radiation in these doses the viable fraction, equal on average to 5% of the control, formed an ectopic focus containing fewer than $0.5 \cdot 10^6$ cells, which is close to the sensitivity of the method. This objection applies to some extent to results obtained with AD also. Meanwhile it was found unexpectedly that AD itself, if injected into intact bone marrow donors, increases the size of the foci of ectopic hematopoiesis formed by their bone marrow, possibly due to the lower sensitivity of precursors treated with AD to the transfer procedure.

For this reason direct proof that mechanisms of DNA repair participate in postradiation recovery of precursor cells of the hematopoietic stroma, observed in this investigation, necessitates approaches of a different kind.

LITERATURE CITED

1. A. G. Konoplyannikov, Dokl. Akad. Nauk SSSR, 206, No. 4, 978 (1972).
2. O. A. Konoplyannikova and A. G.Konoplyannikov, Radiobiologiya, 13, No. 4, 531 (1973).
3. T. V. Todriya, Byull. Éksp. Biol. Med., No. 11, 584 (1978).
4. M. Elkind, K. Kamper, V. Moses, et al., in: Recovery and Repair Mechanisms in Radiobiology [Russian translation], Moscow (1972), pp. 69-94.
5. J. T. Chaffey and S. Hellman, Radiology, 93, 1167 (1969).
6. J. L. Chertkov and O. A. Gurevitch, Radiat. Res., 79, 177 (1979).
7. P. A. Chervenick and D. R. Boggs, Blood, 37, 568 (1971).
8. M. M. Elkind and H. Sutton, Nature, 184, 1293 (1959).
9. J. E. Till and E. A. McCulloch, Radiat. Res., 18, 96 (1963).
10. H. R. Withers and M. M. Elkind, Radiology, 91, 998 (1968).
11. H. R. Withers and M. M. Elkind, Int. J. Radiat. Biol., 17, 261 (1970).

PROLIFERATION OF HEMATOPOIETIC MICROENVIRONMENT PRECURSORS IN LONG-TERM MOUSE BONE MARROW CULTURES

O. A. Gurevich, N. I. Drize, and I. L. Chertkov

UDC 612.419.014.2:612.6:612.119/-085.23

KEY WORDS: stromal precursors; long-term bone marrow culture; proliferation; hydroxyurea.

The hematopoietic microenvironment is stromal in nature [6] and is created by precursor cells capable of transferring it during implantation of hematopoietic tissues. These precursors have been called "hematopoietic microenvironment transferring units" (HMTU) [1]. During equilibrium hematopoiesis the HMTU are in a resting state and their proliferation cannot be revealed by "suicide" methods. However, the transfer procedure mobilizes HMTU into the cycle, and after 48 h (but not after 24 h) their high sensitivity to cytostatics, which lasts about 2 weeks, can be easily demonstrated [5]. This short-term proliferation is sufficient for the formation of the necessary number of new HMTU to enable construction of a focus of ectopic hematopoiesis, corresponding in size to the initial hematopoietic territory.

A method of long-term bone marrow culture has recently been devised [9]. Maintenance of hematopoiesis in culture depends critically on the construction of a layer of adherent cells, mainly stromal in origin [3], which plays the role of hematopoietic microenvironment. HMTU are contained in the sublayer of long-term bone marrow cultures [2]. Their proliferative state is unknown. Meanwhile the process of sublayer construction is analogous in many respects to the formation of a focus of ectopic hematopoiesis.

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. 98, No. 11, pp. 612-614, November, 1984. Original article submitted September 27, 1983.

In connection with the facts described above it was interesting to study mitotic activity of HMTU after explantation of bone marrow into long-term culture, and the investigation described below was carried out for this purpose.

EXPERIMENTAL METHOD

Experiments were carried out on male C57BL/6 (B6) and (CBA × C57BL/6)F_1 (CBF_1) mice aged 8-16 weeks at the beginning of the investigation. Long-term bone marrow culture was conducted as described previously [2]. Femoral bone marrow of the mice was flushed out with 10 ml of complete nutrient medium into a 25-cm^2 plastic flask and cultured at 33°C with daily change of half of the medium. The composition of the complete medium was: Fisher's medium with 20% horse serum, enriched with 1% L-glutamine, 10^{-6} M hydrocortisone hemisuccinate, and antibiotics. For "suicide" hydroxyurea (HU) was used, and was added to the culture in a concentration of 13 mM (1 mg/ml) for a period of between 2 h and 7 days. To stop the action of HU the sublayer of the culture was washed three times in medium 199 with 2% embryonic calf serum, 5 ml each time. HMTU in the culture were determined by counting cells in the focus of ectopic hematopoiesis formed in the course of 1-1.5 months after implantation of the sublayer of the culture beneath the renal capsule of syngeneic normal mice or of mice previously irradiated in a dose of 11-13 Gy and restored with syngeneic bone marrow [1].

EXPERIMENTAL RESULTS

On implantation of the sublayer of control cultures into unirradiated recipients the size of the foci formed was $0.3 \cdot 10^6$ to $1.3 \cdot 10^6$ nucleated cells. The same sublayer, in irradiated mice, formed substantially larger foci: $17 \cdot 10^6$ to $22 \cdot 10^6$ cells (Table 1). Intermittent treatment (2 h) with HU had no effect on HMTU in the 2-week cultures, in which formation of the sublayer was close to completion. In one-week cultures, in which the sublayer was in a stage of active growth [8], HU damaged 60-80% of the HMTU.

The results agreed for testing on both intact and irradiated recipients (Table 1, I-III). Hence it can be concluded that in the process of sublayer construction stromal precursors functioning during transfer in the unirradiated recipient, and their more mature progeny, which participate in the formation of the focus of ectopic hematopoiesis in irradiated recipients [7], proliferate actively. However, it must be recalled that intermittent treatment with HU revealed proliferation only of cells with a short mitotic cycle. To discover any possible proliferation of stromal precursors with a long generation time, HU was kept in the culture until the time of change of the medium. HU is known to be relatively stable in culture and to block entry of the cells into the synthetic period only temporarily [4]. When kept in the medium for 3-6 days, HU destroyed the stromal precursors of cultures aged from 2 to 11 days virtually completely (Table 1, III).

On addition to a one-day culture the effect was weaker. Consequently, the results confirm that stromal precursors proliferate in culture during sublayer construction. Moreover, it can be tentatively suggested that their transition into the state of active proliferation does not take place immediately, but requires at least 1 day. However, the absence of full data on the pharmacokinetics of HU in culture makes a more definite conclusion impossible, for it is uncertain which fraction of the precursors is destroyed by HU in the course of 2 days and more after its addition. Accordingly, the time of action of HU was restricted to 1 day. Even with this exposure, HU did not act on HMTU of two-four-week cultures, confirming absence of proliferation of these cells in the formed sublayer (Table 1, IV, V). Sensitivity of HMTU to HU was sharply increased after only 2-3 days in culture. On the 1st day the stromal precursors still remained in the resting state, as shown by their insensitivity at this time to the action of HU (Table 1, V).

Implantation of bone marrow *in vivo*, leading to construction of a new hematopoietic microenvironment and to the appearance of a focus of ectopic hematopoiesis [5], or its explantation *in vitro*, leading to the construction of a hematopoietic microenvironment in the form of a sublayer of adherent cells, are thus accompanied by absolutely identical changes in the proliferative state of the stromal precursors: During the first 24 h after transfer they remain in a state of mitotic rest, during the second day the precursors are mobilized into the cycle, largely synchronously. Their high proliferative activity is manifested for only 2 weeks. Later, despite continued growth of the focus of ectopic hematopoiesis and the unceasing morphological and functional changes in the sublayer of the cultures, the stromal precursors do not proliferate, at least to a degree which exceeds sensitivity of "suicide" methods.

TABLE 1. Sensitivity of Stromal Bone Marrow Precursors to Hydroxyurea Depending on Duration of Culture and of Exposure to Cytostatic

Experiment	Line of mice	Duration of exposure to HU	Time of addition to culture	Age of culture, weeks	HU	Unirradiated recipients			Irradiated recipients		
						number of foci/number of implants	size of foci $\times 10^{-6}$	"suicide"	number of foci/number of implants	size of foci $\times 10^{-6}$	"suicide"
I	B6	2h	2 weeks	2	—	2/2	0,7	0% (stimulation)	2/2	22,5	0% (stimulation)
					+	2/2	1,6		2/2	35,0	
II	B6	2h	1 weeks	3	—	6/6	0,3	83%	—	—	—
					+	4/4	0,05				
			2 weeks	3	+	3/4	0,3	0%	—	—	—
III	B6	6days	1 day	4	—	—	—	—	4/4	17,4	
					+	—	—	—	4/4	9,5	61%
		3 days	4 days	4	+	—	—	—	0/2		100%
		7 days	7 days	4	+	—	—	—	0/4		100%
		2 h	7 days	4	+	—	—	—	3/4	8,9	64%
		3 days	11 days	4	+	—	—	—	3/4	0,3	98%
IV	CBF_1	1 days	2 weeks	2	—	2/2	1,3	0% (stimulation)	2/2	21,8	0% (stimulation)
					+	2/4	14,5		2/2	51,3	
			4 weeks	4	—	2/2	1,1	0% (stimulation)	1/1	16,7	7%
		1 days			+	1/1	2,4		2/2	15,6	—
V	CBF_1	1 days		3	—	2/2	0,4	—	—	—	—
			1 day	3	+	2/2	2,8	0% (stimulation)	—	—	—
			2 days	3	+	1/1	0,05	88%	—	—	—
			3 days	3	+	2/2	0,13	31%	—	—	—
			15 days	3	+	1/1	1,9	0% (stimulation)	—	—	—

This agrees completely with the absence of increase in the number of stromal precursors both *in vivo* and *in vitro* after the first 3 weeks of construction of the microenvironment [5, 7].

It is difficult to judge the intensity of proliferation of the stromal precursors in culture not only because the number of cultures studied was too small for quantitative evaluation, but also because, as was unexpectedly discovered, the action of HU on a nonproliferating population of stromal precursors gives rise to an "absurd effect" [10], and the experimental foci are larger than the controls (in Table 1 results of this kind are qualified as "stimulation"). Regardless of the cause (increased efficiency of HMTU transfer under the influence of HU, their easier detachment from the substrate under the influence of the cytostatic, which may make the transfer procedure less traumatic, and so on), this effect of HU makes the quantitative evaluation of its action on cells in the synthetic period of the cycle more difficult.

The results show that Dexter bone marrow cultures are an adequate model with which to study not only hematopoietic, but also stromal precursor cells.

LITERATURE CITED

1. O. A. Gurevich and I. L. Chertkov, Probl. Gematol., No. 8, 43 (1980).
2. O. A. Gurevich, N. I. Drize, G. A. Udalov, et al., Probl. Gematol., No. 7, 7 (1982).
3. A. T. Ivanov-Smolenskii, O. A. Gurevich, R. S. Samoilova, et al., Probl. Gematol., No. 7, 25 (1982).
4. B. R. Bhuyan, T. J. Fraser, L. G. Gray, et al., Cancer Res., 33, 888 (1973).
5. I. L. Chertkov and O. A. Gurevich (J. L. Chertkov and O. A. Gurevitch), Cell. Tissue Kinet., 13, 534 (1980).
6. I. L.Chertkov (J. L. Chertkov), O. A. Gurevich (O. A. Gurevitch), and G. A. Udalov, Exp. Hematol., 8, 770 (1980).
7. I. L. Chertkov, N. I. Drize, O. A. Gurevich (J. L. Chetkov, N. J. Drize, O. A. Gurevitch), et al., Exp. Hematol., 11, 231 (1983).
8. I. L. Chertkov, N. I. Drize, and O. A. Gurevich (J. L. Chertkov, N. J. Drize, and O. A. Gurevitch), Exp. Hematol., 8, 244 (1980).
9. T. M. Dexter, T. D. Allen, and L. G. Lajtha, J. Cell. Physiol., 91, 335 (1977).
10. S. Hozout and H. Vallerot, Cell. Tissue Kinet., 10, 569 (1977).