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QUANTITATIVE DETERMINATION OF TRANSPLANTABLE HEMOATOPOIETIC STEM CELLS BY THE LIMITING DILUTIONS METHOD

N. L. Samoilina and G. A. Udalov

UDC 615.361.419.03:616.419-089.843].876.9

KEY WORDS: hematopoietic stem cell, transplantation.

Totipotent hematopoietic stem cells (THSC), giving rise to all categories of cells of the hematopoietic and immune systems, have received less study than all other categories of colony-forming hematopoietic precursors, including polypotent myeloid precursors (CFU-s), which can easily be determined quantitatively, for direct methods of their quantitative analysis are not yet available. An attempt at a direct quantitative approach was undertaken by Boggs and co-workers [2], who used the limiting dilutions method [10] during transplantation of normal bone marrow into unirradiated genetically anemic W/W^V mice. We tested the limiting dilution method during transplantation of bone marrow into lethally irradiated recipients, assuming that restoration of hematopoiesis by donors' cells and, consequently, survival of the recipient, are possible if even only one THSC should find its way to hematopoietic territories.

EXPERIMENTAL METHOD

As recipients we used female (CBA × C57B1/6)F_1 (CBF_1) mice aged 5-10 months. The donors were CBF_1 or (C57B1/6 × CBA/T6/F_1 ($BCT6F_1$) mice, and their age and sex are given in Table 1. Total irradiation was carried out on the IPK (^{137}Cs) apparatus with a dose rate of 19 Gy/min, in two sessions separated by an interval of 3 h; the total dose was 15.2 Gy. Under these conditions not a single control mouse, not receiving bone marrow cells, survived more than 18 days. The mice were kept under ordinary conditions. After irradiation they were given acidified water (pH 3.5) and antibiotics with their food (polymyxin sulfate and monomycin, 500,000 U/500 g of each). Bone marrow cells were taken from the femur by flushing out with Hanks' solution. A suspension of single cells was obtained by passing the suspension repeatedly through a fine needle. After enumeration of the nucleated cells and dilution, the cell suspensions were injected intravenously into recipients 1-2 h after irradiation in 2 or 3 doses, at least 10 mice being used for each dose. Survival of the irradited mice was monitored for 28 days or more. Proliferation of the donors' cells in the bone marrow, spleen, and thymus was determined by the presence of the T6-chromosome in metaphase plats. To describe the THSC which, on transplantation, repopulated the irradiated recipient, and consequently ensured its survival after lethan irradiation, we used the functional term "hematopoiesis restoring unit" (HRU) [1]. The principle of the limiting dilutions method as applied to determination of hematopoietic precursors was described previously [1-3]. Briefly, the probability that a sample of homogeneous cell suspension does not contain HRU is determined by the equation $P_0 = e^{-KX}$, where K is the fraction of HRU in 10^6 cells and X the dose of cells injected (in millions). P_0 denotes the proportion of samples not containing HRU. This will be identical (in the case under discussion) with the proportion of mice which did not survive. The concentration of HRU in the sample is determined by the equation $K = -\ln P_0/X$.

EXPERIMENTAL RESULTS

Examples of determination of the HRU fraction in bone marrow samples from healthy mice are given in Table 1. In three bone marrow samples from female CBF_1 mice we obtained values

All-Union Hemotologic Scientific Center, Ministry of Health of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR A. I. Vorob'ev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 110, No. 10, pp. 421-423, October, 1990. Original article submitted December 29, 1989.

 0007-4888/90/0010-1418$12.50

TABLE 1. Determination of HRU in Mouse Bone Marrow

Expt. No.	Mouse	Number of cells injected, $\times 10^{-6}$ (x)	Fraction of mice not sur-	Concentration of HRU in 10^6 K	Concentration of HRU in 10^6 mean M ± m
1	CBF1 females (4.5 months)	0	1		
		0,01	0,9	10,5	
		0,05	0,8	4,5	
		0,2	0,3	6	7±1,8
2	CBF1 females (9 months)	0	1		
		0,05	0,5	14	
		0,2	0,3	6	10±4
		1	0		
3	CBF1 females (12 months)	0	1		
		0,05	0,5	13,9	
		0,2	0,1	11,5	12,7±1,2
4	BCT6F1 females (12 months)	0	1		
		0,025	0,9	4,2	
		0,05	0,2	32	
		0,075	0,2	21,5	19,2±8,1
5	BCT6F1 males (4.5 months)	0	1		
		0,0125	0,5	55,4	
		0,05	0,2	32	43,8±11,4
		0,2	0		

Legend. Arithmetic mean values from determinations for 2 to 3 doses of cells, with standard deviation of arithmetic mean, given in Tables 1 and 2.

TABLE 2. Dependence of Fraction of Nonsurviving Mice (P_0) and Value of HRU Fraction (K) on Time after Transplantation

Expt. No.	Number of cells injected, $\times 10^{-6}$	P_0 weeks 3	4	5	6	7	8	10	K weeks 4	8	10
3	0,05	0,5	0,5	0,5	0,6	0,6	0,6	0,9	13,9	10,2	2,1
	0,2	0,1	0,1	0,1	0,1	0,2	0,2	0,3	11,5	8	6
	1	0,1	0,1	0,1	0,1	0,1	0,1	0,1			2,3
							$m \pm M$		12,7±1,2	9,1±1,1	3,5±1,3
4	0,025	0,6	0,9	1					4,2		
	0,05	0,2	0,2	0,2	0,3	0,7	0,9		32	2,1	
	0,075	0,2	0,2	0,2	0,2	0,4	0,5		21,5	9,3	
							$m \pm M$		19,2±8,1	5,7±3,6	

TABLE 3. Repopulation of Recipients' Hematopoietic Organs by Donors' Cells

Number of cells injected, $\times 10^{-6}$	Day after injection	Percent of metaphase with T6-chromosome bone marrow	spleen	thymus
0,1	48	100	100	—
0,1	366	100	—	—
0,075	59	100	100	100
0,05	36	100	100	—
0,05	59	87	90	100

Legend. —) No data available.

of HRU of between 7 and 12.7 per 10^6 cells, and in two bone marrow samples from $BCT6F_1$ mice, we obtained values of 19.2 (for yearling females) and 43.8 (for males aged 4.5 months). These values of HRU concentration were obtained by recording survival of the animals after 4 weeks. Long-term monitoring of survival of the mice showed that the fraction of nonsurvivors could increase with time. As will be clear from the data in Table 2, the increase in P_0 was inversely proportional to the dose of cells injected. With sufficiently large doses of cells (10^6 or more) the value of P_0 did not change with time. Repopulation of the donors' cells in the bone marrow, spleen, and thymus was confirmed by the presence of the T6-chromosome in the dividing cells (Table 3).

The results confirmed the expected exponential dependence of survival of the recipients on the number of stem cells injected. Knowing the concentration of HRU in the test population, the dose of cells ensuring this particular level of survival of the recipients, namely ED_{50} or ED_{90}, can be calculated. Although the possibility cannot be ruled out that

the concentrations of transplantable hematopoietic stem cells (HSC) can differ in mice of different strains or of different sex and age, nevertheless our results can be compared with those obtained by other methods. Using a model of treatment of mice with hereditary macrocytic anemia, Boggs and co-workers [2] obtained a higher concentration of "treating cells" than we did ($10/10^5$). However, Nakano and co-workers, using a sensitive method of monitoring labeled donors' hemoglobin, found on the same model a lower concentration [$(7\text{-}14)/10^6$] precursors restoring erythropoiesis of the anemic recipients. They showed that these precursors can differentiate also into lymphoid cells, i.e., they are true HSC [9]. By estimating the number of primitive HSC (PHSC), forming clones after transplantation of a mixture of congeneic labeled bone marrow cells, using a binomial equation, a value of the order $(5\text{-}10)/10^6$ ws obtained for the concentration of PHSC in C57/6 and C57B1/10 mice [4]. This value is identical with that we found for CBF_1 mice by transplantation, using the limiting dilutions method.

After injection of small doses of bone marrow, used in the limiting dilutions method, we observed an increase in the fraction of nonsurviving animals with time. The negative correlation observed between this increase and the dose of injected cells suggests that it is connected with the number and properties of the HRU injected. There is evidence of the heterogeneity of PHSC for proliferative potential [4, 7]. If this is confirmed, the number of HRU with different levels of proliferative potential can be determined by recording the fraction of nonsurviving recipients at different times after transplantation (Table 2).

All things considered, the method of quantitative analysis of transplantable THSC (HRU) offers good prospects for the study of the THSC fraction.

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