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A STIMULATOR OF PROLIFERATION OF SPLEEN COLONY-FORMING CELLS (CFU-S) IN THE BONE MARROW OF IRRADIATED RATS

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The presence and activity of a spleen colony - forming cell (CFU-S) proliferation stimulator was investigated in rat bone marrow after irradiation. Wistar rats were treated with 5 Gy X-irradiation and the CFU-S proliferation stimulator was detected in regenerating bone marrow on day 5 after irradiation. The dose dependent increase in cytosine arabinoside induced cell death of normal mouse bone marrow day 8 CFU-S was found when the 30-50 kDa extract fraction of media conditioned by regenerating rat bone marrow cells was tested for the activity of a CFU-S proliferation stimulator. The results demonstrate the existence of a CFU-S proliferation stimulator in rat bone marrow similar to that originally found as a macrophage product in regenerating mouse bone marrow (Lords type of Stimulator). The CFU-S proliferation stimulator activity was not associated with the presence of interleukin - 1, 2, or 6 like activites in the material tested.

Key words: Stimulator of CFU-S proliferation, CFU-S proliferation following irradiation, specificity of CFU-S regulators.

INTRODUCTION

The haemopoietic system comprises a continuum of cells with a defined age structure. Regulatory molecules influencing growth and development of stem and progenitor cells in vitro are available and include a variety of colony stimulating factors, interleukins and lymphokines (Dexter et al. 1990). The stem cell population is proliferatively quiescent in constitutive haemopoiesis (Lajtha et al. 1969) and there is ample evidence for the existence of regulatory molecules locally produced and acting in the haemopoietic microenvironment (Moore 1990). The proliferative status of stem cells is regulated by a balance of stimulator and inhibitor activity on the stem cell population (Lord 1986a).It has been demonstrated that in bone marrow regenerating after irradiation treatment the increased proliferation rate is associated wirh the presence of a stimulator of stem cell proliferation (Frindel et al. 1976, Lord et al. 1977). The relationship of the CFU-S proliferation stimulator present in the bone marrow after cytotoxic

damage to defined cytokines produced by the haemopoietic microenvironment and possibly influencing CFU-S proliferation is not known. The CFU-S proliferation stimulator is produced by the bone marow monocyte/macrophage population of cells (Wright and Lorimore 1987). We have recently demonstrated that the CFU-S proliferation stimulator found in immunodeficient mouse bone marrow after irradiaton or cyclophosphamide treatment is not related to the presence of interleukins - 1, 2, 6 (IL-1, IL-2, IL-6) or inhibitors of IL-1 or IL-6 (Milenković et al. 1991). It is possible that the existence of a specific CFU-S proliferation stimulator unrelated to major monokines is a common biological phenomenon. Therefore the presence and activity of CFU-S proliferation stimulator was investigated in this paper in the bone marrow of irradiated rats.

MATERIALS AND METHODS

Experimental animals and procedures. The experiments were performed on male Wistar rats weighing 180-200g. Regenerating bone marrow cells after sublethal irradiation of the rats were used for preparation of bone marrow cell conditioned media and fractionated material to be tested for the presence and activity of CFU-S proliferation stimulator. Male CBA/H mice weighing 20-25 g were used as donors of bone marrow cells for the testing of activity of CFU-S proliferation stimulator in vitro and as irradiated recipients in the CFU-S assay. The number of nucleated bone marrow cells was determined using conventional methods.

Preparation of fractions from bone marrow conditioned media. Conditioned media (CM) were prepared as described previously (Milenković et al. 1987) using the method described originally for mouse bone marrow (Lord et al. 1977). The rats were subjected to a sublethal dose of 5 Gy X-irradiation (Philips RT 305, 300 kV 10 mA, dose rate 0.959 Gy/min). Regenerating bone marrow cells on day 5 after irradiation were used for preparation of conditioned media (CM-Ir) to be tested for the presence of CFU-S proliferation stimulator. Washed bone marrow cells were suspended in Dulbecco's modification of Eagles medium (DMEM) and incubated at 37° C for 2 h in an atmosphere of 5 % CO_2 in air.The cells were removed by centrifugation and the supernatant was sterilized and stored at -20° C until tested. Media conditioned by normal bone marrow cells (CM-N) prepared in the same way were used as a control. The activity of CFU-S proliferation stimulator was determined in the 30-50 kDa fraction of bone marrow CM. CM-Ir or CM-N were fractionated by Amicon Diaflo molecular weight ultrafiltration into molecular weight ranges. The fraction retained on the PM 30 filter after previous filtration of CM-Ir through the XM 50 filter was designated as regenerating bone marrow extract (RBME-III) according to Lord et al. (1977). Material of the same molecular weight range was also prepared from CM-N and designated as NBME-III. The fractionated materials were lyophilized and stored at -20° C until tested.

Assay of CFU-S proliferation stimulator. Diferences in sensitivity of mouse CFU-S subpopulations to stimulator and inhibitor of CFU-S proliferation have been described (Wright et al. 1985) and therefore the effects of rat bone marrow

CM and RBME-III were tested on mouse day eight CFU-S (CFU-Sd8) as a sensitive population for detecting CFU-S stimulator activity. Conditioned media (CM-Ir and CM-N) were tested undiluted. RBME-III was dissolved in DMEM and increasing concentrations tested as indicated in the Results section. The presence and activity of CFU-S proliferation stimulator was estimated on the basis of an increased proportion of normal mouse bone marrow SFU-Sd8 killed by cytosine arabinoside (Ara-C) after in vitro treatment with the material tested. The method used was essentially as described previously (Milenković et al. 1987,1991). Duplicate aliquots of normal bone marrow cell suspensions (5 x 10^6 /ml) in DMEM with 15 % fetal calf serum were incubated at 37° C in an atmosphere of 5 % CO2 in air for 2h with one millilitre of material tested. To one of the duplicate aliquots 40 μg Ara-C (Cytosar, Upjohn) was added and incubated for the last 1 h. A control normal bone marrow cell suspension without material tested was also incubated with and without Ara-C. At completion of the incubation period cells were washed in DMEM and appropriately diluted suspensions made for assay of CFU-Sd8. The reduction in colony formation by the cells from samples treated with Ara-C was proportional to the number of CFU-S in DNA synthesis (suicide) and therefore represented an indirect measure of the proportion of proliferating CFU-Sd8 in DNA synthesis. Due to the large intrinsic errors in the suicide techniques, only the values of more than 25 % kill by Ara-C were considered to represent the effect of CFU-S stimulator (Lord 1986b).

Spleen colony assay. The method of CFU-S determination was basically that of Till and McCulloch (1961), as described previously (Milenković et al. 1983). The recipient mice were irradiated with 9 Gy, 300 kV X-rays at a dose rate of 0.959 Gy/min. Groups of 8-22 recipients were then injected with an appropriate number of bone marrow cells to give countable numbers of spleen colonies. The spleens of the recipients were removed 8 days later, fixed in Telleyesniczky's fluid for spleen colony counting. The number of CFU-Sd8 was calculated per 10^5 cells.

Granulocyte-macrophage progenitor assay. The number of granulocyte-macrophage colony forming units (CFU-GM) derived colonies was determined in rat femoral bone marrow using the methylcellulose culture technique as described previously (Rolović et al. 1990). The culture mixture containing 5 x 10^4 or 1 x 10^5 nucleated cells, 10 % FCS, and 10 % horse serum with 5 % rat lung-conditioned medium derived colony-stimulating activity and 0,8 % methyl-cellulose was cultivated in 35 mm Greiner petri dishes for 7 days at 37° C in a humidified atmosphere containing 5 % CO2 in air. The number of colonies (> 50 cells) was scored using an inverted microscope and calculated per total number of femoral bone marrow nucleated cells. The number of CFU-GM after irradiation was expressed as a percentage of the CFU-GM found in rats not irradiated.

Assays for cytokine biological activites. IL-1 activity in RBME-III and NBME-III was measured by 3H thymidine ((3H)TdR) incorporation into the IL-1 sensitive D10.S cell line, a subclone of the murine T-helper cell line D 10.6.4.1 described by Orencole and Dinarello (1989) (kindly provided by Dr C.A. Dinarello). D10.S

cells were tested at 1 x 10^4 cells per culture and different dilutions of human recombinant IL-1, (rIL-1, Genzyme), RBME-III or NBME-III were added. Cultures were incubated for 72 h at 37° C and 5 % CO_2, pulsed for 18 h with (3H)TdR and then harvested and counted.

IL-2 activity was tested using a proliferation assay of Con A - induced T-cell blasts, as described previously (Lukić et al. 1987). Briefly, T cell blasts (2 x 10_4 cells in 0,2 ml final volume were cultured for 48 h (in 96 - well flat - bottomed microculture plates) as triplicate cultures in medium with serial log 2 dilutions of human recombinant IL-2 (rIL-2, Cetus, Emerwille, CA) or different dilutions of RBME-III and NBME-III. IL-2 driven proliferation was determined by pulsing cells with (^{3}H)TdR for the final 8 h of culture.

For determination of IL-6 activity in RBME-III and NBME-III, an IL-6 dependent murine hybridoma cell line B9 (kindly provided by Dr Aarden) was used (Aarden et al. 1987, Helle et al. 1988). The cells were grown in RPMI 1640 medium containing 5 % FCS, 5 x 10^{-5} M 2 - mercaptoethanol, 100 U/ml garamycim ad 1 % of a supernatant source of IL-6 (supernatant of rat peritoneal cells cultured for 24 h with 5 ng/ml lipopolysaccharide). The B9 assay was carried out in flat - bottomed microtitre plates in a volume of 200 ml. A total of 5 x 10^3 B9 cells/well were cultured in the presence of various concentrations of RBME-III or NBME-III or rIL-6.Proliferation was measured by a (3H)TdR pulse from 64-72 h.

Statistical analysis. The differences in percentage of CFU-Sd8 killed by Ara-C was evaluated by Student's t-test.

RESULTS

The treatment of normal mouse bone marrow cells in vitro with the rat CM assayed here did not influence the colony yield in the CFU-Sd8 assay. About 10 % of CFU- Sd8 in normal mouse bone marrow were in DNA synthesis. The treatment of normal mouse bone marrow cells in vitro with CM from regenerating rat bone marrow after 5 Gy irradiation increased the percent of CFU-Sd8 in DNA synthesis (Table 1). This was not found after the treatment with CM prepared from normal rat bone marrow.

Table 1. The effect of media conditioned by rat bone marrow cells regenerating after irradiation and normal bone marrow cells on the proliferation of CFU-Sd8 in normal mouse bone marrow

	CFU-Sd8/10^5 cells		% in DNA synthesis (% killed by Ara-C)
	without Ara-C	with Ara-C	
Control	41.5 ± 3.0	35.3 ± 3.2	12.4 ± 3.0
CM-Ir	43.2 ± 2.6	27.2 ± 1.9	30.5 ± 3.5***
CM-N	31.1 ± 2.0	32.0 ± 2.2	not detectable

CM-Ir = medium conditioned by rat bone marrow cells on day 5 after 5 Gy X irradiation; Ara-C = cytosine arabinoside, 40 μg / 5 x 10^6 incubated cells; Results are mean values ± SE of three experiments; three mice per donor group, eight animals per recipient group;*** = $p < 0.001$ in comparison to the control values.

The presence of a CFU-S stimulator in the RBME- III is shown in Table 2. A significant increase in the proportion of CFU-Sd8 killed by Ara-C was found with 12.5 to 50 μg/ml of RBME-III. In contrast CFU-S stimulator activity was not detected in NBME-III at the same dose range of extract tested.

Table 2. The effect of rat RBME-III prepared after irradiation and NBME-III on proliferation of CFU-Sd8 in normal mouse bone marrow

	dose	SFU-Sd8/10^5 cells		% in DNA synthesis
	μg/ml	without Ara-C	with Ara-C	(killed by Ara-C)
Control	0	33.4 ± 2.7	29.2 ± 2.0	10.3 ± 5.7
RBME-III	12.5	38.9 ± 1.1	26.2 ± 1.2	32.5 ± 3.2*
	25.0	30.3 ± 1.9	18.0 ± 1.5	40.3 ± 4.8**
	50.0	27.1 ± 2.0	19.3 ± 2.5	28.9 ± 9.3*
Control	0	34.3 ± 2.2	30.0 ± 1.2	12.3 ± 3.7
NBME-III	6.2	28.0 ± 3.7	26.0 ± 2.1	7.0 ± 2.6
	12.5	31.4 ± 1.2	25.9 ± 1.5	16.8 ± 5.0
	25.0	32.8 ± 2.1	29.4 ± 2.1	10.4 ± 6.6

RBME-III = extract fraction (30-50 kDa) prepared from media conditioned by regenerating rat bone marrow cells; Results are mean values ± SE of three experiments, three mice per donor group, eight animals per recipient group;* = $p<0.05$;** = $p<0.01$ in comparison to the control values.

RBME-III prepared from irradiated rats was titrated in two-fold dilutions in the B9 assay to determine the possible presence of IL-6 activity (Figure 1). There was no proliferation of B9 cells cultured in the presence of the samples

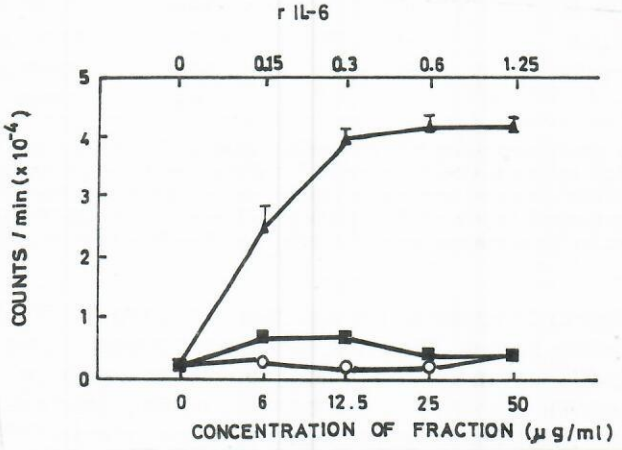

Figure 1. Quantification of interleukin — 6 (IL-6) activity in 30-50 kDa extract fraction prepared from (o) regenerating rat bone marrow cells (RBME-III) or (■) normal bone marrow sells (NBME-III) in B9 assay. The control response to rIL-6 of B9 cells (▲) cultured for 72 h is also shown. (3H)TdR was added at 64 h. The results are expressed as counts/min ± SE of triplicate cultures.

tested, demonstrating the absence of IL-6 like activity in RBME-III. Neither was IL-1 like activity detectable as determined by proliferation of the IL-1 sensitive D10.S cell line (Table 3). Finally there was no IL-2 activity in the range of doses tested in RBME-III from irradiated rat bone marrow (Table 4) as evalued by the proliferative response of T-cell blasts. It appears that the observed stimulatory effects of RBME-III on CFU-S proliferation are not due to the presence of IL-1, IL-2, or IL-6 even in the unpurified material tested.

Table 3. IL-1 activity in rat RBME-III prepared after irradiation and control NBME-III

	RBME-III	NBME-III	Human rIL-1	
reciprocal dilution	(^{3}H)TdR incorporation counts/min		pg/ml	(^{3}H)TdR incorporation counts/min
1	466 ± 39	267 ± 29	25	8172 ± 556
10	279 ± 58	302 ± 19	12	10935 ± 211
50	286 ± 51	267 ± 58	6	7475 ± 337

IL-1 activity was determined using the D10.S cell line; The spontaneous (^{3}H)TdR uptake of D10.S was 786 ± 121 counts/min; RBME-III = extract fraction (30-50kDa) prepared from media conditioned by regenerating bone marrow cells of rats sacrificed on day 5 after 5 Gy X-irradiation; NBME-III = extract fraction (30-50 kDa) prepared from media conditioned by bone marrow cells of normal rats; Results are mean values ± SE of triplicate cultures.

Table 4. IL-2 activity in rat RBME-III prepared after irradiation and control NBME-III

	RBME-III	NBME-III		Human rIL-2
reciprocal dilution	(^{3}H) TdR incorporation counts/min		pg/ml	(^{3}H)TdR incorporation counts/min
1	267 ± 32	285 ± 4	50.0	60785 ± 584
10	214 ± 9	263 ± 22	25.0	61249 ± 1243
50	221 ± 24	262 ± 17	12.5	30079 ± 2933
			6.2	15842 ± 893

IL-2 activity was determined using the proliferation assay of Con A-induced T-cell blasts; The spontaneous (^{3}H)TdR uptake of Con A - induced T cell blasts was 264±10 counts; RBME-III = extract fraction (30-50 kDa) prepared from media conditioned by regenerating bone marrow cells of rats sacrificed on day 5 after 5 Gy X-irradiation; NBME-III = Extract fraction (30-50 kDa) prepared from media conditioned by bone marrow cells of normal rats; Results are mean values ±SE of triplicate cultures.

It is not possible to demonstrate the age structure of the rat CFU-S population in a rat to rat assay system. Therefore, in this work instead of rat CFU-Sd8 the number of CFU-GM was measured in bone marrow as a population of cells known to have a similar response in number and cycling after irradiation as CFU-S (Tejero et al. 1988).The changes within rat bone marrow CFU-GM observed at different time intervals after irradiation are presented in Figure 2. The number of CFU-GM was drastically reduced 24 h after 5 Gy irradiation and was followed by the beginning of spontaneous regeneration. Five days after irradiation, at the time when bone marrow cells were used for the preparation

of CM the number of CFU-GM was only 12 % of that found in unirradiated control animals. It could be assumed that this low number of CFU-GM was associated with the high cycling rate of CFU-S and CFU-GM.

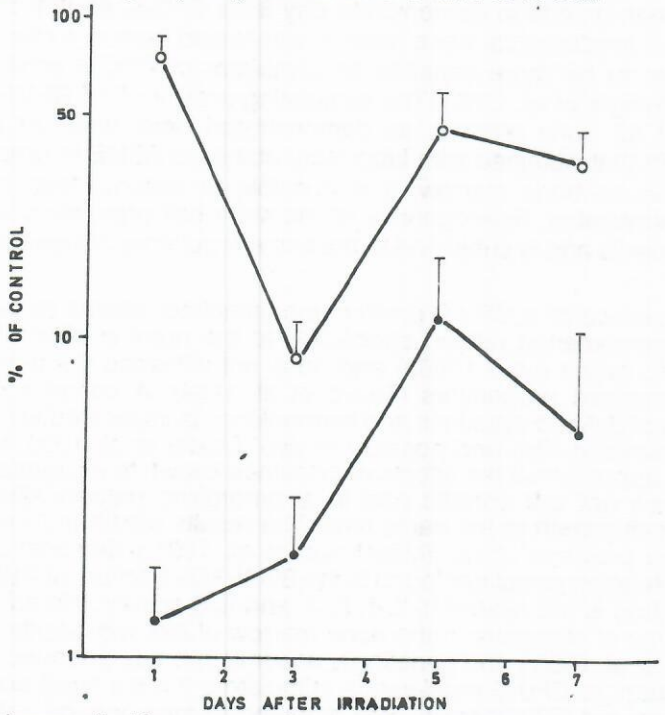

Figure 2. The changes of rat bone marrow CFU-GM (•) and number of nucleated cells (o) after 5 Gy X-irradiation. The results are presented as percentage of corresponding control values in unirradiated rats, mean ± SE of 3-9 rats per group.

DISCUSSION

The results presented here demonstrate the presence of a CFU-S proliferation stimulator in rat bone marrow regenerating after irradiation, as has been originally found in mouse bone marrow (Lord et al. 1977). The CFU-S proliferation stimulator found in rat bone marrow significantly increased the proportion of mouse bone marrow CFU-Sd8 in DNA syntesis. The same type of stimulator has been also found in human bone marrow and human fetal liver (Cork et al. 1982, Oishi et al. 1987) and was tested on mouse bone marrow CFU-S indicating that this type of CFU-S proliferation stimulator is not species specific.

Although an assay for determination of rat CFU-S has been described (Comas and Byrd 1967) the standard rat to rat spleen colony assay with unsorted bone marrow cells is faced with a number of difficulties. The population of rat bone marrow CFU-S (McCarthy and Hale 1990) enriched to a similar extend as mouse CFU-S (Lord and Spooncer 1986, Spangrude et al. 1988), as well as unsorted bone marrow cells form only day 12 spleen colonies in irradiated

recipients (CFU-Sd12). Heterogeneity within the rat spleen colony-forming cell population similar to that found in mice has only been described when irradiated mice are used as recipients of rat bone marrow cells (Martens et al. 1986). We have also been unable to demonstrate day 8 rat CFU-S, so that the activity of RBME-III from irradiated rat bone marrow was tested here in a mouse CFU-Sd8 assay known to be more sensitive to stimulator of CFU-S proliferation than CFU-Sd12 (Wright et al. 1985). The stimulating activity of rat RBME-III obtained in irradiated rat bone marrow, as demonstrated here, was not substantially different from that obtained with laboratory standard RBME-III preparared from irradiated mouse bone marrow. It is possible to assume that, although not readily demonstrable, heterogeniety of the stem cell population also exists in rat haemopoiesis and is subjected to the same regulatory influences in proliferation.

The existece of a CFU-S proliferation stimulator seems to be a common biological phenomenon related specifically to the number of stem cells in the haemopoietic organ (Lord 1986a) and does not influence the number and cycling of committed progenitors (Tejero et al. 1984). A complex of regulatory interactions of defined cytokines and lymphokines is implicated in the regulation of haemopoiesis in vitro and possibly in vivo (Dexter et al. 1990, Moore 1990). However, it appears that the effects of cytokines known to influence proliferation or CFU-S are not cell specific and in a synergistic pattern affect stem and progenitor cell growth at the same time. The results presented here as well as those in our previous paper (Milenković et al. 1991.) demonstarate that the CFU-S proliferation stimulator found in the 30-50 kDa fraction of RBME-III (Lord's type stimulator) is not related to IL-1, IL-2, and IL-6 activity. We have also found the same type of stimulator in the bone marrow of rats with sterile inflammation (Milenković et al. 1993.) and genetically anaemic b/b rats (manuscript in prepartion). Furthermore CFU-S proliferation stimulators (Lord's type) so far tested in our laboratory did not synergize with granulocyte-macrophage colony stimulating factor (GM-CSF) or erythropoietin (Epo) in CFU-GM or erythroid progenitors (BFU-E and CFU-E) formation in vitro (data not shown). It is therefore possible that the unpurified 30-50 kDa fraction of RBME-III contains specific molecules influencing CFU-S proliferation distinct from the already defined interleukins, colony stimulating factors and probably stem cell factor (Zsebo 1990). Further experiments with more purified molecules of CFU-S proliferation stimulator are needed to demonstrate this specificity.

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STIMULATOR PROLIFERACIJE PLURIPOTENTNIH MATIČNIH ĆELIJA HEMATOPOEZE (CFU-S) U KOSTNOJ SRŽI ZRAČENIH PACOVA

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SADRŽAJ

U kostnoj srži pacova soja Wistar određivano je prisustvo i aktivnost stimulatora proliferacije CFU-S peti dan nakon podvrgavanja životinja subletalnoj dozi X zračenja (5 Gy). Prisustvo stimulatora je određivano na osnovu porasta suicida CFU-S izazvanog citozin-arabinozidom nakon inkubacije ćelija kostne srži normalnog miša sa frakcijom od 30 do 50 kDa dobijenom iz medijuma kondiciniranog ćelijama kostne srži zračenih pacova. Testirani materijal, koji je pokazao aktivnost stimulatora proliferacije CFU-S nije sadržavao merljive aktivnosti IL-1, IL-2 i IL-6 što isključuje učešće ovih citokina u pokazanom stimulatornom efektu. Rezultati pokazuju postojanje stimulatora proliferacije CFU-S u kostnoj srži pacova čija aktivnost se ne može pripisati IL-1 i IL-6 i koji je sličan stimulatoru nađenom u kostnoj srži miša, za koji je pokazano da je produkt makrofaga.