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A cellular analysis of long-term haematopoietic damage in mice after repeated treatment with cyclophosphamide

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Summary. Following repeated treatment of mice with cyclophosphamide (5 × 200 mg/kg) it was found that slight, but significant, residual marrow damage persisted for at least half the lifespan of the animals. This long-term damage occurred despite preferential sparing of those multipotential haematopoietic cells (CFU-S) that had a high self-renewal capacity after each step of the multistep regimen and despite a smaller CFU-S kill after each successive dose. The damage was characterized by low mean numbers of CFU-S and stromal colony-forming cells (CFU-F) which were around 70% of control values.

Examination of individual animals revealed that the majority had slightly subnormal numbers of CFU-S and CFU-F, with only a few suffering a more severe injury, including 8% of mice with clinical hypoplasia or myelodysplasia.

Introduction

The use of chemotherapy in patients who have a good probability of long-term survival has prompted the search for long-term residual injury in several tissues. Damage to cerebral, cardiac, pulmonary, endocrine, and gonadal tissue is well recognised. Although the haematopoietic tissue is one of the dose-limiting tissues with regard to acute damage by chemotherapeutic agents, its extensive capacity for repopulation often results in apparently full reconstitution of the functional capacity of the tissue. However, it is well established in experimental systems that normal numbers of mature functional haematopoietic cells can be produced even where there is considerable damage in earlier cell populations (reviewed in [28]). This has also been seen in some patients in long-term haematological remission after acute lymphoblastic leukaemia and lymphoma, who have normal bone marrow cellularity and normal numbers of white blood cells (WBC) in spite of a severe and long-lasting reduction in numbers of granulocyte-macrophage colony-forming cells (GM-CFC) [13, 22, 25]. In experimental systems in mice, such damage is manifested as a reduction in the numbers of multipotential spleen colony-forming cells (CFU-S) as well as of GM-CFC after treatment with repeated doses of busulphan or X-rays. This damage persists for more than a year and after a long latency period, which may be at least one-third of the lifespan of the mice, may be apparent at the level of peripheral blood cells in decreased numbers of WBC or a fall in the haematocrit [14, 23].

There may also be alterations in the self-renewal capacity of the CFU-S. Transplantation experiments have shown a reduction in the ability of treated CFU-S to produce new CFU-S after busulphan but not after cyclophosphamide [30]. Also, serial transplantation experiments show a more pronounced decline in CFU-S numbers for busulphan-treated than for control marrow [2]. In the same series of experiments, marrow from cyclophosphamide-treated donors was no different from control. In contrast, other workers have reported subnormal numbers of CFU-S up to 6 months after cyclophosphamide treatment [31], and also a microenvironmental defect which was repaired in about 4 months [9].

Measurements of the numbers of a type of bone marrow stromal cells (CFU-F) showed moderate decrease after repeated doses of cyclophosphamide, which recovered to normal 3 months after cessation of treatment [31].

Here we report long-term studies in mice on the effect of repeated doses of cyclophosphamide. The treatment caused persisting damage to haematopoietic and stromal bone marrow cells, which was not corrected for at least 20 months and which in a minority of mice resulted in myelodysplastic syndromes.

Materials and methods

Mice. The mice used were female C57Bl/6 × DBA/2 ($B6D2F_1$), which were produced and maintained in the animal unit of the Paterson Laboratories. At the beginning of all experiments the mice were aged between 8 and 12 weeks. At different times after treatment mice were killed, the femora were dissected, and single cell suspensions were prepared as described previously [27].

CFU-S assay. The method of Till and McCulloch [29] was used with modification. Mice were irradiated with 13.5 Gy from a ^{60}Co source at a dose rate of 0.0141 Gy/min, and then within 4 h received test cells injected i.v., in 0.2 ml Fischer's medium (Flow Labs); they were killed by cervical dislocation 8 or 11 days later. When either normal or 15- to 600-day CP-treated mice were used as marrow don-

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ors then 5×10^4 cells were injected. When marrow taken 24 h after CP was used 4×10^5 cells were injected. The spleens were fixed in Bouin's solution, and macroscopic colonies were counted. Each experimental group consisted of three to five donor mice. Where individual mice were studied this is explicity stated in the *Results* section. There were ten recipient mice per group.

To assay the self-renewal capacity of CFU-S, the mean number of CFU-S generated in the spleen colonies was measured [19]. Mice (primary recipients) left until 11 days after injection were killed, after which the spleens were removed and the spleen cells suspended in medium. An aliquot of this suspension was then injected into further irradiated mice (secondary recipients). The mice were killed and the spleens were removed 8 days later. Previous experiments have shown that the numbers of spleen colonies in the secondary recipients remain constant between 8 and 11 days when bone marrow from control or cyclophosphamide-treated mice is assayed [21]. The spleens were removed and fixed in Bouin's solution, and the colonies were then counted. The numbers of colonies on the spleens of the primary and secondary recipients and the known injected fraction of total spleen cells were used to calculate the number of CFU-S produced on average in one of the original 11-day colonies.

CFU-F assay. Colonies of stromal cells were grown using modifications of the original technique [11, 32]. Although these colonies contained macrophage and endothelial cells in addition to fibroblastic cell types, the name CFU-F is retained for historical reasons, to identify the procedure. Marrow cells were seeded in triplicate at 5×10^6 in 5 ml alpha medium (Gibco) supplemented with 20% preselected fetal calf serum (Flow) in a 25-cm^2 growing surface plastic tissue culture flask (Nunc); the flasks were gassed with 5% CO_2 in air and incubated for 7 days at 37 °C. The colonies were then stained and counted.

Haematocrit. Cardiac blood was collected from each treated mouse in duplicated heparinised capillary tubes. Each tube was heat-sealed and spun in a Hawksley Microhaematocrit centrifuge before being read directly as percentage packed red cells by volume.

Injection of CP. Cyclophosphamide powder (Farmitalia Carlo Erba Ltd) was dissolved in isotonic saline and injected i.p. into preweighed mice at the volume of 0.01 ml per g body weight, to give a final dose of 200 mg per kg body weight. Doses were given singly or repeatedly at 2-week intervals for up to five doses per mouse. Control animals received injections of saline.

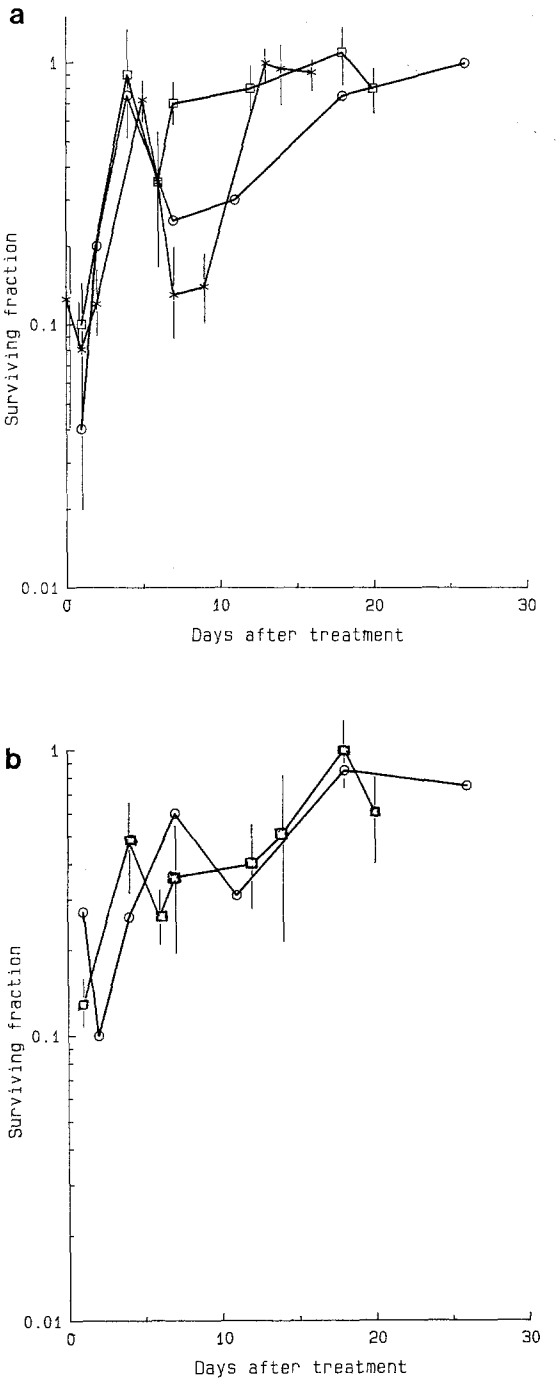

Fig. 1a, b. Recovery of femoral 8-day CFU-S **(a)** and CFU-F **(b)** colonies after the first (-o-) and fifth (-□-) doses of 200 mg/kg (±1 SE) or the first dose of 220 mg/kg (-X-), as fractions of the numbers in controls

Results

Recovery after single or repeated doses

The recovery of femoral CFU-S after the initial depletion caused by CP is shown in Fig. 1 a. Comparison of the recovery from the first and fifth doses shows similarities in the timing and level of the initial abortive peak in numbers at 4 days after treatment (Fig. 1 a). There then follows a period in which the recovery curves diverge somewhat, showing higher numbers at intermediate times in the recovery after the fifth dose than after the first dose. The return to normal levels was similar in both situations, however. Normal levels of GM-CFC were reached at about the same time (data not shown). The recovery of CFU-F after the fifth dose of 200 mg/kg was similar to that after a single dose of 220 mg/kg, with normal values reached at about 20 days (Fig. 1 b).

Each successive increment of the treatment regimen spared increasing numbers of CFU-S (Fig. 2) in measurements taken 24 h after each injected dose.

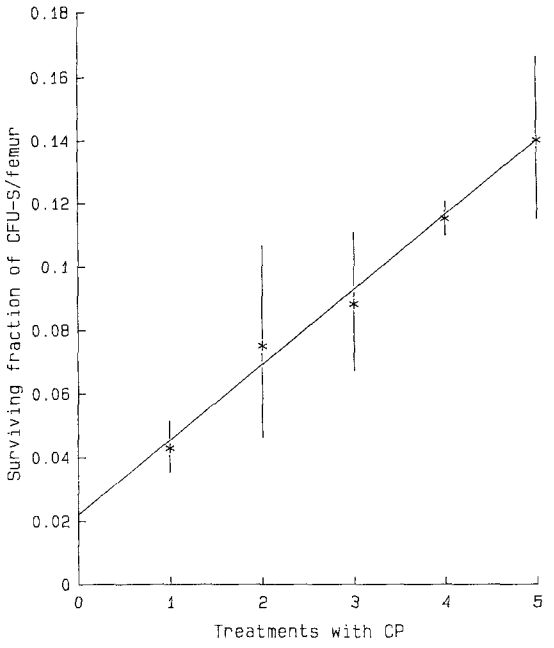

Fig. 2. The surviving fraction of CFU-S 24 h after one to five doses of CP 200 mg/kg. Each *point* is the mean of four experiments ± 1 SE. Correlation coefficient of straight line, $r = 0.993$

Changes in the self-reproduction capacity of the CFU-S

The number of colonies counted in the spleens of recipient mice increased between 8 and 11 days when they had received injections of bone marrow from mice treated with CP 24 previously (Table 1). However, the increase in counts of spleen colonies derived from marrow of mice which had been treated with CP 15 to 600 days earlier gave a normal ratio of day 11 to day 8 colonies, i.e., a ratio of 1.1–1.2 (Table 1).

One day after drug administration the ability of the surviving CFU-S to generate further spleen colonies was markedly increased over control levels (Table 2). This situation recurred after each step of the multidose regimen. These numbers are to be compared with the number of CFU-S per colony measured at an equivalent time in a group of mice which had not received the further increment in dose, in which the number of CFU-S per colony was normal or moderately subnormal. There was no dose effect with increasing number of doses (Table 2).

Long-term effects of cycloposphamide treatment

In the long term after CP, reduced numbers of both CFU-S and CFU-F were measured in the femora. The mean values of 37 individual determinations were 72% and 70% of control in CFU-S and CFU-F, respectively. This defect in both the haematopoietic and stromal colony-forming cells was stable for at least 600 days after the last increment of the five-dose regimen.

In determinations in individual mice many treated mice showed subnormal numbers of CFU-S, whilst some mice had control levels, leading to a skewed distribution of femoral CFU-S compared with the control animals (Fig. 3a). A similar though less marked shift in CFU-F was seen (Fig. 3b).

The mean peripheral blood haematocrit was lower in the CP-treated mice. However, this represented a situation where most mice had normal levels, with a minority of mice showing subnormal levels (Fig. 4). Such abnormal

Table 1. Numbers of day-8 and day-11 spleen colonies from normal and cyclophosphamide treated mice

Time after treatment	Number of experiments	Number of colonies		Ratio	Day 11
		Day 8	Day 11		Day 8
Control	15	9.8+0.7	10.7+0.5[a]	1.1	
24 h	15	12.3+2.1	20.4+1.4[b]	1.7	
15 days	5	6.0+1.1	7.0+0.9[a]	1.2	
60–600 days	3	4.2+0.9	4.5+0.9[a]	1.1	

[a] Per 5×10^4 injected marrow cells
[b] Per 4×10^5 injected marrow cells

Table 2. The effects of five repeated 200-mg/kg doses of CP on femoral 8-day CFU-S

Time after treatment (days)	CFU-S per femur[a]			CFU-S per colony[b]		
	0	1	15	0	1	15
Dose						
0	4843±25)	–	–	43±13	–	–
1×200	–	207± 83	3042±633	–	400±64	70± 7
2×200	–	180± 67	3269±637	–	265±28	55±11
3×200	–	281±173	4854±907	–	251±13	24±16
4×200	–	507± 14	4771±205	–	376±71	79± 4
5×200	–	697±225	–	–	163±42	–

[a] Mean of four experiments ± 1 standard error
[b] The mean number of CFU-S per colony for untreated mice was 79±11 (1 SE) in experiments

Table 3. Mice with values different from both control and treatment group means (TGMs) after five CP doses of 200 mg/kg

Days after 5th dose of CP	Haemato-crit %	Cells[a]		GM-CFC[a]		CFU-F[a]		CFU-S[a]	
		Abnormal mouse	TGM	Abnormal mouse	TGM	Abnormal mouse	TGM	Abnormal mouse	TGM
59	32	98	100	196	80	96	70	99	60[b]
59	43	91	100	44	80	55	70	30	60[c]
74	46	51	100	37	105	20	72	15	115[c]
124	24	ND	–	ND	–	54	80	34	91[c,d]
144	32	100	100	ND	–	37	80	43	91[c]

These were 5 mice among 63 checked

[a] Expressed as numbers per femur as percentage of number in age-matched controls

[b] Possibly preleukaemic

[c] Possibly microenvironmental and stem cell defect

[d] Hypoplastic anaemia

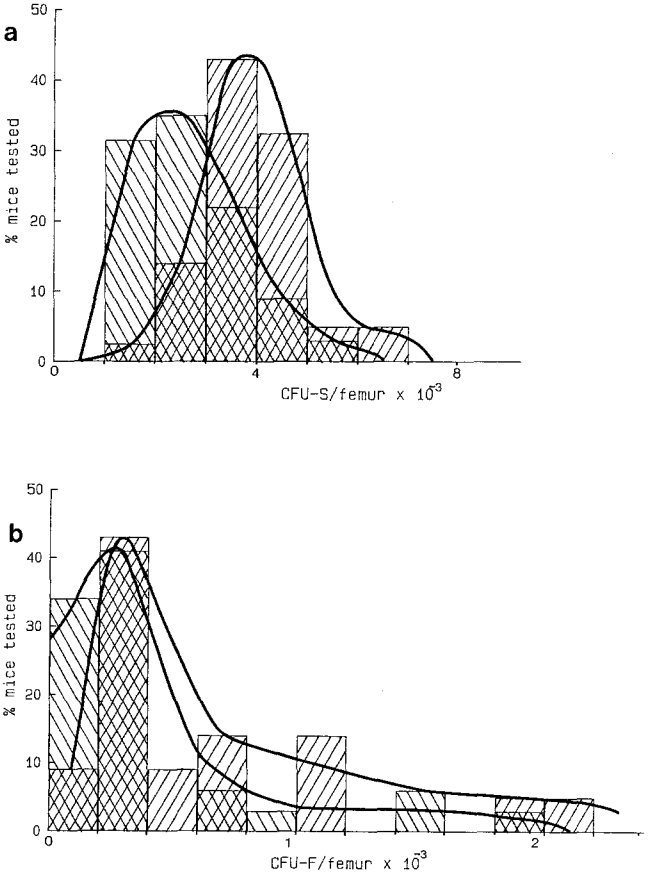

Fig. 3a, b. Distribution of CFU-S **(a)** and CFU-F **(b)** per femur in individual mice between 30 days and 15 months after treatment. Mean control values were 3860±220 (1 SE) CFU-S per femur (n=21) and 630±110 CFU-F per femur (n=21), versus 2760±80 CFU-S per femur (n=37) and 440±80 CFU-F per femur (n=33) in CP-treated mice. □, CP treated mice; □, controls

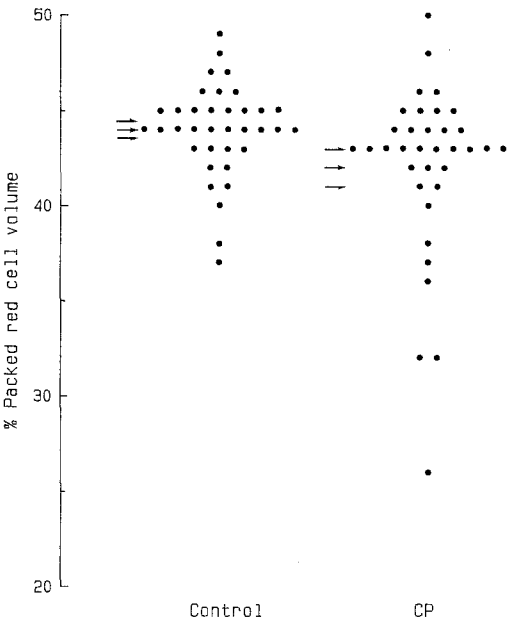

Fig. 4. Haematocrits of controls and treated mice studied at the same time intervals as given for Fig. 3. → mean ±1 SD

mice were detected not only in the haematocrit values, but also in results obtained with the colony assays, where combinations of various deficiencies were observed in 8% of the mice studied individually. However, the mean values for the treated groups show less marked differences with the untreated controls (Table 3).

Discussion

Acute effects of CP

Previous reports have shown that administration of high doses of cyclophosphamide cause major acute damage to the blood forming tissues of murine bone marrow (reviewed in [18]) and to the haematopoietic environment [1, 10]. As expected, administration of CP caused a profound reduction in the femoral CFU-S (Fig. 1), but the numbers normalised rapidly. The abortive rise seen at day 4 after CP has been reported previously after 250 mg/kg [3, 4]. Recovery from a fifth dose was slightly better than recovery from the first dose of CP, due to a smaller fall in numbers after the abortive rise at day 4. The recovery was very rapid, resulting in control levels at about 14–18 days, whether from the first or from the fifth dose, indicating that recovery was not worsened by the repeated-dose regi-

men. One possible reason for this was the increasing fraction of CFU-S surviving after successive doses. Whether this increasing fraction was the result of expansion of a CFU-S subpopulation which was more resistant to CP or of altered metabolism of the drug is not known. It has been discussed elsewhere [7] that the reduced kill of CFU-S by a second administration of CP is a result of the altered cycling status of the population. Though no examination has been performed here upon the number of CFU-S in cycle, it is unlikely that an increased fraction of the CFU-S population is in cycle when the population size has already normalised. However, the serum half-life of CP is markedly reduced after multiple treatment [8, 12]. Therefore, the reduced kill probably reflects a reduced effective dose of CP to the marrow.

Numerical evaluations of the CFU-S population concealed the existence of qualitative changes in this compartment, though no significant reduction in CFU-S/colony was reported after eight injections (1 per week) of CP 160 mg/kg [30]. Examination of the mean number of CFU-S/colony 1 day after treatment with CP revealed a high average number of daughter CFU-S produced from the CFU-S surviving treatment (Table. 2). Concurrent with the selection of CFU-S with high self-renewal capacity, there was an increase in the number of CFU-S which formed colonies after 11 days with respect to the numbers of CFU-S which formed colonies after 8 days. These data agree with the concept that CFU-S which form colonies at later times have a higher self-renewal capacity, inferred both from examination of colony growth from normal marrow [20] and from studies involving the use of cytotoxins, such as 5-fluorouracil [16], and are consistent with a model of CP action in which the less primitive CFU-S are more sensitive to the action of CP. This interpretation is also supported by the relative sparing of day 12 with respect to day 8 CFU-S reported after in vitro treatment of mouse marrow with 4-hydroperoxy-CP, an activated derivative of CP [26], and also other activated derivatives of CP [6].

Longer term effects of CP

From the studies upon the cells which survive CP, it may be speculated that little long-term damage would be expected since the highest quality CFU-S (i.e., those which form colonies at day 11, and which have higher self-reproduction capacity) selectively survive the treatment and are in theory capable of reconstituting the normal CFU-S population. However, the CFU-S population shows some abnormalities: data obtained from mice a long time after CP treatment showed a slight but definite deficit in the numbers of femoral CFU-S (Fig. 3) and a decrease in the numbers of CFU-S per colony, which is maintained for several months [33]. The study of individual mice provides more information: the overall picture is one in which many mice were in a much worse condition than controls. This situation is clearly seen in the shift of the distribution of CFU-S numbers in the femur (Fig. 3).

In haematocrit determinations the overall mean is lower in the treated group than in the controls, due to the existence of a minority of mice with severely abnormal values (Fig. 4). Such abnormal mice were rare, at about 8% of the animals studied. Close examination of five of these severely abnormal mice revealed possibly three different manifestations of residual damage (Table 3). The first, seen in only one mouse, may be a preleukaemic state in which the numbers of CFU-S and CFU-F are normal, but elevated numbers of GM-CFC and a reduced haematocrit may indicate some myeloid preleukaemic disorder. The second type of damage, noted in two mice, appears similar to that seen by Morley and Blake [23] after treatment with busulphan. Lowered CFU-S, GM-CFC, and CFU-F were noted, though the haematocrit was normal. This condition may indicate a profound residual dysfunction in the environment and the stem cell populations, which is also seen in the committed precursor GM-CFC. This intermediate type of damage may precede that seen in the last two mice in which reduced numbers of CFU-S- GM-CFC, and CFU-F were seen but in association with anaemia. The reason why these few mice suffered more profound damage than the majority is not clear, but may be linked to the complex metabolism of CP. CP is both enzymatically and nonenzymatically altered in vivo to produce derivatives of varying cytotoxicity [15]. Also there may be damage to regulatory cell populations [17, 24].

In summary, we have documented long-term damage after repeated CP treatment, which is manifested by subnormal numbers of haematopoietic and stromal colony-forming cells. This damage appears to be stable for periods in excess of half the normal lifespan, and may be manifested as haematopoietic dysplasia or hypoplasia in a minority of mice. These long-term effects are seen despite the preferential survival of CFU-S of high self-renewal capacity shortly after treatment and a smaller acute kill of CFU-S by each successive dose.

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