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Original Articles

Haematopoietic Late Effects of Prolonged Bleomycin Treatment in Mice

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Summary. *In two studies, haematopoietic late effects of prolonged bleomycin treatment were evaluated in mice given serial injections of 21 mg bleomycin/m² weekly for 31 and 44 weeks, respectively. Femoral bone marrow cellularity measured at 43, 45, and 49 weeks after discontinuation of the drug in the first and after 20 weeks in the second study was found to be significantly ($P < 0.05$) lower in the treated mice than in the controls. CFU-S, BFU-E, and CFU-C contents were also reduced in the treated bone marrow, but with the exception of CFU-S in the second study, differences from control values were not significant. Additional long-term bone marrow cultures performed in the second study revealed no marked changes in the marrow proliferative activity and the self-renewal of stem cells to explain the reduced marrow cellularity and stem cell content. These last findings might, therefore, be due to a decrease in femoral size with less marrow content in the treated mice, since measurements of the tibial weights in both groups showed that the bones in the treated animals were significantly ($P < 0.05$) lighter than those in the controls.*

Introduction

Bleomycin (BLM) is an antineoplastic agent with moderate acute effects on haematopoiesis [9]. The drug, however, has been shown to be able to produce chromosomal changes in bone marrow cells [4] and to induce recruitment of resting cells into the cell cycle [7]. Furthermore, proliferating haematopoietic stem cells have been reported to be more sensitive to BLM than nonproliferating cells [22]. It is therefore possible that if given repeatedly for a longer period of time, the drug could produce changes in the susceptibility of stem cells or in the structure of stem cell populations, leading to cumulative toxicities or late effects on haematopoiesis.

Large doses of BLM given once a day for 3 days were found to induce a slight but protracted reduction of marrow compartment size of pluripotential haematopoietic stem cells (CFU-S) in mice [2]. When the animals were treated with small doses of the drug for many weeks, the marrow CFU-S number declined severely during the initial phase of treatment, but recovered slowly to the normal level despite continued drug administration [3]. Furthermore, the proliferative activity of the bone marrow and the self-renewal behaviour of CFU-S and granulocytic precursor cells (CFU-C) were observed not to be markedly affected during the course of the prolonged treatment with BLM [18]. However, no investigations have been yet carried out to consider the possible haematopoietic late effects of such treatment. The aim of the present study was to evaluate quantitative and qualitative changes of the bone marrow after repeated doses of BLM, using conventional stem cell assays [14, 15, 20] in combination with the long-term bone marrow culture technique [10].

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Materials and Methods

Animals and Drug Administration. F_1 (CBA × C 57 BL) female mice 8–12 weeks of age and weighing 20–25 g were used for BLM treatment. BLM (Mack Illert, F.R.G.) was appropriately dissolved in 0.5 ml 0.9% NaCl and given IP. Control animals received the same volume of diluent. Single-cell suspensions were prepared from flushed femoral marrow of five mice per group per point in the first study and of 10–12 mice in each group in the second study.

CFU-S, CFU-C and BFU-E Assays. CFU-S were assayed using the method of Till and McCulloch [20]. F_1 (CBA × C 57 BL) male mice, at least 10 mice per group per point, were used as recipients and exposed to 7.5 Gy total body irradiation (X-ray machine, rate 1.43 Gy/min, focal distance 40 cm) before injection of 4×10^4 nucleated cells. Macroscopic surface colonies in the spleens were counted 9 days later. CFU-C were assayed according to the method of Bradley and Metcalf [6] as modified by Iscove et al. [14]. Mouse heart-conditioned medium was used as a source of colony-stimulating activity [8]. Early erythroid progenitor cells (BFU-E) were assayed using the technique of Iscove et al. [15] as modified by Murphy and Sullivan [17]. Sheep plasma erythropoietin (Connaught) was added to the cultures at a concentration of 2 U/ml. BFU-E colonies were stained using an improved benzidine staining technique [12]. CFU-C and BFU-E cultures were set up in triplicate.

Long-Term Bone Marrow Cultures. Cultures were set up as described by Dexter et al. [10] and maintained at 37° C in a humidified atmosphere with 5% CO_2 in air. Re-inoculation of adherent layers was performed in the 4th week. Thereafter, suspension cells obtained at weekly demidepopulations were counted, centrifuged, and resuspended in alpha-medium at a suitable concentration for cytological analysis and CFU-S, CFU-C, and BFU-E assays. In each experiment, pooled cells from six cultures were used per group per point.

Statistical Analysis. Significance of differences between the results from control and BLM-treated groups was tested by Student's *t*-test for two means.

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Table 1. Late effects of prolonged bleomycin (BLM) treatment on the bone marrow cellularity and stem cell content in mice[a]

	Duration of BLM[b] treatment (weeks)	Time of marrow examination (weeks after drug cessation)	Total nucleated cells/femur ($\times 10^6$)		CFU-S/femur		CFU-C/femur		BFU-E/femur	
			Control	BLM	Control	BLM	Control	BLM	Control	BLM
Study I	31	43	22.31 ± 0.08	18.61* ± 0.1	4,379 ± 266	3,557 ± 331	25,589 ± 3,804	20,380 ± 4,423	NA[c]	NA
		45	22.66 ± 0.04	18.45* ± 0.07	4,446 ± 700	3,971 ± 799	35,118 ± 3,111	30,767 ± 2,385	NA	NA
		49	22.69 ± 0.01	18.38* ± 0.13	5,077 ± 371	3,885 ± 463	25,676 ± 5,196	16,380 ± 100	1,834 ± 740	1,181 ± 115
Study II	44	20	21.39 ± 2.12	14.11* ± 0.9	5,758 ± 368	3,664* ± 337	31,324 ± 2,659	22,894 ± 5,800	1,281 ± 564	1,028 ± 173

[a] Data represent the means ± standard errors of two separate experiments
[b] 21 mg/m^2 weekly
[c] NA, Data not available
* Significantly different from control ($P < 0.05$)

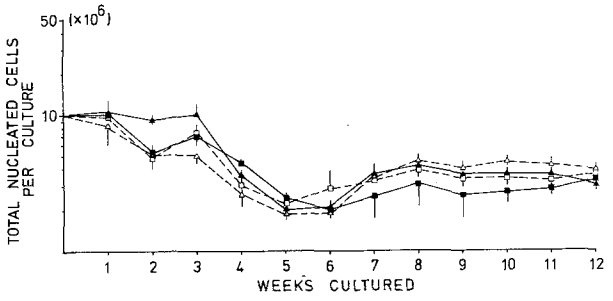


Fig. 1. Production of nucleated cells in long-term bone marrow cultures containing marrow-derived adherent layers from bleomycin-treated or untreated mice, each randomly reseeded with bone marrow cells from treated or control animals. The former were given 21 mg BLM/m^2 weekly for 44 weeks. Cultures were set up 20 weeks after drug cessation. Results represent the means ± SE of two separate experiments, in each of which six cultures per group were used. (■) BLM adherent + BLM marrow; (□) BLM adherent + control marrow; (▲) control adherent + BLM marrow; (△) control adhrent + control marrow

Results

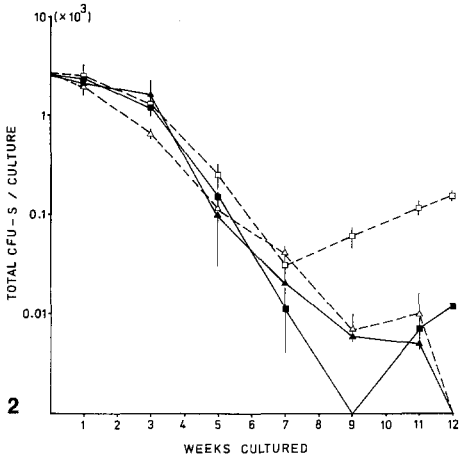
In two studies, mice received serial injections of 21 mg/m^2 (= 7 mg/kg) weekly for 31 and 44 weeks, respectively. This single dose of BLM is approximately one-fifth to one-quarter of the LD_{10} for the animals used [13], and when the conversion factor of Freireich et al. [11] is applied it is seen to be comparable to a single dose of 40–50 mg BLM given to a human weighing 70 kg. Femoral marrow cellularity and contents of CFU-S, CFU-C, and BFU-E were measured 43, 45, and 49 weeks after discontinuation of the drug in the first and after 20 weeks in the second study. At all experimental points there was a significant ($P < 0.05$) reduction of the marrow cellularity in the treated animals compared with the controls (Table 1). CFU-S, CFU-C, and BFU-E contents were also reduced in the treated bone marrow, but with the exception of CFU-S in the second study, the differences from the control values were not significant. Marrow differentials performed in the first study revealed comparable distributions of mature and immature cells in the treated and untreated mice.

In the second study, additional long-term bone marrow cultures were used to evaluate possible changes in the marrow proliferative activity, in the self-renewal behaviour of stem cells, and in the haematopoietic micro-environment. Bone marrow-derived adherent layers from treated and untreated mice were each randomly reseeded with bone marrow cells from treated or control animals. Cultures were maintained for 12 weeks. Total nucleated cell count and concentrations of CFU-C and BFU-E per culture were determined at weekly intervals and CFU-S every 2 weeks.

No persistent differences were found in nucleated cell formation between the different culture groups throughout the culture period (Fig. 1). Proliferative activity of nucleated cells was observed to be most vigorous between the 2nd and 3rd weeks and from the 5th week up to the termination of cultures. In all culture groups, the nonattached cells collected during the first 4–5 weeks consisted of up to 60% granulocyes at different stages of maturation, and thereafter of macrophages.

Self-renewal of CFU-S was found to be most marked during the first 3 weeks in all culture combinations (Fig. 2). Afterwards, in cultures combining marrow-derived adherent layers from the control mice and marrow cells from the treated or control animals, the number of CFU-S declined progressively, and the cells disappeared in the 12th week of culture. A more rapid decrease of CFU-S concentration was found in cultures containing both adherent layers and marrow cells from the treated mice. In this culture group, the CFU-S disappeared as soon as the 9th week, but the cells reappeared in the 11th week and showed increasing proliferative activity. In cultures combining marrow-derived adherent layers from the treated mice and marrow cells from the controls, the CFU-S number only declined during the first 7 weeks. Thereafter, this culture group produced an increasing number of CFU-S, with significant ($P < 0.05$) differences from the numbers present in the other culture combinations.

In all culture groups, the BFU-E and CFU-C showed a similarly high proliferative activity during the first 2 weeks, with an overshoot of the cell concentrations above the inoculated levels (Fig. 3 and 4). Afterwards, the cell numbers declined rapidly and the cells disappeared by the 7th to the 8th week. From the 4th to the 6th week, the cultures containing marrow-derived adherent layers from the treated mice and marrow cells from the controls produced higher numbers of BFU-E with partly significant ($P < 0.05$) differences from the numbers present in the other culture combinations, but these



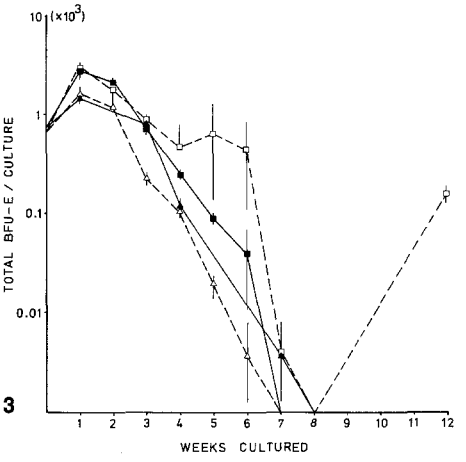


Fig. 2. CFU-S proliferation in long-term bone marrow cultures containing marrow-derived adherent layers from bleomycin-treated or untreated mice, each randomly reseeded with bone marrow cells from treated or control animals. Results correspond to the data presented in Fig. 1. (■) BLM adherent + BLM marrow; (□) BLM adherent + control marrow; (▲) control adherent + BLM marrow; (△) control adherent + control marrow

Fig. 3. BFU-E growth in long-term bone marrow cultures containing marrow-derived adherent layers from bleomycin-treated or untreated mice, each randomly reseeded with bone marrow cells from treated or control animals. Results correspond to the data presented in Fig. 1. (■) BLM adherent + BLM marrow; (□) BLM adherent + control marrow; (▲) control adherent + BLM marrow; (△) control adherent + control marrow

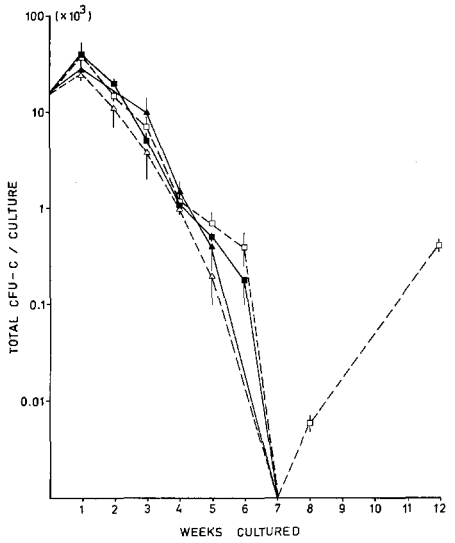


Fig. 4. CFU-C growth in long-term bone marrow cultures containing marrow-derived adherent layers from bleomycin-treated or untreated mice, each randomly reseeded with bone marrow cells from treated or control animals. Results correspond to the data presented in Fig. 1. (■) BLM adherent + BLM marrow; (□) BLM adherent + control marrow; (▲) control adherent + BLM marrow; (△) control adherent + control marrow

Table 2. Comparison of tibial weight and length between bleomycin[a] treated and untreated mice

	Control[b]	Bleomycin[b]
Weight (mg)	57.1 ± 1.0 (23)	49.9* ± 1.0 (18)
Length (mm)	18.2 ± 0.3 (23)	17.7 ± 0.3 (18)

[a] Animals received weekly injections of 21 mg BLM/m² for 44 weeks. Measurements of tibial weight and length were performed 20 weeks after drug cessation

[b] Figures in parentheses give the numbers of tibia examined

* Significantly ($P < 0.05$) different from the control

differences did not persist; and from the 6th to the 7th week the BFU-E concentration dropped sharply to the levels given in the other culture groups. Thereafter, however, the BFU-E and CFU-C were only detectable in the former culture combination.

Discussion

The data presented suggest that prolonged treatment with BLM at the given dose could eventually result in a significant decrease of marrow cellularity, and stem cell content in mice. Additionally, these effects appear to depend upon the duration of treatment, considering the extent to which the marrow nucleated cell count and CFU-S pool were reduced in the first and in the second study. However, no apparent changes were found in the distribution of morphologically recognizable cells, in the marrow proliferative activity, or in the self-renewal of stem cells, which would explain the reduced marrow cellularity and stem cell content. In contrast, marrow-derived adherent layers from the treated mice appeared to be more active in supporting the maintenance of stem cells than those from the controls. Other mechanisms, like growth abnormalities in the treated mice, might therefore be responsible for the reduction of the marrow cell concentrations. Individual measurements of tibial weights and lengths performed in the second study showed that the bones of the treated mice weighed on average significantly ($P < 0.05$) less than those of the controls (Table 2). Skeletal retardations have been reported in children treated for long periods with cytotoxic agents [1, 19]. These children, however, showed compensatory growth after therapy was discontinued. In our studies, the mice treated with BLM were in a similarly vulnerable phase of their growth during the prolonged drug administration, but the animals were treated for a relatively longer period of time in view of the different lifespans of mice and man. Thus, the growth activity of the animals might have been completed before the drug was discontinued. Whether the data presented here suggest growth abnormalities remains an open question. However, with

regard to the reduction of the bone weight in the treated mice, it may be that the reduced femoral content of marrow nucleated cells and stem cells was due to a reduced femoral size and marrow volume and not to a lowering of marrow cell concentration as such.

A number of cytotoxic agents such as busulfan, L-phenylalanine mustard and chlorambucil have been shown to produce persisting depletion of stem cell compartment sizes, and particularly of repopulating ability, when given repeatedly [5, 21]. In view of our experimental results, BLM appears to be less harmful than these agents, since the stem cells did not seem to be affected qualitatively after prolonged treatment with this agent.

Marrow-derived adherent layers in liquid cultures have been shown to be essential for the maintenance of stem cells, and to allow the growth of cells responsible for some properties attributed to a haematopoietic micro-environment [10, 23]. If this is considered together with our data, it seems possible that the haematopoietic micro-environment in the BLM-treated mice was primed to support the self-renewal of stem cells. Such an assumption, however, remains speculative and deserves further studies evaluating the adherent layers quantitatively by measurement of adherent colonies in the layers derived from the bone marrow of the treated and untreated animals [16, 23].

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