The results show that migrating stem cells do not differ from settled cells in the probability of divergent differentiation, e.g., myeloid and lymphoid, as can be deduced from the identical fraction of the two subpopulations of CFUs in the bone marrow and spleen, where myeloid hematopoiesis predominates during regeneration after irradiation, compared with the thymus, where differentiation is entirely lymphoid. Meanwhile, in their ability to support themselves, migrating CFUs are significantly inferior to settled. Hence it follows that migrating CFUs are not members of a single population of CFUs that have accidentally entered the circulation, but are a special subpopulation of stem cells with reduced proliferative potential. Possibly only stem cells which have already passed through a process of clonal aging, removed from the bone marrow as spent precursors, migrate into the blood stream. The problem of whether a special structural organization is present in the bone marrow to produce displacement of the stem cells during aging into regions from which migration into the circulation is easier requires further study.

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INVESTIGATION OF ERYTHROID PRECURSORS BY MOUSE BONE MARROW CULTURE IN A PLASMA CLOT

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Two types of erythroid precursors were isolated during culture of mouse bone marrow in a plasma clot in the presence of mouse serum and without the addition of exogenous erythropoietin to the medium. The first type of precursor was more similar in its characteristics to the erythroid colony-forming unit described previously. The second type of precursor was an erythroid burst-forming unit, similar in its properties to that described previously. The optimal concentration of mouse serum in the culture medium was 10-15%. The clonal nature of the colonies and bursts described is confirmed by the linear relationship between their number and the cell concentrations in culture.

KEY WORDS: erythroid colony-forming unit; erythroid burst-forming unit; erythropoietin; mouse serum.

Methods of culture of hematopoietic tissue in semisolid media whereby precursors of cells of the erythroid series at different levels of differentiation can be identified have recently been developed [1, 5, 6, 9]. The most mature of them, the erythroid colony-forming unit (CFU-E) gives rise to colonies consisting of 8-32 erythroid cells after culture for 48 h in the presence of low concentrations of erythropoietin. The least mature precursor, the erythroid burst-forming unit (BFU-E), forms either isolated colonies consisting of 64 to 10,000 erythroid cells or colonies consisting of several small cell clusters after culture for 8 h.

This paper describes a system by means of which two precursors can be discovered: One forms erythroid colonies of 8-32 cells on the third day, the other forms erythroid bursts on the fifth day in culture.

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TABLE 1. Dependence of Appearance of Erythroid Precursors on Presence of Mouse Serum and Erythropoietin in Culture Medium

Samples (addition to medium)	Number of precursors per 10^5 bone marrow cells			
	CFU-E		BFU-E	
	$M \pm \sigma$	n	$M \pm \sigma$	n
control	2,8±1,4	9	0,2±0,03	9
NMS added to medium %				
5	10,6±1,9	9	4,4±0,78	8
10	17,4±2,74	9	7,4±0,96	8
15	16,8±5,38	5	7,0±1,34	4
20	7,0±2,42	8	1,8±1,22	8
30	0	3	0	3
AMS added to medium, %				
5	12,5±3,9	7	3,1±2,0	6
10	13,6±4,1	7	5,4±2,8	6
15	16,8±6,8	4	11,7±7,0	3
20	3,0±1,2	7	1,2±1,5	6
30	0	2	0	2
Erythropoietin, IU/ml				
0,3	3,8±2,37	6	1,1±0,66	4
3	19,1±5,17	4	4,0±1,56	4
AMS 10%+erythropoietin 3,0 IU/ml	11,36±5,0	6	3,6±1,72	5

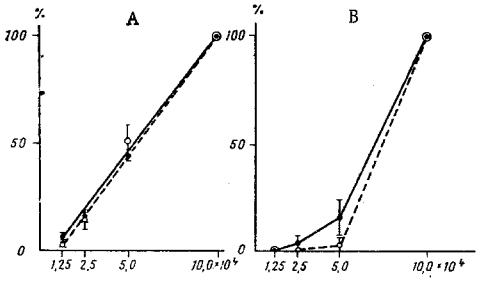

Fig. 1. Number of colonies and bursts as a function of cell concentration in culture of mouse bone marrow. A) In medium containing 10% mouse serum; B) in control without mouse serum. Continuous line, number of colonies; broken line, number of bursts. Abscissa, cell concentration in culture; ordinate, number of colonies and bursts (in % of number of colonies and bursts formed with cell concentration of $10.0 \cdot 10^4$/ml, taken as 100%).

EXPERIMENTAL METHOD

Bone marrow from female (CBA × C57BL)F_1 mice aged 8-12 weeks was suspended in NCTC-109 medium with 2% inactivated embryonic calf serum (ECS) and cultured in medium of the following composition: 40% NCTC-109, 40% ECS, 10% suspension of bone marrow cells, 10% citrated bovine plasma, and antibiotics (100 units/ml penicillin and 50 μg/ml streptomycin). Either various doses of erythropoietin or various concentrations of serum from intact mice (normal mouse serum – NMS) or of mice bled 24 h previously (anemic mouse serum – AMS), with a corresponding decrease in the dose of ECS in the culture medium, were added to the experimental samples. A standard preparation of erythropoietin with a specific activity of 100 IU/mg was obtained from the National Institutes of Health, USA. Some of this preparation was further purified on Sephadex G-100 [5].* The two preparations were dissolved in culture medium with 10% ECS and tested for activity in vivo with respect to incorporation of ^{59}Fe into erythrocytes of polycythemic mice [3].

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Culture was carried out in microplates. Each compartment contained 0.1 ml of the cell suspension in culture medium and one sample was shared among 4 to 6 compartments. The microplates were incubated in an atmosphere with 5% CO_2 in air, 100% humidity, and at 37°C. The cultures were examined daily for 10 days in an inverted microscope (magnification 63). The erythroid colonies were identified after staining with benzidine [2].

EXPERIMENTAL RESULTS

The quantitative results are given in Table 1. Colonies consisting of 8-32 erythroid (benzidine-positive) cells, breaking up during the next few days, appeared in the control samples without erythropoietin and mouse serum on the 3rd day of culture. Virtually no bursts were present. In the samples with NMS the number of 3-day colonies increased statistically significantly, and on the 5th day of culture bursts of two types appeared: I) isolated clusters of 64 or more erythroid cells and II) groups consisting of several 8-32-cell clusters. During the next 2 days both types of bursts were destroyed.

As Table 1 shows, the cloning efficiency was maximal in medium containing 10-15% mouse serum. The effect of NMS and AMS was the same both qualitatively and quantitatively.

In a concentration of 0.3 IU/ml erythropoietin virtually did not stimulate the formation of 3-day colonies, although under these circumstances there was a significant increase (compared with the control) in the number of 5-day bursts. Only in a concentration ten times greater (3 IU/ml) did erythropoietin induce the formation of 3-day colonies to the same degree as mouse serum, but under these circumstances the number of bursts was 33-50% smaller than in the samples with the optimal concentration of mouse serum. Combined addition of 10% AMS and 3 IU/ml erythropoietin causes little change from the action of AMS alone.

The number of colonies and bursts increased as a linear function with an increase in cell concentrations from $1.25 \cdot 10^4$ to 10^5 in 0.1 ml (Fig. 1) during culture in medium containing mouse serum, but in the control tests without mouse serum the corresponding function was not linear.

The culture system described above thus revealed two different types of erythroid precursors in mouse bone marrow. One type formed colonies of 8-32 cells on the 3rd day and closely resembled in its characteristics the CFU-E described previously. The other precursor, forming bursts on the 5th day, was a BFU-E, although evidently more mature than the BFU-E appearing on the 8th day of culture [1, 6]. The clonal nature of the colonies and bursts observed was confirmed by the fact that their number was a linear function of cell concentration.

However, the two types of precursors differed from those described in the literature [1, 4-6, 8, 9] in their ability to form colonies and bursts in a system not containing exogenous erythropoietin. Addition of erythropoietin to the culture, moreover, did not increase the cloning efficiency; however, this was not due to toxicity of the preparation, for the presence of additionally purified erythropoietin in the culture medium likewise did not increase the number of colonies and bursts formed. This is further supported by the fact that erythropoietin causes no change in the effectiveness of mouse serum when both were added together to the medium.

The mechanism of the inducing action of mouse serum on colony and burst formation is not yet absolutely clear. The identical effect of normal serum and of anemic serum containing an increased quantity of endogenous erythropoietin, and also the low level of effectiveness of exogenous erythropoietin suggests that erythropoietin-independent erythroid precursors may be found in this system. This hypothesis is in harmony with the description of an erythropoietin-independent erythroid precursor discovered during culture of bone marrow and of mouse embryonic liver cells in the absence of erythropoietin, when spleen cells were used as the conditioning factor [7]. It may be that in the suggested system the mouse serum also is simply a conditioning factor in the medium. At the same time there is another possibility to take into account, namely that the precursors described above, on the contrary, are hypersensitive to erythropoietin and respond completely even to small doses of the hormone such as are present in normal serum. Investigations with neutralization of endogenous erythropoietin in the culture medium are necessary to rule out this suggestion.

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EFFECT OF THE THYMUS ON PRECURSOR CELLS OF THE HEMATOPOIETIC STROMA

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It was shown by transplantation of a fragment of bone marrow beneath the capsule of the kidney that thymectomy reduces the number of cells constructing the hematopoietic microenvironment. Transplantation of the thymus abolishes the effect of thymectomy.

KEY WORDS: hematopoietic microenvironment; precursor cells; heterotopic focus of hematopoiesis; thymectomy; stroma of hematopoietic organ.

According to several recent reports the thymus influences not only differentiation of the T lymphocytes, but also earlier hematopoietic cells. Although data in the literature are contradictory, it can be concluded that thymectomy, whether neonatal or in the adult animal, modifies endocolonization and the number of hematopoietic stem cells (HSC) in the body [1-3, 11, 15], reduces the proliferative activity of HSC [8, 9], and disturbs the ability of the HSC to regenerate after sublethal radiation injury [4]. In all these cases it is not yet clear whether the results of thymectomy are due to its direct effect on hematopoietic cells or whether they can be partly attributed to its action on the hematopoietic microenvironment, controlling proliferation and differentiation of HSC [5].

It was therefore decided to study the effect of thymectomy and subsequent transplantation of the thymus on the function of cells transmitting the hematopoietic microenvironment.

EXPERIMENTAL METHOD

Female CBA and (C57BL × CBA)F_1 mice were used.

Thymectomy was performed on mice at the age of 8-10 weeks under hexobarbital anesthesia [7]. For mock thymectomy the same operation was performed but without aspiration of the thymus.

The mice were irradiated with ^{137}Cs γ rays (dose rate 21 rad/min) in a dose of 1300 rad. After irradiation the animals received an intravenous injection of syngeneic embryonic liver cells (age of the embryo 14.5 days) in a dose of $12 \cdot 10^6$ cells.

One lobe of neonatal mouse thymus was transplanted into thymectomized syngeneic recipients beneath the kidney capsule.

A focus of heterotopic hematopoiesis was obtained by transplanting a fragment of femoral marrow beneath the kidney capsule of a syngeneic recipient. Under these conditions the hematopoietic cells leave the implant and the stromal precursor cells form the hematopoietic microenvironment, which is secondarily colonized by the recipient's hematpoietic cells [10]. The size of the developing focus, proportional to the

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