample was then analysed using a model S + IV Coulter counter. A reduced dose of 140 mg/kg carboplatin was used in this experiment to allow animals given carboplatin alone to survive the duration of the experiment.

Tumour growth delay studies

The benzpyrene-induced fibrosarcoma FS13, obained from Dr. S. Eccles, Institute of Cancer Research (ICR) was implanted bilaterally into the shaved flanks of recipient, syngeneic CBA mice. Tumour growth was monitored by measuring tumour volume with calipers. As tumours do not usually grow spherically but as oblate spheroids, a good estimate of tumour volume was obtained by measuring the longest diameter of the tumour (L) and the diameter at right angles to it (D) and applying the formula $V = \pi L \times D^2/6$. Vo represents the volume of the tumour at the beginning of the experiment and Vt the volume at subsequent time t [13]. Growth delay was measured as the horizontal separation in time between treatment groups and control once the tumours had begun to grow exponentially again after treatment. A dose of 120 mg/kg carboplatin was used to allow the mice receiving carboplatin alone to survive the duration of the experiment. This group would otherwise have died on day six after treatment which is too early to assess a growth delay. The priming dose was 200 mg/kg cyclophosphamide and was given 2 days before the carboplatin.

RESULTS

Survival

All the mice given 170 mg/kg carboplatin died, and the mean survival was 6.4 ± .2 days (Table 1). The survival pattern was unaltered if 200 mg/kg cyclophosphamide was given at the same time as the carboplatin. However, if cyclophosphamide was given 1–4 days before carboplatin, survival was improved. The greatest improvement in survival was observed when cyclophosphamide was given 1–2 days before carboplatin.

CFUs assay

Table 2 shows that 1 day after the mice had received 170 mg/kg carboplatin the CFUs content of their femurs was reduced to less than 1 per femur in both primed and unprimed animals. Four days after carboplatin treatment there were still only 2.5 CFUs per femur in the group receiving carboplatin alone. However, in those animals that

Table 1. Thirty-day survival of mice given a 200 mg/kg *cyclophosphamide prime at various times before* 170 mg/kg *carboplatin*

Group primed	Survival	Mean day of death of non-survivors (+ 1 S.E.)
Unprimed control	0/10	6.4 ± 0.2
On same day as carboplatin	0/10	6.8 ± 0.2
1 day before carboplatin	10/10	N/A
2 days before carboplatin	8/10	16.5 ± 10.5
3 days before carboplatin	2/10	7.0 ± 1.2
4 days before carboplatin	1/10	11.1 ± 1.6

N/A = not applicable.

Table 2. The effect of priming with 200 mg/kg *cyclophosphamide* 2 days *before* 170 mg/kg *carboplatin on the regeneration of pluripotent haemopoietic stem cells (CFUs)*

Group	CFUs/femur 1 day after carboplatin	CFUs/femur 4 days after carboplatin
Carboplatin alone	0.7	2.5 ± 1.9
Cyclophosphamide alone	0.7	42.0 ± 2.2*
Cyclophosphamide two days before carboplatin	0.5	44.8 ± 4.3*

Normally there are 4500 ± 350 CFUs/femur. Four donors and five recipients were used per group. Results are expressed as mean ± S.E.
* = $P < .001$, difference from control group by Student's *t*-test.

received either cyclophosphamide alone or cyclophosphamide 2 days before carboplatin, the CFUs had recovered to over 40 CFUs per femur.

Peripheral blood counts and body weight

After 140 mg/kg carboplatin the white cell count fell gradually to a nadir at 8 days (Fig. 1a). There was no such decline in white cell number in primed animals indeed, there was a leukocytosis reaching a maximum of twice normal on day 8.

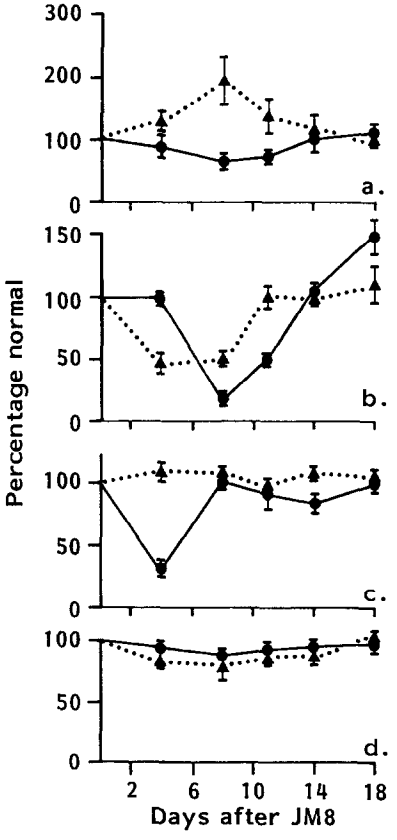

Fig. 1. Peripheral leukocyte count (a), *platelet count* (b), *red cell count* (c) *and body weight* (d). 140 mg/kg *carboplatin* (●), 200 mg/kg *cyclophosphamide* 2 days *before* 140 mg/kg *carboplatin* (▲). *Time scale refers to the time of carboplatin administration. Five animals were used per group.*

The platelet count fell earlier in the primed animals than in those who received carboplatin alone. However, recovery was earlier as well and cell counts were back to normal in 10 days compared to 14 days in the unprimed animals (Fig. 1b).

There was no decline in the red cell numbers in primed mice but for mice that received carboplatin alone there was a sharp decline in numbers on day 3 with recovery on day 7 (Fig. 1c). This is consistent with previous findings that carboplatin destroys red cells and that red cell transfusions are sometimes necessary in unprimed animals to permit survival [14].

Body weight was slightly, but not significantly, decreased in the primed animals compared with the unprimed group and recovery was complete by day 18 (Fig. 1d).

Tissue distribution of carboplatin following priming

The concentration of platinum on days 1 and 4 in plasma, blood, liver, jejunum and spleen of primed and unprimed mice was not significantly different (Table 3). The concentration of platinum in the muscle on day 1, kidney on day 4 and skin on days 1 and 4 was significantly higher in the primed mice. Blood urea nitrogen (BUN) and kidney weights were the same for primed and unprimed groups on day 1 but by day 4 BUN was significantly higher ($P < .001$) and kidney weight significantly reduced ($P < .01$) in the primed group (Table 4).

Histology

All animals showed normal kidney, heart, lung, liver, and brain histology, this being independent of treatment or time of sampling. Splenic megakaryocytes were present in normal numbers 24 hr after carboplatin treatment but were absent by 4 days. In both primed and unprimed groups, the

Table 3. Tissue distribution of carboplatin following a priming dose of 200 mg/kg *cyclophosphamide*

Tissue/Fluid	Platinum concentration (μg Pt/ml or μg Pt/g)			
	Day 1		Day 4	
	Control	Primed	Control	Primed
Plasma	0.65 ± 0.06	0.81 ± 0.07	0.22 ± 0.05	0.33 ± 0.06
Blood	2.3 ± 0.2	2.9 ± 0.3	1.6 ± 0.2	1.7 ± 0.1
Liver	5.6 ± 0.7	6.0 ± 0.3	5.8 ± 1.0	6.9 ± 0.6
Kidney	7.4 ± 0.9	8.4 ± 0.7	3.9 ± 0.2	5.8 ± 0.6*
Jejunum	1.8 ± 0.3	2.5 ± 0.2	0.75 ± 0.19	0.58 ± 0.09
Spleen	2.0 ± 0.5	3.4 ± 0.6	N.D.	N.D.
Muscle	0.96 ± 0.08	1.5 ± 0.2*	0.93 ± 0.17	1.6 ± 0.4
Skin	2.4 ± 0.4	5.3 ± 0.7*	3.2 ± 0.2	4.0 ± 0.3*

Five animals per group. Results are presented as mean ± S.E. N.D. = not determined. * = $P < 0.05$, difference from control group by Student's *t*-test.

Table 4. Blood urea nitrogen (BUN) (units) levels and left kidney weight (mg)

	Day 1		Day 4	
	BUN	wt	BUN	wt
170 mg/kg carboplatin	15.9 ± 2.2	137.2 ± 6.1	15.2 ± 0.5	127.6 ± 4.8
200 mg/kg cyclophosphamide two days before 170 mg/kg carboplatin	15.1 ± 1.6	132.2 ± 3.6	25.7 ± 0.7*	105.4 ± 4.3*

Five animals per group. Results presented as means ± S.E. * = $P < .01$, difference from control by Student's *t*-test.

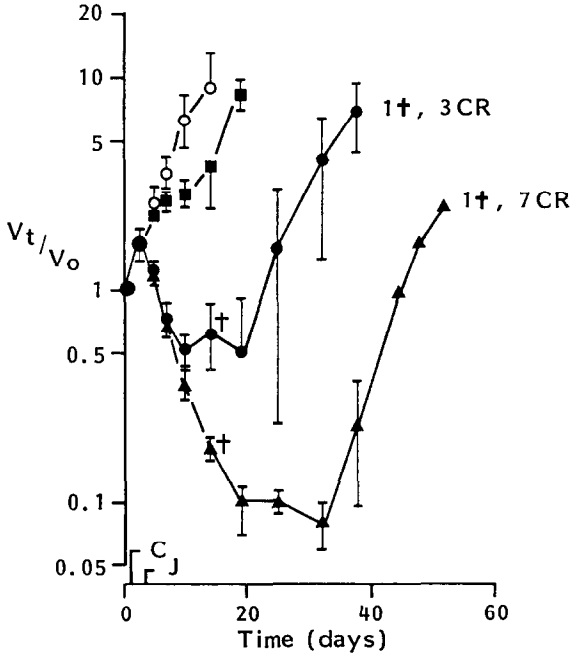

Fig. 2. Tumour growth delay. Untreated control (○), 120 mg/kg *carboplatin* (■), 200 mg/kg *cyclophosphamide* (●) 200 mg/kg *cyclophosphamide* 2 days *before* 120 mg/kg *carboplatin* (▲). Vo = *volume of tumour at the beginning of the experiment,* Vt = *the volume at subsequent time* t. *On the time scale C marks the time of administration of the cyclophosphamide and J that of carboplatin.* † = *death,* CR = *complete remission.*

small intestine exhibited normal histological appearances except for the presence of numerous individually necrotic cells in the crypts. These were more prominent 24 hr post carboplatin than after 4 days, while the number of mitoses within crypts was reduced in all groups 24 hr post carboplatin but slightly raised on day 4. There were no significant differences between the primed and unprimed groups.

Tumour growth delays

A dose of 120 mg/kg carboplatin produced a growth delay of about 8 days (Fig. 2). Tumours in mice that were primed before treatment exhibited a growth delay of 45 days, with one death and seven complete remissions and those that only received the cyclophosphamide priming showed a growth delay of 22 days, with one death and three complete remissions.

DISCUSSION

We have shown that the survival of mice after high-dose carboplatin is improved by prior administration of cyclophosphamide and that this improvement in survival is associated with a more rapid regeneration of pluripotent stem cells in the marrow of these primed animals. The distribution

of carboplatin was largely unaltered as the result of priming except in three organs, the kidney, the skin and muscle. In these sites, tissue levels of carboplatin were elevated rather than depressed and therefore the sparing of normal tissues from the cytotoxic effects of the drugs cannot be attributed to a lowering of tissue drug levels. Histological examination of these three organs from primed animals failed to show any tissue damage associated with raised tissue levels of carboplatin. Recovery of marrow stem cells is slow after they have been exposed to cytotoxic agents such as radiation, melphalan, busulphan and the nitrosoureas [6, 9, 15, 16]. In patients this can lead to long periods of life-threatening pancytopoenia. The administration of cyclophosphamide 1 or 2 days before any of these cytotoxic agents shortens the recovery time of marrow stem cells [6]. In this report we have shown that carboplatin-induced myelosuppression in animals may be similarly overcome by a priming dose of cyclophosphamide. Interestingly, in this series of experiments the combination of cyclophosphamide and carboplatin had an additive anti-tumour effect thus increasing the therapeutic gain still further. In previous experiments [10] additive anti-tumour effects have been seen in situations where the priming dose has reduced the normal tissue toxicity of the second anti-tumour agent. In one series of experiments these authors deliberately exploited this concommitent increase in tumouricidal effect and reduction in normal tissue toxicity [13].

The precise mechanism underlying the priming phenomenon is not yet understood but it may involve the production of acute phase proteins in response to the priming dose, since some are known to stimulate bone marrow [17]. Acute phase proteins probably do not have a direct protective effect on marrow stem cells since the CFUs count immediately after high-dose therapy is the same in both primed and unprimed animals (Table 2 and [6]). It is the subsequent recovery pattern which is altered by priming.

Tumour tissue does not appear to be affected by priming (Fig. 2 and [10]) whereas those normal tissues which are most sensitive to cytotoxic damage derive particular benefit from priming. It is the cytotoxic damage to these that is often dose limiting. There is thus a gain in therapeutic index if priming is used because conventional doses of anticancer drugs can be given with fewer side effects or doses can be escalated to well above those normally tolerated.

Priming in mice against other cytotoxic drugs has been reviewed [10]. The translation from mouse to man has not been an easy one. We showed that cyclophosphamide priming in mice reduced the gut toxicity of high dose melphalan [4]. Other workers [18] found this prime inconsistent. Hedley *et al.* [19] showed in patients that 300 mg/m^2 cyclophosphamide given before 140 mg/m^2 melphalan hastened the haemopoietic recovery. Using the same priming doses but treating outpatients, Spitzer and his colleagues [20] could not demonstrate a beneficial effect of cyclophosphamide priming before 20–30 mg/m^2 of melphalan. This is quite a low dose and priming is generally not seen under these circumstances, however higher doses could not be given because of previous chemotherapy. Evans [7] showed that cyclophosphamide before high dose cyclophosphamide was a protective manoeuvre in mice, but Harland and his colleages [21] could not repeat the finding in the clinic but this may have been due to the inclusion in the challenge dose of several other cytotoxic agents.

Carboplatin is an active anticancer drug in man at a conventional dosage of 400 mg/m^2 and the demonstration of cyclophosphamide's ability to prime animals against the toxic effects of high doses of this drug has encouraged us to commence randomised trial study of cyclophosphamide priming before high dose carboplatin (1.6 g/m^2) in patients with lung cancer.

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