POSSIBILITY OF ARTIFACT WHEN DETERMINING HEMATOPOIETIC STEM CELL PROLIFERATION BY CFU-S SUICIDE METHODS

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UDC 612.419.014.2:612.119:612.6

KEY WORDS: hematopoietic stem cell; proliferation; "suicide" of stem cells; [^{3}H]-thymidine; hydroxyurea; cytosine arabinoside.

The low level of stem cell proliferation during equilibrium hematopoiesis and its sharp increase on the occurrence of various hematopoietic stresses, such as during regeneration after sublethal irradiation or after transplantation into a lethally irradiated recipient, were established about 20 years ago [2] and have repeatedly been confirmed [1]. All these results were obtained by determining "suicide" of CFU-S with the aid of phase-specific cytostatics. However it was recently shown [6] that not only hematopoietic stem cells, but also their more mature offspring can produce colonies in the spleen. These more mature cells give rise to transient colonies 7-8 days after injection, which disappear by the 10th-11th day. With ordinary methods of fixation of the spleen (7-9 days after transplantation), colonies produced only by some stem cells, and also by their progenies, are recorded. After 11 days, all stem cells entering the spleen give rise to macroscopic colonies, and the colonies produced by the more mature precursors themselves mature and the cells composing them pass out into the circulation. In this case transient colonies will fail to be recorded. Proliferative activity of stem cells and their offspring can evidently differ in different situations, and consequently the method of determining stem cells in the form of CFU-S organically carries with it the possibility of serious artefact.

To test this possibility, it was decided to study CFU-S proliferation by different "suicide" methods in two situations, namely in sublethally irradiated mice and in lethally irradiated recipients into which hematopoietic cells were injected intravenously. The usual method of determination of CFU-S was used and was corrected by recording the number of colonies 11 days after injection of the cells.

EXPERIMENTAL METHOD

Female (CBA × C57BL/6)F_1 mice aged 8-12 weeks were used. The animals were irradiated by ^{137}Cs γ-rays on the IPK apparatus with a dose rate of 0.25 Gy/min. Mice irradiated 3 days before sacrifice in a dose of 2 Gy, or mice irradiated in a dose of 13 Gy and restored by injection of bone marrow in a dose of 1/5 femur equivalent, were used as donors of bone marrow. In the latter case hematopoietic cells were obtained from the spleen 7 or 11 days after irradiation. For *in vivo* "suicide" the donors of the hematopoietic cells were given [^{3}H]thymidine by intravenous injection in a dose of 0.3-3 mCi/mouse (specific activity 12-16 Ci/mmole) 25 min before sacrifice, or hydroxyurea (1 mg/g body weight) by intraperitoneal injection 2 h before sacrifice, or cytosine arabinoside (1 mg/g) or methotrexate (0.25 mg/g) by intraperitoneal injection 4 h before sacrifice. For *in vitro* "suicide" cells were incubated at 37°C in Hanks' solution with [^{3}H]thymidine (100-200 μCi/ml) for 30 min or in medium No. 199 with glutamine and 2% embryonic calf serum with hydroxyurea (1 mg/ml) for 2 h. Cells undergoing "suicide" and control cells were injected intravenously into recipients (16 in a group), irradiated in a dose of 13 Gy. In half of the recipients colonies were counted 7 days, and in the other half 11 days after injection of the cells.

Central Research Institute of Hematology and Blood Transfusion, Ministry of Health of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. A. Fedorov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 96, No. 12, pp. 81-83, December, 1983. Original article submitted November 18, 1982.

 0007-4888/83/9612-1754$07.50

TABLE 1. Effect of Time of Recording Colonies on Magnitude of *in Vivo* "Suicide" of Hematopoietic Stem Cells from Sublethally Irradiated Mice

Dose of bone marrow (in femur equivalents)	Method of "suicide"	Recording after 7 days		Recording after 11 days	
		number of colonies per spleen	"suicide," percent	number of colonies per spleen	"suicide," percent
		Experiment I			
1/100	—	17,0±1,4		7,3±0,9	
1/100	Hydroxyurea	8,5±2,2	50	9,2±1,3	0
		Experiment II			
1/100	—	17,6±4,3		7,0±0,4	
1/100	[^{3}H]Thymidine, 1.5 mCi	10,8±0,6	39	7,4±1,4	0
1/100	[^{3}H]Thymidine, 0.3 mCi	12,4±1,8	30	8,6±1,2	0
1/100	Hydroxyurea	10,2±1,5	42	7,8±0,7	0
1/100	Cytosine arabinoside	11,0±2,0	38	7,4±1,4	0
		$M\pm m$	40±3,2		

TABLE 2. Effect of Time of Recording Colonies on Degree of "Suicide" of Hematopoietic Cells from Lethally Irradiated Mice in Early Stages after Injection of Bone Marrow Cells

Time of taking cells from donors after irradiation, days	Dose of spleen cells (in splenic equivalents)	Method of "suicide"	Recording after 7 days		Recording after 11 days	
			number of colonies per spleen	"suicide," percent	number of colonies per spleen	"suicide," percent
		Experiment 1				
11	1/1000	—	11,0±1,6		10,0±1,0	
11	1/1000	In vivo —Hydroxyurea	6,5±0,7	41	4,0±2,0	60
11	1/1000	In vivo —Cytosine arabinoside	10,5±0,9	5	7,2±1,7	28
11	1/1000	In vivo —Methotrexate	1,5±1,3	86	7,3±0,4	27
11	1/1000	—	8,4±2,0		6,3±1,5	
11	1/1000	In vitro —[^{3}H] Thymidine	5,6±0,9	36	3,0±0,7	52
		Experiment 2				
7	1/75	—	5,2±1,1		1,7±0,6	
7	1/75	In vitro —[^{3}H] Thymidine	2,4±0,5	54	1,3±0,9	24
7	1/75		2,5±0,5		1,3±0,4	
7	1/75	In vitro —Hydroxyurea	2,1±0,3	16	0,7±0,4	46
7	1/75	In vivo,	20,1±3,9		19,5±3,0	
7	1/75	In vivo — [^{3}H] Thymidine	18,0±2,0	10	5,3±1,3	73
7	1/75	In vivo — Hydroxyurea	11,9±1,8	41	8,3±1,1	57
7	1/75	In vivo —Cytosine arabinoside	11,6±0,9	46	7,5±1,2	62
11	1/250	—	40,1±3,1		25,2±1,1	
11	1/250	In vivo — Hydroxyurea	21,8±2,0	46	13,2±1,4	48
11	1/250	In vivo — Cytosine arabinoside	19,5±2,7	51	10,0±1,0	63
		$M\pm m$	39±6,9		49±5,1	

EXPERIMENTAL RESULTS

The number of CFU-S in the femoral marrow of sublethally irradiated mice 3 days after irradiation was 20 and 13% of the initial value in experiment 1, when colonies were counted 7 and 11 days after injection of the cells respectively, and 17 and 15% in experiment 2. Hence it follows that the total number of colonies, counted at different times, shows only a negligible change: Transient colonies disappearing from the 7th through the 11th days were largely compensated by newly appearing splenic colonies formed by CFU-S forming clones slowly. The data in Table 1 show that CFU-S in sublethally irradiated mice do not proliferate when determined by the corrected method. The increased demand for hematopoiesis after sublethal irradiation is satisfied by increased proliferation of precursors more mature than the stem cell. It is this increased proliferation that is recorded when CFU-S are counted by the usual method. Consequently, published results showing intensification of stem cell proliferation after sublethal irradiation [4] are an artefact due to a defect in the method of determining CFU-S in the usual arrangement.

It was a different matter with the lethally irradiated animals (Table 2). Hematopoietic stem cells transplanted into them in fact proliferated actively both in the logarithmic phase of growth (7 days after transplantation), when their number in the spleen was about half the normal level, and in the over-shoot phase (11 days), when their number in the spleen was 3.3 times above normal. Clearly the results differ considerably, both between individual experiments with different methods of "suicide," and also in the same experiment, using the same method but counting the colonies at a different time. This great scatter is characteristic of certain statistical problems arising during the use of "suicide" methods [7]. However, the average results did not differ significantly — when colonies were recorded both after 7 days and after 11 days, on average about half of the stem cells at every given moment were in the synthetic period of the cell cycle. At the time studied (7-11 days after restoration of lethally irradiated mice by syngeneic bone marrow) the stem cells were in a phase of intensive growth and the doubling time was about 1 day (in 4 days the number of CFU-S in the spleen increased sevenfold). This intensive growth is easily recorded by the relatively insensitive "suicide" methods. Meanwhile, after sublethal irradiation, the increase in the number of CFU-S took place more slowly (doubling time about 5 days) and their proliferation could not be detected by "suicide" methods.

On the whole these results revealed for the first time an organic defect in determination of proliferation of hematopoietic stem cells by "suicide" methods in the classical arrangement. Accordingly other cases when increased stem cell proliferation has been reported and, in particular, in long-term bone marrow cultures after a change of medium [3], in short-term cultures under the influence of humoral stimulators [5], and so on, need to be studied by the corrected method also. At present the possibility cannot be ruled out that in these cases also the hematopoietic precursor cells recorded were more mature than CFU-S.

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