Agar Cultures of Human Clonogenic Hemopoietic Precursor Cells for Early Diagnosis of Some Myeloproliferative Diseases

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Growth characteristics of human hemopoietic cells in erythremia and chronic myeloid leukemia were studied using agar cultures with and without hemopoietic growth factors. Agar cultures, similarly to cultures on other semisolid media (plasma clot, methylcellulose) can be used for early differential diagnosis of polycythemia vera (erythremia) and secondary erythrocytosis: erythremia, but not erythrocytosis, is characterized by spontaneous (erythropoietin-independent) formation of colonies from erythrocyte precursor cells. Spontaneous colony formation from granulocyte-macrophage precursor cells can serve as an important test for early diagnosis of chronic myeloid leukemia. The study of colony formation from granulocyte-macrophage precursors and of the capacity of bone marrow cells to form colonies from hemopoietic stromal precursor cells revealed new characteristics of the studied myeloproliferative diseases. Presumably, spontaneous colony formation from erythrocytic and myeloid precursors should be regarded as a sign of tumor transformation of the studied hemopoietic cells.

Key Words: *agar cultures; erythroid, myeloid and stromal precursors; spontaneous colony formation; chronic myeloproliferative diseases; early diagnosis*

Development of new methods for *in vitro* cloning of hemopoietic cells provided ample data and extended our knowledge on the mechanisms of hemopoiesis in health and disease [1,4,5]. Stem cells are important objects in the analysis of hemopoiesis and its regulation, moreover their practical value for the diagnosis of hemopoiesis disorders, prediction the disease course, and monitoring of the treatment efficiency is no longer doubted.

The growth of clone colonies from unipotent committed precursor giving rise to only one hemopoietic stem and from polypotent precursor cells forming colonies containing cells of two and more hemopoietic stems was demonstrated in primary human and animal cell cultures. Clonal growth is provided by immobilization of single-cell suspension in semisolid media (agar, methylcellulose, plasma clot) and addition of a corresponding stimulators to the nutrient medium (colony-stimulating factor or hemopoietic growth factor). Clonal growth from hemopoietic stromal precursor cells was also demonstrated (CFU-F) [6].

Here we studied primary clonal cultures of human hemopoietic cells and their use for the early diagnosis (before clinical manifestation of disease) of some myeloproliferative diseases, primarily erythremia and chronic myeloid leukemia.

MATERIALS AND METHODS

The study was carried out on hemopoietic cells of 144 patients treated at Medical Radiology Research Center

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in Obninsk and Hematological Research Center in Moscow. Bone marrow specimens (0.5-1.0 ml) and venous blood (5 ml) served as the source of hemopoietic cells. The material was collected under sterile conditions and placed in tubes with heparin (100 U/ml bone marrow and 30 U/ml peripheral blood). After erythrocyte sedimentation (1-2 h at 18-20°C) the supernatant was collected with a Pasteur pipette, the cells were washed with medium 199 by centrifugation at 1000 rpm, and resuspended in McCoy 5A medium. The number of nucleated cells in the suspension was evaluated using 5% acetic acid, and the suspension was diluted with McCoy 5A medium to a concentration needed for their explantation into the culture. Cloning of granulocyte-macrophage precursor cells from the bone marrow and peripheral blood was carried out in a modified [3] bilayer agar culture system [8] (stimulated cultures). For evaluation of spontaneous colony formation from erythrocyte (CFU-E, BFU-E), granulocyte-macrophage (CFU-GM), and polypotent (CFU-GEMM, granulocyte, erythrocyte, macrophage, and myelokaryocyte precursors) precursor cells we used a monolayer agar culture without hemopoietic growth factors. CFU-F cloning was carried out in monolayer cultures as described previously [6]. Human Bone marrow CFU-F from healthy subjects and patients with hemoblastosis were characterized in detail previously [2].

Donor peripheral blood leukocytes (10^6/ml medium) placed in the lower layer (in 2 ml) of 0.5% agar were used as the source of colony-stimulating factor in our system. The upper layer of nutrient medium (1 ml) with 0.3% agar contained the test hemopoietic cells. CFU-GM colonies appeared after 10-14 days (Fig. 1, *a*).

Spontaneous colony formation from CFU-E was evaluated 5-7 days after seeding, although erythremia and secondary erythrocytosis could be tentatively discriminated as early as on day 3 of culturing by the appearance of erythronormocyte clusters consisting of 8-16 cells. In case of erythremia the dishes were stuffed with erythrocyte colonies from CFU-E, which became more cellular by days 5-7 (Fig 2, *a*).

Spontaneous formation of colonies from CFU-GM or CFU-GEMM was evaluated 10-14 days after seeding. By this term solitary CFU-E colonies were seen in agar cultures, while granulocyte (CFU-GM) or mixed (CFU-GEMM) colonies reached their maximum size and usually contained more than 1000 cells. A culture dish with CFU-GEMM colonies derived from a patient with erythremia is shown in Fig. 2, *b*. Cell morphology in these colonies was studied after staining for lipids (Fig. 3).

Spontaneous colonies of CFU-GM did not visually differ from colonies of stimulated CFU-GM (Fig. 1, *a*).

Statistical analysis of the growth of colonies formed by different types of precursor cells was carried out using routine methods of variation statistics with calculation of the means and standard error ($M \pm m$) and Student's t test for comparison of the parameters in different groups of patients.

RESULTS

In the control, no spontaneous growth of CFU-E, CFU-GEMM, or CFU-GM colonies was observed. In stimulated cultures the yield of colonies from CFU-GM was 12.0±1.2 per 10^6 peripheral blood leukocytes and

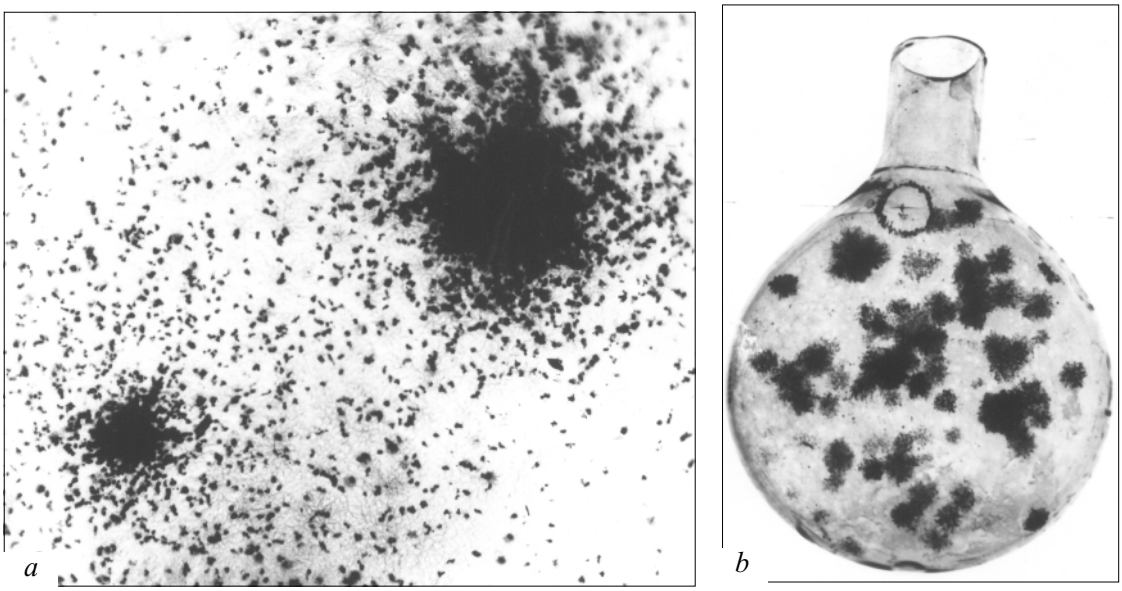

Fig. 1. Colonies formed by descendants of bone marrow granulocyte-macrophage (*a*) and stromal (*b*) precursor cells.

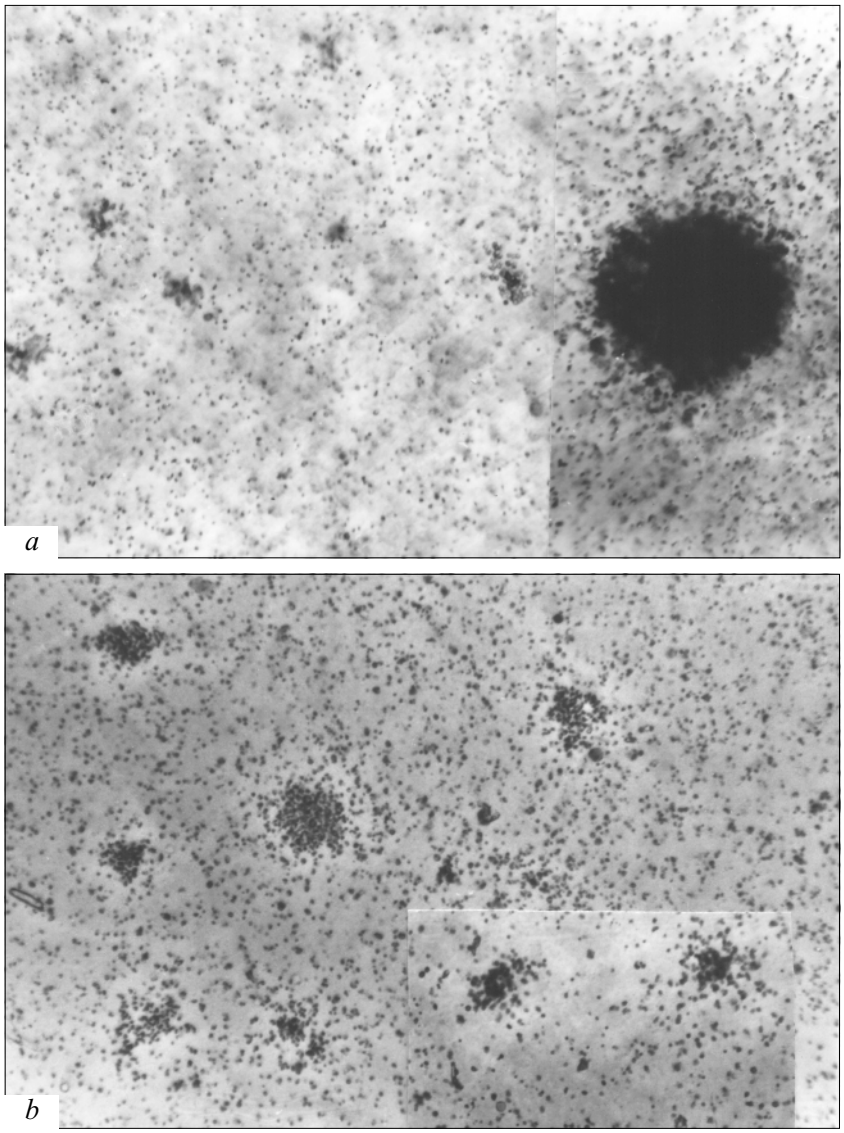

Fig. 2. Spontaneous colonies from erythrocytic precursor cells (*a*) and mixed colonies from erythro- and myelopoiesis precursor cells (*b*) in human bone marrow agar cultures, ×70. *a*) 6-day culture, numerous small clusters of erythronormocytes, large and solid colony of erythronormocytes; *b*) 7-day culture.

52.4±3.5 per 10^5 myelokaryocytes. Colony-forming capacity of CFU-F present among bone marrow cells was 49.0±3.5 per 10^6 myelokaryocytes.

In primary patients with lymphosarcoma and 26 patients with Hodgkin's disease the yield of colonies in stimulated agar cultures from CFU-GM and bone marrow CFU-F before treatment little differed from control values (subgroup A), while in 24 patients with Hodgkin's disease (subgroup B) these values increased compared to the control and patients with stage A Hodgkin's disease (Table 1).

Erythremia (patients aged 16-80 years, in 3 patients the study was carried out twice) was characterized by spontaneous growth of colonies from CFU-E (in 100% cases) and BFU-E constituting 10% erythrocyte colonies in the culture (Table 2). The increase in the yield of colonies from these precursors indicated the progress of the disease and was paralleled by an increase in CFU-GM content in the bone marrow and peripheral blood, evaluated by stimulated cultures (panmyelosis phase) and activation of bone marrow stroma (increased colony growth from bone marrow CFU-F).

It is noteworthy that 10 of 34 patients with erythremia aged 16-34 years (erythremia rejuvenation). These patients should be included in the risk group and regularly examined. In one patient (No. 1 in Table 3, male aged 26 years) the disease progressed with the involvement of small arteries, development of ischemia and necrosis of fingers. The disease progressed very rapidly in this patient. Three years before this young man was examined for transitory erythrocytosis, the examinations including the method of cell culturing. No clinical manifestations of the disease were detected at that examination, except spontaneous colony formation from CFU-E. The patient was consulted at the Hematological Research Center and the diagnosis of erythremia made on the basis of the me-

TABLE 1. Growth of Hemopoietic and Stromal Precursor Cells in Stimulated Cultures

Group		Colony growth		
		from CFU-GM		from CFU-F, per 10^6 myelokaryocytes
		per 10^6 leukocytes	per 10^5 myelokaryocytes	
Donors (n=19), $M\pm m$		12.0±1.2	52.4±3.5	49.0±3.5
Hodgkin's disease, primary patients				
before treatment	subgroup A (n=24), $M\pm m$	9.9±0.8	59.1±3.6	87.6±8.2
	subgroup B (n=26), $M\pm m$	14.5±1.8	77.3±7.5	105.9±24.9
Lymphosarcoma, primary patients (n=9),	$M\pm m$	16.8±2.3	59.2±7.2	59.0±6.3
Erythremia (n=34)	$M\pm m$ (n)	19.0±7.1 (20)	86.0±8.8 (22)	92.7±9.2 (21)
	range	4-118	33-187	47-200
Erythrocytosis				
secondary (n=15)	$M\pm m$	12.5±4.8	52.9±9.1	70.0±14.2
	range	4-30	18-87	34-99
in liquidators (n=7)	$M\pm m$	—	68±21	91±42
	range	—	43-118	54-159
Chronic myeloid leukemia (n=10)	$M\pm m$	121.6±23.9	175.5±6.6	95.0±10.1
	range	4.7-243.7	133.8-200.0	49-150

thod of cell culturing was not confirmed. However, after one year clinical manifestations of erythremia progressed, and repeated tests with cell culturing (Table 2) showed the disease progress (Table 3).

No spontaneous colony formation from erythrocyte precursors was observed in secondary erythrocytosis (22 cases, 7 of these Chernobyl accident liquidators). In these patients the content of CFU-GM in the bone marrow and peripheral blood evaluated by the growth of stimulated agar cultures and the yield of colonies from bone marrow CFU-F were virtually normal (Table 1).

Manifest stage of chronic myeloid leukemia was characterized by increased number of CFU-GM in the bone marrow and peripheral blood (which is in line with published reports) and spontaneous colony growth from CFU-GM (Table 1). Signs of activation of bone marrow stroma were detected in some patients.

Thus, our findings suggest that spontaneous (in the absence of hemopoietic growth factor in culture) colony formation from CFU-E or CFU-GM indicates tumor transformation of the studied clonogenic hemopoietic precursor cells. In contrast to foreign scientists used colony formation from CFU-E in plasma culture for differential diagnosis of early clinically manifest stages of polycythemia vera and secondary erythrocytosis [7,9], we used agar cultures for this purpose. Our studies showed that erythremia was characterized

TABLE 2. Spontaneous Growth of Colonies from 10^5 Explanted Human Bone Marrow Cells

Group		CFU-E	CFU-GEMM	CFU-GM
Donors (n=19)		0	0	0
Lymphosarcoma, primary patients (n=9)		0	0	0
Erythremia (n=34)	$M\pm m$ (n)	256.5±25.8 (34)	100.0±10.0 (2)	9.0±6.6 (14)
	range	300-600	95-105	20-100
Erythrocytosis	secondary (n=15)	0	0	0
	in liquidators (n=7)	0	0	0
Chronic myeloid leukemia (n=10)	$M\pm m$	0	0	220.7±46.0
	range	—	—	40-500

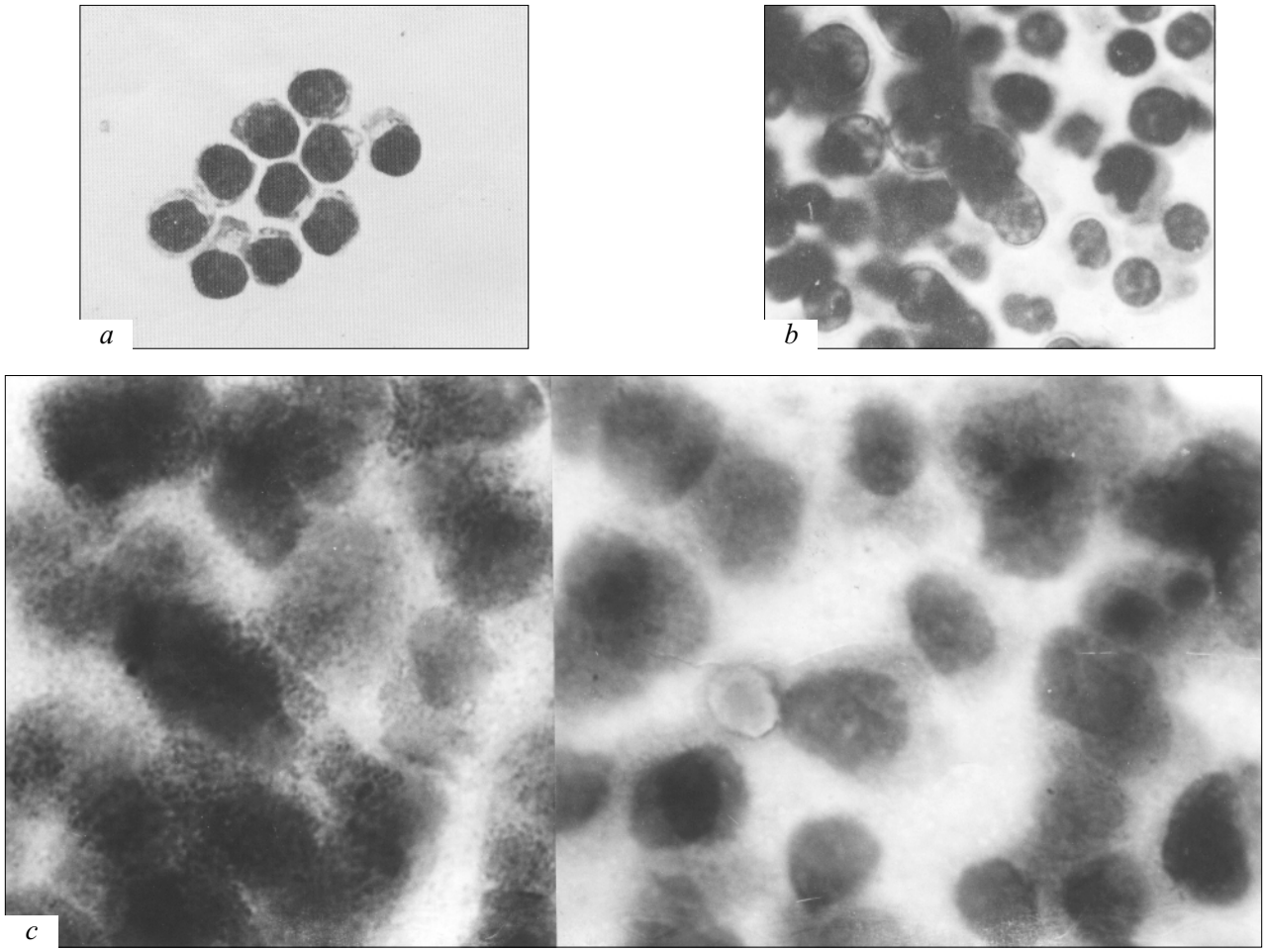

Fig. 3. Morphological composition of fragments from erythrocyte colonies (*a*) and burst-forming (*b*) units and erythro- and myelocytic CFU (*c*) in human bone marrow cultures, ×600 (*a*, *b*), ×1800 (*c*). *a*, *b*) erythronormocytes; *c*) myeloid and erythrocytic cells.

by spontaneous growth of colonies from CFU-E (in 100% cases), BFU-E (in 50% cases), CFU-GM (in 31% cases), and CFU-GEMM (in 6% cases). The progress of erythremia was associated with increased colony growth from spontaneous CFU-E paralleled by accumulation of CFU-GM in the bone marrow and peripheral blood (panmyelosis phase), as well as by activation of the bone marrow stroma (increased colo-

TABLE 3. Time Course of Hemopoietic and Stromal Precursor Cell Growth in Culture in Patients with Erythremia

Colony growth	Patient No. 1		Patient No. 2	
	initially	in 1 year	initially	in 2 weeks
Spontaneous from 10^5 explanted bone marrow cells				
CFU-E	120	300*	600	600
CFU-GM	0	0	388	383
In stimulated cultures				
from CFU-GM				
per 10^6 leukocytes	13	28*	7	8
per 10^5 myelokaryocytes	49	62*	111	91
from CFU-F per 10^6 myelokaryocytes	105	200*	119	89

Note. *$p<0.05$ compared to the initial level.

ny growth from bone marrow CFU-F). Spontaneous growth of colonies from CFU-GM in patients with erythremia confirms the involvement of the myeloid stem in the tumor process, along with the erythrocytic stem, which to a certain measure is responsible for the development of panmyelosis phase and polycythemia. Hyperplasia of the spleen also contributes to the development of polycythemia in this disease, because it serves as the source of the excess of circulating CFU-GM. One more mechanism of enhanced production of circulating CFU-GM is activation of CFU-GM proliferation, evaluated by the growth of colonies in stimulated cultures. Spontaneous growth of mixed colonies from CFU-GEMM observed in 2 cases can be regarded as the development of early stage of erythromyelosis, but not polycythemia vera, because this growth was associated with a low level of spontaneous colony formation from CFU-E.

Spontaneous colony formation from CFU-GM seems to be an important test for the early diagnosis of chronic myeloid leukemia. It is particularly important in cases when the cytogenetic marker of this diseases (Ph' chromosome) is not detected. A new characteristic of CFU-GM in myeloid leukemia, first detected in this study, extends our knowledge on the nature of this disease and is important for hematology.

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