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Pure and mixed erythroid colony formation *in vitro* stimulated by spleen conditioned medium with no detectable erythropoietin

(fetal liver culture/agar culture/hemopoietic colonies/cloning)

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ABSTRACT **Cells from CBA fetal mouse liver formed pure or mixed erythroid colonies in semisolid agar culture after stimulation by medium conditioned by pokeweed mitogen-stimulated mouse spleen cells. In general shape, the erythroid colonies resembled typical 7-day single or multiple (burst) colonies. However one-third to one-half contained, in addition to erythroid cells, macrophages and neutrophils and, less commonly, megakaryocytes or eosinophils. Culture of micromanipulated single colony-forming cells showed these erythroid colonies to be clones. Colony-forming cells declined in frequency with advancing fetal age, but low numbers were detectable in adult bone marrow. Assays of spleen conditioned medium in polycythemic mice failed to detect erythropoietin; the cloning system may detect a fetal type of erythropoietin-independent, erythropoietic cell since few were detected in adult marrow.**

One of the few universally accepted dogmas in hematology is that erythropoiesis *in vivo* is controlled by the glycoprotein humoral regulator, erythropoietin. Erythroid colony formation by precursors in the fetal liver or marrow has been described in plasma gel and methylcellulose cultures (1–4). In agreement with earlier work *in vivo*, the proliferation of erythropoietic cells *in vitro* appears to be dependent on a factor tentatively characterized as erythropoietin (5). However, the present studies show that large erythroid colonies can be grown in agar cultures of fetal mouse cells after stimulation by pokeweed mitogen-stimulated spleen conditioned medium containing no detectable erythropoietin.

MATERIALS AND METHODS

Fetal Cultures. Liver cells from fetal CBA mice aged 11–18 days (day of plugging = day 0) and cells from adult mice were cultured in 35-mm plastic petri dishes. The agar medium used was Dulbecco's modified Eagle's medium with 20% heat-inactivated human plasma in 0.3% agar (6).

Sufficient fetal cells were added to give a concentration from 2000–100,000 nucleated cells (excluding yolk sac erythroblasts) per ml, and 1-ml volumes of the cell suspension in agar medium were pipetted into culture dishes containing 0.2 ml of spleen conditioned medium.

Preparation of Spleen Conditioned Medium. C57BL spleen cells were incubated for 7 days at a concentration of 2×10^6 cells per ml in RPMI-1640 containing 5% heat-inactivated human plasma and 0.05 ml of a 1:15 dilution of pokeweed mitogen per ml of culture medium (Grand Island Biological Co., New York). After incubation, the media were centrifuged for 10 min at $3000 \times g$. The supernatant fluid was then harvested and filtered through Millipore filters.

Scoring of Cultures. Cultures were scored on day 7 for erythroid colonies using an Olympus dissection microscope and semi-indirect lighting at ×35 magnification. Red or pink aggregates containing more than 50 cells were scored as erythroid colonies. In addition, the cultures usually contained degenerating erythroid colonies with a characteristic, fuzzy, outline. These were counted separately. The cultures also developed more numerous neutrophil and/or macrophage colonies and occasional colonies of eosinophils and megakaryocytes. These were scored by the usual criteria.

The cellular composition of the colonies was determined by picking off individual colonies, smearing them on glass slides, and staining them with benzidine and Giemsa (7). Sequential colonies were sampled from specially prepared cultures of low cell numbers so that no more than 10 to 20 colonies developed per dish. For most colonies, differential counts were performed on at least 200 to 500 cells.

Micromanipulation and Culture of Single Cells. Cultures of 2000 11-day CBA fetal liver cells were prepared with 0.2 ml of spleen conditioned medium. At 12–24 hr, dividing cells (two- to six-cell clusters) were identified microscopically and individual cells were separated with a fine, glass, orally controlled pipette. Individual cells were each transferred to a marked area in a new culture dish containing 1.5 ml of agar medium and spleen conditioned medium but no cells. After transfer, the marked areas were rechecked to ensure that only a single cell had been transferred. Then the dishes were incubated for a further 6 days.

Erythropoietin. Human urinary erythropoietin (Fraction E 6-3-15 LSL, fractionated by gel filtration and affinity chromatography on concanavalin A-Sepharose, 80 units/mg) was supplied by P. P. Dukes on behalf of the Division of Blood Diseases and Resources of the National Heart, Lung and Blood Institute.

RESULTS

Erythroid Colony Formation in Cultures Stimulated by Conditioned Medium. Previous studies have shown that pokeweed mitogen-stimulated spleen conditioned medium can stimulate the formation by mouse bone marrow cells not only of neutrophil-macrophage colonies, but also of eosinophil and megakaryocyte colonies (6, 8). Fetal liver suspensions contain progenitor (colony-forming) cells of these types; cultures of 20,000 12-day CBA fetal liver cells containing 0.2 ml of conditioned medium developed large numbers of neutrophil-macrophage colonies and occasional eosinophil and megakaryocyte colonies.

However, the most spectacular feature of the cultures was the development of large, red erythroid colonies between the

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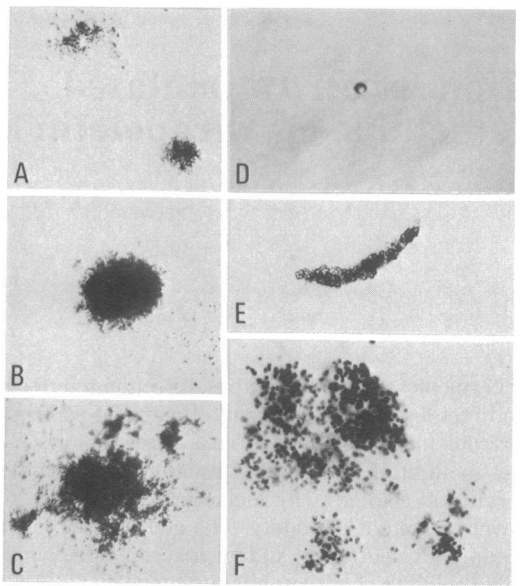

FIG. 1. (*A–C*) Unstained 7-day cultures of 12-day CBA fetal liver cells (×24) showing: *A*, two degenerating erythroid colonies; *B*, a single red erythroid colony; and *C* a multiple (burst) erythroid colony. (*D*) A transferred single cell for cloning (×80). (*E*) A mixed erythroid colony grown from a single cell; note growth of colony around circumference of implantation hole (×32). (*F*) Portion of a multiple (burst) erythroid colony grown from a single cell (Orcein stain, ×50).

more numerous granulocytic and macrophage colonies. These red colonies first became apparent at 2–4 days of incubation. Some increased in size to form large single or multiple (burst) colonies (Fig. 1 *B* and *C*), but in others the erythroid cells died at 4–6 days and the colonies were visible at 7 days as characteristically ragged and pale pink or colorless. These latter colonies were scored separately as degenerating erythroid colonies (Fig. 1*A*). The ratio of degenerating to viable erythroid colonies increased if the conditioned medium was diluted or exhibited only weak colony stimulating activity. No erythroid colonies developed in cultures lacking conditioned medium.

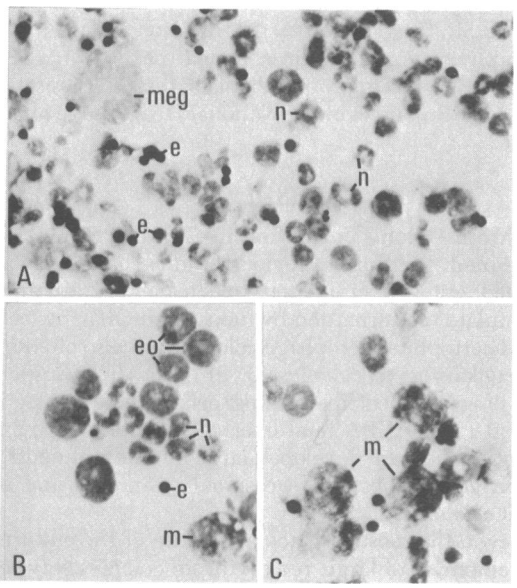

FIG. 2. Three views of cells from a single mixed erythroid colony. e, Nucleated erythroid cell; n, neutrophil; eo, eosinophil; m, macrophage; meg, megakaryocyte. (Benzidine-Giemsa stain.) *A*, ×120; *B* and *C*, ×190.

Table 1. Cytological analysis of mixed erythroid colonies grown from 12-day CBA fetal liver cells*

	No. of cells						
Colony	Erythroid	Macrophage	Neutrophil	Eosinophil	Blast or unidentifiable	Megakaryocyte	Total†
E 169	405	57	33	0	5	0	500
E 171	54	35	0	1	33	0	123
E 174	299	132	0	0	60	9	500
E 175	413	71	0	0	43	2	529
E 176	240	55	191	0	14	0	500
E 177	88	19	0	0	16	0	123
E 178	166	20	0	0	1	0	187
E 180	340	147	0	0	13	0	500
E 181	93	29	0	0	9	0	131
E 186	405	70	2	0	23	0	500
E 188	204	76	0	0	20	0	300
E 194	122	12	15	0	1	0	150
E 196	176	24	1	0	1	0	202
E 197	233	266	0	0	1	0	500
E 203	352	89	7	0	52	0	500
E 205	349	123	0	0	28	0	500
E 206	124	25	0	0	35	15	199
E 208	348	128	0	0	24	0	500
E 210	59	48	389	0	4	0	500
E 211	287	91	0	0	123	0	501
E 212	381	7	116	82	7	8	601
E 214	442	26	3	2	26	1	500
E 216	239	48	0	0	69	0	356
E 221	180	10	152	0	8	0	350

* Fifty sequential red colonies were sampled, of which 26 contained only erythroid cells or fewer than 10% other cells.
† Number of sequential cells typed, *not* total number in colony.

Red colonies classified as "erythroid" were composed of nucleated normoblasts at various stages of differentiation with occasional reticulocytes and nonnucleated mature erythroid cells. The largest colonies contained 1000–5000 typical benzidine-positive erythroid cells. In a survey of 296 sequential colonies growing in uncrowded cultures of 11- and 12-day CBA fetal liver cells, 45% were composed of macrophages, 6% of neutrophils, 15% of a mixture of neutrophils and macrophages, 2% of eosinophils, and 32% contained erythroid cells as the major or only population.

A most unusual feature was the fact that many colonies containing a major population of erythroid cells also contained additional hemopoietic cells of other types (Fig. 2). Analysis of 50 sequential red colonies containing erythroid cells (Table 1) showed that 24 (48%) contained, in addition, a significant content (arbitrarily defined as >10%) of other hemopoietic cells. As shown in Table 1, the most common combination was erythroid cells and macrophages with or without neutrophils. The distinction of megakaryocytes from macrophages was not easy in many of these preparations and the frequency of megakaryocytes could be higher than indicated in Table 1. Electron microscopic examination (D. Metcalf, G. R. Johnson, and T. Mandel, unpublished data) has documented that some mixed erythroid colonies can contain at least five different populations—erythroid, macrophage, neutrophil, eosinophil, and megakaryocyte.

Apart from the fact that some of the erythroid colonies contained other hemopoietic cells, on size and general morphology the 7-day erythroid colonies corresponded exactly to the burst colonies reported by others to form from erythroid

Table 2. Frequency of erythroid colony-forming cells in CBA liver*

Age, days	No. of cell pools tested	Colony-forming cells/10^5 cells†		
		Nonerythroid‡	Erythroid	Degenerating erythroid
		Fetal liver		
11	3	2259 ± 1190	649 ± 604	1138 ± 1020
12	12	547 ± 366	160 ± 160	122 ± 22
13	7	362 ± 243	37 ± 25	57 ± 44
14	3	310 ± 71	24 ± 24	4
17–18	5	113 ± 87	2 ± 2	10 ± 8
		Neonatal liver		
<7	4	72 ± 72	1 ± 1	3 ± 3
7–14	3	42 ± 58	1 ± 1	3 ± 3
14–21	2	0	0	0
		Adult bone marrow		
180	12	178 ± 83	1 ± 1	3 ± 3

* Cultures contained 2,000–100,000 nucleated cells and were stimulated by 0.2 ml of pokeweed mitogen-stimulated spleen conditioned medium. Colonies were scored on day 7.

† Mean calculated numbers of colonies per 10^5 nucleated cells ± SD.

‡ Mainly neutrophil and/or macrophage colonies with occasional eosinophil or megakaryocyte colonies.

precursors (BFU-E) after stimulation by high concentrations of 1–10 units of erythropoietin per ml (3, 9).

Frequency of Erythroid Colony-Forming Cells. As shown in Table 2, the frequency of 7-day erythroid colony-forming cells was highest in 11- and 12-day fetal livers. The frequency declined with advancing age, although a few colony-forming cells were still demonstrable up to 3 weeks after birth. Erythroid colony-forming cells were also detected in 10- to 13-day yolk sacs, 10- to 18-day fetal peripheral blood, and 17- to 18-day fetal spleen and bone marrow. In cultures of adult CBA marrow cells, a few erythroid colonies were also found (Table 2). Coculture of adult bone marrow cells with fetal liver cells did not inhibit the development of the expected number of fetal liver-derived erythroid colonies.

Clonal Origin of Colonies. Titration of the number of fetal liver cells added to each culture dish showed that a linear relationship, passing through zero, existed between the number of erythroid colonies developing and the number of cells cultured from 1000 to 80,000 cells per culture dish. Where few cells were cultured, single pure or mixed erythroid colonies developed in cultures that contained no other proliferating cells; this was highly suggestive that such colonies were clones derived from single cells. Single cell cloning studies were performed to eliminate the possibilities (*a*) that the observed erythroid colony formation resulted from the production of erythropoietin by some other fetal liver cells in the culture dish, and (*b*) that mixed colonies were derived from the fusion of two unrelated colonies.

Since developing erythroid colonies could not be distinguished from other colonies at 12–24 hr of incubation, only some of the transferred cells could be expected to generate erythroid cells. From 289 single cells transferred (Fig. 1*D*), 23 progressively growing colonies were obtained, although many of the other transferred cells survived or gave rise to smaller aggregates of less than 50 cells. Two colonies were composed wholly of macrophages and two wholly of neutrophils, and three contained mixtures of both cell types, confirming the known common ancestry of these two cell types. Seven colonies were composed only of erythroid cells and three of these were multiple (burst) erythroid colonies. One of these is shown in Fig. 1*F*. Seven were mixed erythroid colonies containing other hemopoietic cells. The example shown in Fig. 1*E* contained 306 cells (145 erythroid cells, 141 neutrophils, 4 macrophages, and 16 megakaryocytes). The other six contained 492–2500 cells. All contained neutrophils and macrophages, and four also contained megakaryocytes.

Table 3. *In vivo* assay for erythropoietin in spleen conditioned medium using hypertransfused C57BL mice

Total material injected	^{59}Fe counts/min*	
	Peripheral blood	Spleen
RPMI-1640 medium used to prepare conditioned medium, 0.8 ml	484 ± 75	940 ± 130
Heat-inactivated human plasma used in agar cultures, 0.8 ml	371 ± 149	879 ± 133
Spleen conditioned medium, 0.8 ml	418 ± 59	900 ± 172
Erythropoietin, 2 units	18,852 ± 3043	12,916 ± 4726
Erythropoietin, 2 units, plus spleen conditioned medium, 0.8 ml	14,154 ± 5388	10,261 ± 1476

Mean values ± SD are given.

* Six 3-month-old C57BL mice were used per group. All mice were injected on days 1, 2, 3, and 5 with 0.5 ml of packed C57BL red cells. Mean hematocrits on day 7 were above 75%. Mice were injected on days 7 and 8 with 0.4 ml of test substance and on day 9, with 0.5 μCi of ^{59}Fe in 0.2 ml of saline. Twenty-four hours later, cpm were measured in 0.4 ml of whole blood and whole spleen.

Assays for Erythropoietin. With hypertransfused, polycythemic C57BL mice no erythropoietin was detected in spleen conditioned medium or in the human plasma used either in the agar cultures or in the production of the conditioned medium as measured either by a reticulocyte response or by 24-hr uptake of ^{59}Fe (Table 3). The conditioned media did not interfere with the ability of injected erythropoietin to stimulate erythropoiesis in the test animals. The mice used were capable of exhibiting a readily measurable response to the lowest dose of erythropoietin tested (0.2 unit). This implies that in cultures stimulated by 0.1 ml of conditioned medium, even if erythropoietin was present in the conditioned medium, less than 0.025 unit of erythropoietin could have been added to each culture dish.

In repeated tests, addition of up to 4 units of erythropoietin per ml alone to agar cultures of 20,000 CBA fetal liver cells or adult bone marrow cells invariably failed to stimulate the formation of any 7-day erythroid colonies and, in fact, no colonies of any type developed. Mixture of low concentrations of erythropoietin with conditioned medium did not alter the number or appearance of colonies developing with conditioned medium alone. However, mixture of 1–4 units of erythropoietin with 0.1 ml of spleen conditioned medium approximately doubled the number of viable 7-day erythroid colonies with a corresponding reduction in the frequency of degenerating erythroid colonies (Fig. 3). The combined total of viable plus degenerating erythroid colonies was increased less than 50% by the addition of as much as 4 units of erythropoietin. However, in cultures containing added erythropoietin, erythroid colonies tended to be slightly larger and redder in color than in cultures containing conditioned medium alone.

DISCUSSION

The pokeweed mitogen-stimulated spleen conditioned medium used in these experiments to stimulate erythroid colony formation contained no detectable erythropoietin. This type of conditioned medium has been shown to be able to stimulate adult marrow cells to form colonies of granulocytes, macro-

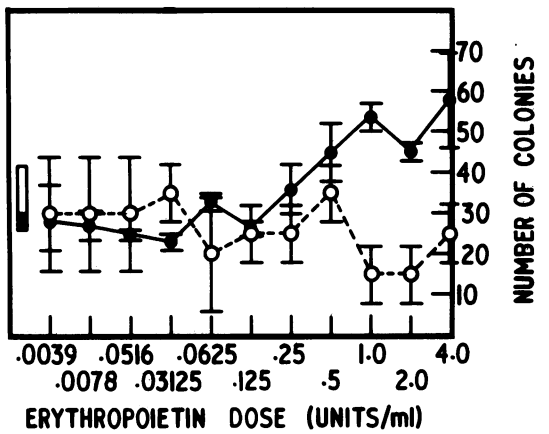

FIG. 3. Effect of adding erythropoietin to spleen conditioned medium on number of 7-day viable (●—●) and degenerating (O- - - - -O) erythroid colonies developing in cultures of 20,000 CBA fetal liver cells. All cultures contained 0.1 ml of spleen conditioned medium. Vertical bar shows range of colonies in cultures with conditioned medium alone. Mean data ± SD.

phages, eosinophils, and megakaryocytes (6, 8). Fractionation studies (10) have shown the existence of four separable, but similar, colony-stimulating factors in this conditioned medium. The material stimulating erythroid colony formation, like the others, is a glycoprotein with an apparent molecular weight of 40,000 and is capable of binding to concanavalin A-Sepharose.

Some erythropoietin must be present in the present type of cultures because of the use of human plasma. It is conceivable therefore that the factor in spleen conditioned medium is not a direct erythropoietic stimulator but merely renders the erythropoietic cells hyperresponsive to the low concentrations of erythropoietin in the medium. However, the experiments in which erythropoietin was mixed with conditioned medium (Fig. 3) lend little support to this interpretation since no major potentiation was observed.

Fetal liver cells have been reported to produce erythropoietin (11). However, the intervention of another fetal liver cell in producing erythropoietin, and thus stimulating colony formation, has been excluded by the present experiments, which showed that single cells in isolation can form erythroid colonies when stimulated by spleen conditioned medium.

Erythropoietic cells in the present agar cultures appear curiously unresponsive to erythropoietin as assessed by 7-day colony formation. Such cultures can, however, develop large numbers of 48-hr (CFU-E) erythroid colonies, but only after the addition of the very high concentration of 1–4 units of erythropoietin (G. R. Johnson and D. Metcalf, unpublished data).

Although some 48-hr erythroid colony formation by CFU-E appears possible in plasma gel cultures in the absence of added erythropoietin (12), such cells require only low concentrations of erythropoietin for stimulation (9). The formation of burst colonies requires very high concentrations of erythropoietin (9). The present experiments demonstrate the formation of such colonies in the presence of erythropoietin levels that must be at least 100-fold lower.

The cells forming the present erythroid colonies have three novel features: (*a*) they respond to a factor in spleen conditioned medium not demonstrable by *in vivo* assays as being erythropoietin, (*b*) the cells occur mainly in fetal tissues, and (*c*) a high proportion of the erythroid colonies contain other hemopoietic cells. It may be therefore that a special type of erythroid progenitor cell has been detected in the present experiments that is not primarily regulated by erythropoietin.

A passing reference was made to megakaryocytes in an erythroid colony (13); however, in this paper we have described mixed erythroid colonies. From our experience it seems likely that the erythroid colonies shown by Gregory in figure 1 *D* and *F* of ref. 9 also contain mixed populations, although no description of the colony cells was given. From the single cell cloning experiments, the cells forming mixed erythroid colonies are clearly multipotential and may be members of the multipotential stem cell compartment in hemopoietic tissues.

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