Acta Veterinaria (Beograd), Vol. 45. No. 5-6, 261-268, 1995.

UDK 619:612.619:612.41

THE NUMBER AND PROLIFERATION ACTIVITY OF RAT BONE MARROW SPLEEN COLONY FORMING-CELLS AS DETERMINED IN A "RAT TO MOUSE" ASSAY

Z. IVANOVIĆ and P. MILENKOVIĆ

Institute for Medical Research, 11001 Belgrade, POB 721, Yugoslavia

(Received, 31. August 1995)

The determination of rat hemopoietic stem cells is associated with a number of difficulties. However, spleen colony forming cells (CFU-S) could be successfully determined using lethally irradiated mice as the recipients of the rat bone marrow cells ("rat to mouse" assay). We used a "rat to mouse" CFU-assay to determine the number and proliferation activity of bone marrow CFU-Sd8 in normal Wistar rats. The results presented indicate that the femoral CFU-Sd8 number in Wistar rats was close to literature values reported for BN/Bi/Rij rats. The proliferation activity of rat bone marrow CFU-d8 was very low. On the basis of the same proliferation activity and similar functional properties with murine bone marrow CFU-Sd8 as found earlier, it could be concluded that the rat CFU-Cd8 as determined in the "rat to mause" assay, represents a mature subpopulation of CFU-S.

Key words: rat hemopoietic stem cells, CFU-Sd8, proliferation activity, bone marrow

INTRODUCTION

The first experimental confirmation of the existence of the pluripotent hemopoietic stem cells (HSC) was presented in the classical study of Till and McCulloch (1961), on the basis of the capability of these cells to seed in the spleen of lethally irradiated mice, to proliferate, differentiate and form colonies consisting of cells belonging to all three hemopoietic lineages (Colony Forming Units-Spleen - CFU-S). Today, more than three decades of research after Till and Mc Culloch's discovery, it is generally considered that the HSC assayed by determination of spleen colony-forming cells (in murine hemopoietic tissue) belong to a mature population of HSC, and the cells responsible for long term reconstitution of lethally irradiated recipients represent a less mature i. e primitive population of HSC. The properties of murine CFU-S are well known (reviewed in: Ivanović 1992). The characteristic of CFU-S in the steady state is a low cycling rate (proliferation activity) i. e. more than 90% of these cells are out of the active cell cycle, mainly in the Go phase.

An assay for the determination of CFU-S in rat hemopoietic tissue, using lelthally irradiated rats as recipients of bone marrow cells ("rat to rat assay") has been developed (Comas and Byrd 1961). Although several studies employing this assay were performed (Dunn and Elson 1970, 1970a, 1970b, Dunn 1972), the question of comparability of mouse and rat bone marrow CFU-S subpopulations determined on the basis of spleen colony development with time after BMC injection in "mouse to mouse" and "rat to rat" assays remained unresolved due to difficulties in obtaining rat day 8 CFU-S (CFU-Sd8) colonies. In addition, "rat to rat" assays are expensive and difficult to perform.

Van Bekkum's group determined rat CFU-S subpopulations using mice as recipients of rat bone marrow cells (Van Bekkum 1977, Martens et al. 1986). These studies demonstrated heterogeneity of rat CFU-S as determined on the basis of colony formation in mouse spleen, similar to that established for mouse CFU-S in a "mouse to mouse" assay. Recently, we have successfully employed a "rat to mouse" CFU-S assay for the determination of CFU-S in genetically anemic Belgrade Laboratory (b/b) rats (Ivanović et al 1995), confirming the validity of this assay system.

In this study the number and proliferation activity of normal rat CFU-Sd8 were determined, in order to compare characteristics of the rat CFU-Sd8 population in the "rat to mouse" assay with the published data obtained in the "rat to rat assay" and with the analogous cell population in mouse bone marrow.

MATERIALS AND METHODS

Experimental animals. Wistar rats, of both sexes, weighing 180-220 g and 12 weeks old (Military Medical Academy, Belgrade), were used for obtaining femoral bone marrow cells. CBA/H mice, of both sexes, and 14 to 18 weeks old, were previously lethally irradiated and used as the recipients of rat bone marrow cells in the "rat to mouse" CFU-S assay.

Preparation of bone marrow cell (BMC) suspensions. The rats were killed under ether anesthesia. Femoral BMC from both femurs were flushed out with Dulbecco's modification of Eagle's medium (DMEM), pooled, and the total number of nucleated cells per femur calculated. In some experiments the femurs from three rats were flushed out, BMC pooled, and the mean total number of nucleated cells per femur calculated.

"Rat to mouse" CFU - Sd8 assay. The "rat to mouse" CFU-S assay was perfomed as described previously (Martens et al. 1986, Ivanović et al. 1995). CBA/H mice were lethally irradiated (9Gy X rays, 0.959 Gy/min, RT-305 Philips), and used as recipients of rat BMC. The recipient mice (8 - 15 per group) were injected with $1 - 3 \times 10^5$ rat BMC in 0.2 ml intravenously. The mice were killed eight days later, the spleens were removed, fixed in Telleyseniczky's solution, and the number of macroscopically visible colonies was calculated.

Determination of proliferation activitiy of CFU-Sd8. The proliferation activity was determined on the basis of the proportion of CFU-Sd8 killed after the in vitro treatment of rat bone marrow cells with cytosine arabinoside - Ara-C (Cytosar, Upjohn) (Ara-C suicide) (Wright et al 1985). Duplicate aliquots (1 ml) of rat bone marrow cell suspension (5×10^6 cells/ml) in DMEM with 15% fetal calf serum (FSC, Flow) were incubated at 37^0 C in an atmosphere of 5% CO_2 in air for 1h with or without Ara-C (1 ml, 40 μg). Suspensions of BMC were then injected into lethally irradiated mice for the CFU-Sd8 determination as described. The decrease of colony number in the spleens of recipient mice that received Ara-C-treated rat BMC represented the proportion of rat CFU-S in DNA synthesis (Ivanović et al. 1995).

RESULTS

The number of CFU-Sd8 in the bone marrow of individual rats, as determined in four independent experiments using the "rat to mouse assay" varied from 6708 ± 536 to 11261 ± 1455 (Table 1.) The mean number of CFU-Sd8 per femur was 9015 ± 716. The same range of values was found when the number of CFU-Sd8 was determined in pooled BMC from three rats (from 7458 ± 714 CFU-Sd8/femur to 13305 ± 247 CFU-S/femur, average 9799 ± 814 CFU-S/femur) (Table 1.).

Table 1. The number of CFU-Sd8 in femoral bone marrow of Wistar rats

BMC	exp No	Nucleated cells per femur (x 10^6)	CFU-Sd8/femur	
Individual rats	1.	78.0	6708 ± 536	average: 9015 ± 716
	2.	85.0	11261 ± 1455	
	3.	79.2	10691 ± 798	
	4.	96.6	7406 ± 808	
Pooled from three rats	1.	85.0	10200 ± 597	average: 9799 ± 814
	2.	105.6	13305 ± 247	
	3.	93.6	8236 ± 672	
	4.	78.8	7458 ± 741	
Cumulative data		87.7 ± 3.5	9408 ± 816	

BMC - bone marrow cells. Results are mean ± SE.

The incidence of CFU-Sd8 per 10^5 BMC was about 10.3 ± 3.1 ± 1.1 (5.5 - 14.4) in eight experiments (individual and pooled bone marrow) (Table 2)

The proliferation acitivity of bone marrow CFU-Sd8 was low and varied from 4.4 % to 13.1 % in individual rat BMC samples and from 0% to 12.3% in samples of BMC pooled from three rats. The overall proliferation activity of CFU-Sd8 was 5.6 ± 4.7 % (Table 2).

Table 2. The proliferation activity of CFU-Sd8 in femoral bone marrow of Wistar rats

BMC	Exp No	CFU-Sd8/ 10^5 bone marrow cells -Ara-C		CFU-Sd8/ 10^5 bone marrow cells +Ara-C	Proliferative activity (%)
Individual rats	1.	8.6 ± 2.2	average: 11.0 ± 3.4	8.2 ± 1.8	4.6
	2.	14.4 ± 2.4		12.5 ± 4.0	13.1
	3.	13.5 ± 3.2		12.2 ± 3.2	9.6
	4.	7.7 ± 2.7		6.9 ± 2.3	9.9
Pooled from three rats	1.	12.0 ± 2.2	average: 9.6 ± 3.1	11.5 ± 2.3	4.1
	2.	12.2 ± 0.5		10.7 ± 2.8	12.3
	3.	8.8 ± 2.1		8.8 ± 1.8	0
	4.	5.5 ± 1.7		5.4 ± 1.0	2
Cumulative data		10.3 ± 3.1 ± 1.1			5.6 ± 4.7

BMC-bone marrow cells. Results are mean ± SD.

DISCUSSION

The data presented by Martens et al. (1986) suggested the existence of heterogeneity in the population of rat bone marrow CFU-S related to timing of appearance and disappearance of colonies (from day 6 till day 14), and sensitivity to cytotoxic drugs, i. e. suggesting that rat CFU-Sd8 are more mature (less primitive) than rat CFU-Sd12, as was demonstrated for the analogous murine CFU-S subpopulation (Chertkov & Drize 1984).

The number and incidence of CFU-Sd8 presented here for Wistar rats (9408 ± 816 and 10.3 ± 1.1 respectively) are very similar to the values obtained in the "rat to mouse" CFU-Sd8 assay by Martens et al. (1986) for BN/Bi/Rij rats (8200 ± 862 and 10 respectively). Thus, it seems that different strains of rats have a smilar range of values for number and incidence of CFU-Sd8 in bone marrow.

The seeding efficiency of normal rat bone marrow CFU-Sd8, as was determined recently in a "rat to mouse" assay (3.3%) (Ivanović and Milenković 1995), was in the range of values found for normal mouse CFU-Sd8 (3-6 %) (Hendry 1971; Lahiri and Van Putten 1969). This implies that rat and mouse CFU-S have similar affinity for seeding to the spleen of lethally irradiated mice, i. e. similar properties in respect to their potential for transplantation efficiency. This confirms the validity of the "rat to mouse" technique for determination of mature category CFU-S in rat hemopoietic tissue.

It is generally accepted that the proliferation activity of mouse bone marrow CFU-S is low, i. e. only about 10% of these cells are in the S phase of the cell cycle (Becker et al. 1965; Lajtha et al. 1969; Lord et al. 1974; Milenković et al. 1979; 1987; 1991; 1993; Ivanović et al. 1993), irrespective of the method used for measuring the proliferation acitivity (cell death resulting from uptake of 3H-thymidine or cytosine-arabinoside during the S phase of the cycle). A similar proliferation activity ~15% (Dunn 1972), and 10% (Dunn 1973) - was found for the population of rat CFU-S determined in the rat to rat" assay and confirmed here

for CFU-Sd8 in a "rat to mouse" assay. Rat CFU-Sd8 responded to a stimulator of CFU-S proliferation in vitro at the same dose range (Ivanović et al 1995) as found for mouse CFU-Sd8 (Lord 1977; Milenković et al. 1991, 1993; Ivanović et al. 1993) Since a difference in sensitivity to stimulation with a stimulator of CFU-S proliferation was established for subpopulations of mouse CFU-S (CFU-Sd12 less sensitive than CFU-Sd8) (Wright et al. 1985), it seems that rat CFU-Sd8 as determined in a "rat to mouse" assay are also functionally equivalent to mouse CFU-Sd8. The data presented here and earlier (Ivanović et al. 1995), therefore, demonstrate that the normal rat bone marrow CFU-Sd8, as determined in a "rat to mouse" assay, have similar proliferation characteristics in the steady state as normal mouse bone marrow CFU-Sd8.

The results of this study confirm the existence of an analogy between CFU-S compartments in the bone marrow of rats and mice.

Acknowledgements. The excellent technical assistance of Mrs. M. Dokić is acknowledged. The authors are thankful to Dr M. Dekić, Department of Radiotherapy, Institute of Radiology, Military Medical Academy, Beograd for irradiation of the animals. This work was supported by a grant from the Ministry of Science and Technology of Serbia.

REFERENCES

1. *Becker, AJ., McCulloch, EA., Siminovich, L., Till, JE.* 1965. The effect of differing demands for blood cell production on DNA synthesis by hemopoietic colony-forming cells of mice. *Blood 26,* 296-230.

2. *Chertkov, JL., Drize, NJ.* 1984. Cells forming spleen colonies at 7or 11 days after injection have different proliferation rates. Cell Tissur Kinet. 17, 247-252.

3. *Comas, FV., Byrd, BL.* 1967. Hemopoietic spleen colonies in the rat. *Rad Res. 32,* 355-365.

4. *Dunn, CDR., Elson L.A.* 1970. Quantitative studies of haemopoietic spleen colonies in rats treated with cytotoxic chemicals. *Br. J. Haematol. 19,* 755-761.

5. *Dunn, CDR, Elson, L.A.* 1970a. An apparent discrepancy between the number of colony forming units transplanted and survival of busulphan lethaly treated rats. *J. Cell. Physiol. 76,* 215-220.

6. *Dunn, CDR, Elson, L.A.* 1970b. The comparative effects of busulphan ("Myleran") and aminochlorambucil on haemopoietic colony forming units in the rat. *Cell Tissue Kinet. 3,* 131-137.

7. *Dunn, CDR.* 1972. Cell proliferation and drug resistance in the response of colony-forming units of rat bone marrow to repeat doses of cytotoxic agents. *J. Natl. Canc. Inst. 48,* 639-645.

8. *Dunn,CDR.* 1973. The proliferative capacity of haemopoietic colony forming units in the rat. *Cell Tissue Kinet 6,* 55-60.

9. *Hendry, JH.* 1971. The number of primary transplanted splenic colony-forming cells. *Cell Tissue Kinet. 4,* 217-221.

10. *Ivanović, Z.* 1992. Regulation of pluripotent stem proliferation in sterile inflammation. *MSc. Thesis. Faculty of Science, University of Belgrade.*

11. *Ivanović, Z., Milenković, P., Stojanović, N., Lukić, ML., Kataranovski, M.* 1993. A stimulator of proliferation of spleen colony-forming cells (CFU-S) in the bone marrow of irradiated rats. *Acta Vet. (Beograd) 43,* 95-104.

12. *Ivanović, Z., Milenković, P., Vasiljevski, M., Dekić, M.* 1995. Hemopoietic stem cells in the hereditarily anemic Belgrade Laboratory (b/b) rat. Exp. *Hematol. in press 23, 1218-1223.*

13. *Ivanović, Z., Milenković, P.* 1995. The seeding effciency of normal and hareditarily anemic (b/b)rat bone marrow colony forming units-spleen as determined in a "rat to mouse" assay. Stem cells. 13, in press.

14. *Lahiri, SK., Van Putten, L.M.* 1969. Distributlon and multiplication of colony forming units from marrow and spleen after injection in irradiated mice. *Cell Tissue Kinet. 2,* 21-27.

15. *Lajtha, L.G., Pozzi, LV., Schofield, MF.* 1969. Kinetic properties of haemopoietic stem cells. *Cell Tissue Kinet. 2,* 39-49.

16. *Lord, Bl., Lajtha, LG., Gidali, J.* 1974. Measurement of the kinetic status of bone marrow precursor cells: Three cautinary tales. *Cell Tissue Kinet. 7,* 507-515.

17. *Lord, Bl., Mori, KJ., Wright, EG.* 1977. A stimulator of stem cell proliferatlon in regenerating bone marrow. *Biomed. 27,* 223-226.

18. *Martens, ACM., Van Bekkum, DW., Hagenbeek, A.* 1986. Heterogeneity within the spleen colony-forming cell population in rat bone marrow. *Exp. Hematol. 14,* 714-718.

19. *Milenković, P., Stojanović, N., Jovčić, G., Lukić, M.* 1987. Regeneration of spleen colony-forming cells and granulocyte-monocyte progenitors in T-cell deprived mice treated with cyclophosphamide. *Leuk. Res. 11,* 1099-1103.

20. *Milenković, P., Ivanović, Z., Kataranovski, M., Lukić, M.* 1991. Stimulator of proliferation of spleen colony-forming cells in T cell deprived mice treated with cyclophosphamide or irradiation. *Cell Prolif. 24,* 507-515.

21. *Milenković P., Ivanović, Z., Lukić, ML., Kataranovski, M., Lord, Bl.* 1993. Stimulator of proliferation of spleen colony-forming cells in acute sterile inflammation. *Cell Prolif. 26,* 503-510.

22. *Till, JE., McCulloch, EA.* 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Rad Res. 14,* 213-221.

23. *Van Bekkum, DW.* 1977. The appearance of the multipotential hemopoietic stem cell In: Experimental Hematology Today, J. B. Baum and G.D. Ledney eds. *Springer-Verlag, New York,* p. 3.

24. *Weright, EG., Lorimore, SA., Lord Bl.* 1985. Effect of hemopoietic stem cell proliferation regulators on early and latle spleen colony forming cells. *Cell Tissue Kinet 18,193-199.*

BROJ I PROLIFERATIVNA AKTIVNOST ZRELIJE KATEGORIJE PLURIPOTENTNIH MATIČNIH ĆELIJA HEMATOPOEZE U KOSTNOJ SRŽI PACOVA ODREĐENIH TEHNIKOM "PACOV NA MIŠA"

Z. IVANOVIĆ i P. MILENKOVIĆ

SADRŽAJ

Određivanje matičnih ćelija hematopoeze kod pacova je skopčano sa tehničkim poteškoćama. Ipak, pluripotentne matične ćelije hematopoeze mogu biti uspešno određene metodom koji podrazumeva korišćenje letalno ozračenih miševa kao primaoca pacovske kostne srži, u čijoj slezini rastu hematopoetske kolonije iz matičnih ćelija kostne srži pacova ("pacov na miša" tehnika). U ovom radu je metodom "pacov na miša", određivan broj i proliferativna aktivnost CFU-Sd8 u kostnoj srži Wistar pacova. Ustanovljeno je da je broj CFU-Sd8 u kostnoj srži femura Wistar pacova približan broju ovih ćelija u kostnoj srži BN/Bi/Rij pacova, poznatim iz literature. Proliferativna aktivnost CFU-Sd8 u kostnoj srži Wistar pacova je veoma niska. Na osnovu jednake (niske) proliferativne aktivnosti

i sličnih funkcionalnih osobina koje su ustanovljene ranije, može se zaključiti da CFU-Sd8 kostne srži pacova određene metodom "pacov na miša", kao i CFU-Sd8 kostne srži miša, predstavljaju zreliju kategoriju pluripotentnih matičnih ćelija hematopoeze.