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### Research Article

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### Long-term Marrow Culture of Cells from Patients with Acute Myelogenous Leukemia

Selection in Favor of Normal Phenotypes in Some But Not All Cases

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#### Abstract

Long-term cultures were initiated with leukemic marrow aspirate cells from each of 13 newly diagnosed acute myelogenous leukemia (AML) patients. Initial assessment of the clonogenic potential of the marrow suggested that normal hemopoietic progenitors were reduced in most cases and progenitors of abnormal colonies and clusters were present in 10 cases. Subsequent assays of both nonadherent and adherent fractions of long-term cultures revealed two patterns of progenitor cell behavior. The most common pattern (nine cases) featured the detection after 1-4 wk of near normal numbers of typical erythroid, granulopoietic, and mixed colony-forming progenitor cells. Progenitors of abnormal (blast) colonies and clusters initially demonstrable in eight of these nine cases were, in these cases, not sustained in long-term culture and could not be found after 4 wk. Conversion to cytogenetic normalcy in long-term culture was confirmed in two experiments in this group. The second pattern (four cases) was characterized by the failure of progenitors capable of normal differentiation to become detectable in long-term cultures, and the concomitant maintenance of blast progenitors in the two cases in this group where such cells were initially demonstrable. Although progenitors capable of producing abnormal (blast) colonies or clusters in methylcellulose were not detected in either of the other two experiments, the maintenance for 6 wk of a hypercellular nonadherent blast population in one of these suggested the persisting activity of an "adherent layer-dependent" leukemic progenitor cell. Taken together, these findings indicate a strong correlation between the presence of leukemic blasts and their progenitors and a decreased level of normal hemopoiesis. In addition, the failure of leukemic cells to be maintained in longterm marrow cultures from some (but not all) AML patients suggests new applications of this methodology for studies of early stages of leukemic cell development.

#### Introduction

Acute leukemia is characterized by the appearance in the marrow and blood of a large population of cells that fail to differentiate normally. It is now clear that these represent the clonal progeny of a single transformed cell, although recent evidence indicates that, at least in some patients, this cell may retain to varying degrees the ability to generate apparently

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J. Clin. Invest. © The American Society for Clinical Investigation, Inc. 0021-9738/85/03/961/09 \$1.00 Volume 75, March 1985, 961-969 normal blood elements (1). Studies with short-term clonogenic systems suggest the existence within the abnormal clone of progenitor populations of greater and lesser proliferative ability, analogous in this respect to the various subclasses of progenitor cells that normally maintain blood cell production (2–4). However, little is known about the contribution of these blast cell progenitors to clonal amplification in vivo or how they may suppress the proliferation and differentiation of persisting normal (nonclonal) progenitors.

To approach these questions, we sought to develop a system in which normal and leukemic stem cells might be maintained in culture for extensive periods. Studies in the mouse indicated that normal stem cell maintenance could be achieved for periods of several months under conditions first described by Dexter et al. (5) and now widely referred to as long-term marrow cultures. The key feature of such cultures appears to be the formation of a complex contiguous network of adherent marrow elements which include a variety of hemopoietic (e.g., stem cells and macrophages) and nonhemopoietic components (e.g., fibroblasts) as well as other less wellcharacterized cell types (5-9). Recently, this type of culture system has been successfully adapted for use with normal human marrow to the extent that primitive human pluripotent and committed progenitors can be regularly shown to persist in the adherent layer for periods of 8 wk or more (10, 11).

In an initial series of experiments we set out to examine systematically the population dynamics of normal and neoplastic hemopoietic cell behavior in cultures of marrow from patients with various types of leukemia. Data from a large series of concomitant normal marrow cultures established and maintained using the same procedures (10, 11) provided a basis for comparison. Results for long-term cultures initiated with marrow from patients with chronic myeloid leukemia  $(CML)^{1}$  have been described elsewhere (12, 13). The present report describes our experience with long-term cultures initiated with marrow from newly diagnosed acute myelogenous leukemia (AML) patients and maintained for a minimum period of 6 wk. The findings obtained refer to a series of 13 consecutively accrued patients, for whom, in all but two initial cases, sufficient marrow cells were available to establish at least one long-term marrow culture (i.e.,  $>2 \times 10^7$  cells).

#### Methods

Patients. The clinical and hematological data for the 13 AML cases studied are shown in Table I. For convenience, they have been grouped

<sup>1.</sup> *Abbreviations used in this paper:* AML, acute myelogenous leukemia; BFU-E, burst-forming unit; CFU-C, granulopoietic progenitor; CFU-E, erythropoietic colony-forming unit; CFU-G/E, pluripotent progenitor; CML, chronic myeloid leukemia; FCS, fetal calf serum; LCM, leukocyte-conditioned medium.

Table I. Patient Data

			Peripheral t	blood			Bone marro	w
Case no.	Sex	Age	Hg	Leukocytes	Blasts	Platelets	Blasts	FAB classification
			g/dl	/mm <sup>3</sup>	%	/mm <sup>3</sup>	%	
1	F	60	12	1,600	9	160,000	41	M2
2	F	34	11	8,200	12	122,000	35	M2
3	М	28	9	218,000	87	91,000	87	M4
4	М	67	6	3,400	23	7,000	35	Not classified
5	М	63	8	380,000	97	75,000	82	M5
6	F	70	9.4	700	0	83,000	65	M3
7	F	80	13	14,800	3	180,000	40	M4
8	М	62	6	1,600	5	157,000	35	M2
9	F	7	6	15,000	51	14,000	<del>9</del> 0	<b>M</b> 1
10	F	72	8	5,600	6	6,000	40	M2
11	F	74	8	28,000	96	36,000	<del>9</del> 7	<b>M</b> 1
12*	М	57	8	7,100	60	58,000	44	Not classified
13	F	79	7.3	10,000	10	60,000	47	M2

\* Marrow from this patient was obtained after one course of chemotherapy.

according to whether normal granulocyte colony-forming progenitors were detectable in long-term cultures that had been maintained for more than 4 wk (cases 1–9) or not (cases 10–13). In each of these groups, those cases where progenitors of abnormal (blast) colonies were initially detectable are listed first. 8 of the 13 cases were females; the other 5 were males. The average age was 58 yr (with a range of 7 to 80). All five types of disease according to the French-American British classification (14) are represented. Marrow specimens from 12 of the 13 patients were taken before the initiation of any treatment. The marrow from patient no. 12 was taken after one course of chemotherapy when the peripheral blood still showed 60% blasts. In three patients (nos. 4, 10, and 12), transformation from a preleukemic phase was suspected on the basis of the histological appearance of the marrow.

Marrow specimens were part of aspirates taken for diagnostic purposes and were obtained with informed consent. Each aspirate was collected in a sterile tube containing 800 units of preservative-free heparin.

Long-term cultures. The procedure used to initiate and maintain long-term marrow cultures was the same as that described previously (10). Briefly, aliquots of  $2-3 \times 10^7$  unprocessed aspirate cells were suspended in 8 ml of alpha medium supplemented with extra glutamine, inositol, folic acid, 12.5% fetal calf serum (FCS, Flow Laboratories, Mississauga, Ontario), 12.5% horse serum (Flow Laboratories), 10<sup>-4</sup> M 2-mercaptoethanol, and 10<sup>-6</sup> M hydrocortisone sodium succinate (Sigma Chemical Co., St. Louis, MO) and then placed in 60-mm Falcon tissue culture dishes (Falcon Labware, Oxnard, CA). In most experiments, these were incubated at 37°C for the first 3-4 d and then subsequently kept at 33°C, since parallel studies with control marrows indicated that initiation at 37°C yielded superior long-term progenitor recoveries (11). However, this finding was not appreciated until after several of the experiments described here had been started. Erythrocytes and granulocytes were usually removed on day 3 or 4 by centrifuging the total nonadherent fraction on Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, MD) or Percoll (1.075 g/cm<sup>3</sup>, Pharmacia Fine Chemicals, Uppsala, Sweden) and returning only the light density cells to the cultures. In experiments 5, 10, and 11, erythrocyte and granulocyte contamination of the initial marrow was minimal and this step was omitted. On day 7 and at weekly intervals thereafter, cultures were fed by a complete change of the medium. At the same time, half of the nonadherent cells were also removed.

The total cellularity and progenitor content of the nonadherent fraction was determined weekly. The adherent layer was assessed less frequently since this required termination of a culture. Adherent cells were detached using 0.1% collagenase as described previously (10). As for normal and CML cultures, this procedure allowed all progenitors to be suspended, although ~25% of the other cell types in the adherent layer were not detached.

For two patients (nos. 1 and 8), the initial marrow aspirate contained  $<1.5 \times 10^7$  cells/ml. In our experience, cultures initiated with normal marrow samples of such cellularity yield scanty adherent layers in which long-term maintenance of progenitors is poor. To try and circumvent this problem, marrow cells from these two patients were therefore added to dishes containing preestablished marrow fibroblast "feeders." These were obtained by subculturing primary normal marrow adherent layers three times using alpha medium plus 20% FCS but without other supplements (including horse serum and hydrocortisone) to eliminate residual hemopoiesis as assessed by absence of colony formation in methylcellulose assays. Cultures established on such feeders were subsequently handled as usual.

Colony assay procedure. Erythropoietic colony-forming unit (CFU-E) and burst-forming unit (BFU-E), granulopoietic (CFU-C), and pluripotent (CFU-G/E) progenitors were assayed in 0.8% methylcellulose in Iscove's medium, supplemented with 30% FCS, 1% deionized bovine serum albumin,  $10^{-4}$  M 2-mercaptoethanol, 200 mM L-glutamine, 3-5 units/ml of human urinary erythropoietin (purified in our laboratory to a specific activity of >100 U/mg) (15), and either agar-stimulated human leukocyte-conditioned medium (A-LCM, 16) or phytohemagglutinin-stimulated human leukocyte-conditioned medium (PHA-LCM, 17). A-LCM was sometimes used in initial assays of the original marrow buffy coat at a final concentration of 9%. In all other instances, PHA-LCM was used at a final concentration of 4.5%. Both types of LCMs were pretested and calibrated against a standard preparation and found to be equivalent in their ability to support normal and abnormal colony growth in methylcellulose assays.

Fresh marrow cells were assayed by plating  $2 \times 10^5$  buffy coat cells/1.1 ml of culture. Cells from the nonadherent and adherent fractions were usually plated at a final concentration of  $1 \times 10^5$  cells/1.1 ml of culture. When cytogenetic studies were planned, additional assay replicates were set up.

Dishes were scored twice, first after 9-12 d and again after 17-21 d of incubation as for normal specimens. Erythroid colonies which

appeared morphologically normal in situ on the basis of well-established criteria (16) were categorized and enumerated. This included the separate assessment of large (>8 clusters) and small ( $\leq 8$  clusters) erythroid bursts thought to be derived from primitive and mature subclasses of BFU-E, respectively. Large colonies of (≥500) granulocytes and macrophages could also be readily identified in situ whenever these were present. However, in those assays where morphologically abnormal (blast) clusters and/or larger colonies were obtained, accurate enumeration of morphologically normal but small granulocyte colonies that may also have been present was not feasible. Assessment of CFU-G/E in such assay cultures was also compromised. Detailed morphological studies of abnormal colonies and clusters were not undertaken, but periodic plucking and staining with May-Grunwald Giemsa confirmed their blast cell nature as described by others (2-4). Because of the sometimes diffuse colony morphology, the often highly variable size of these abnormal clusters and colonies, and the persistence of a variable background of blasts in assays of different leukemic marrows, accurate quantitation of abnormal clusters and colonies was also usually difficult. In the present study, we therefore adopted the following semi-quantitative categories: -, no evidence of abnormal (blast) colonies or clusters; +, up to 50 abnormal colonies and/or clusters per assay (2  $\times$  10<sup>5</sup> initial marrow buffy coat cells or 10<sup>5</sup> longterm culture cells); ++, 50-500 abnormal colonies and/or clusters per assay; +++, >500 abnormal colonies and/or clusters per assay.

Cytogenetic studies. Direct preparations of Giemsa-banded marrow metaphases were obtained using standard methods (18). The genotype of primitive progenitors present in long-term marrow cultures was determined by analyzing metaphases from individual large colonies generated in methylcellulose assays of adherent layer cells. Colonies to be analyzed were selected at a stage when they could first be readily identified as erythroid or granulopoietic but still contained a significant number of immature, proliferating cells. Colcemid was added to the assay dishes, and the colonies were then plucked and processed on polylysine-coated slides as previously described (19). A minimum of two metaphases/colony were analyzed after G-banding and in none of the 34 colonies that satisfied this criterion was more than one genotype evident.

Cytogenetic analysis of fresh marrow progenitors was not attempted because of the anticipated very low incidence of morphologically normal colonies of suitable size and the frequent high background of abnormal growth in initial assays (see Table III).

#### Results

*Culture morphology.* For 10 of the 11 cases where long-term marrow cultures were initiated in the standard way, a typical confluent adherent layer formed in which "cobblestone" areas of hemopoietic cells could be consistently discerned (Fig. 1). The two cultures initiated by adding AML marrow cells to preestablished normal "feeders" (see Methods) developed a similar appearance. In one case (no. 11), only a partial adherent layer formed and confluence was not achieved.

Total nucleated cell recovery. A curve showing the average total number of nucleated cells present in the nonadherent fraction of all 13 experiments as a function of time is shown in Fig. 2. The results appear similar to those for control marrow cultures, at least for the first 6 or 7 wk. However, as a group, the AML cultures displayed greater heterogeneity. For example, in three (nos. 10, 11, and 13), nonadherent cell numbers were maintained between weeks 3 and 6 at levels higher than the maximum measured in any of 33 normal marrow cultures (11) during the same interval (i.e.,  $>2-4 \times 10^6$  nonadherent cells/culture). On the other hand, in another AML culture (no. 9), the nonadherent nucleated cell count



Figure 1. Photomicrograph of a 5-wk-old AML adherent layer (from experiment No. 7) showing a typical "cobblestone" area of putative hemopoietic cells against a confluent background of other cell types.



Figure 2. Cellularity of the nonadherent fraction of the 13 long-term AML marrow cultures described here compared as a single group with corresponding values obtained concurrently for long-term cultures of normal human marrow (10, 11). The shaded area encompasses the range of values encountered in normal marrow cultures.

fell to abnormally low levels ( $<10^5$  cells) within the first 2 wk, and never recovered.

Morphological examination of May-Grunwald Giemsastained cytospin preparations of the nonadherent cells from several of the cultures showed that blasts were the predominant cell type in the nonadherent fraction for the first 4 wk and usually persisted for several additional weeks. Evidence of terminally differentiating granulocytes or monocyte/macrophages were also obtained in all instances, although these were clearly not always normal. Data from four experiments (nos. 5, 6, 10, and 13) are shown in Table II.

The number of cells harvested from the adherent layer by collagenase treatment was determined between weeks 4 and 9 in 10 experiments. In general, the values obtained (mean, 2.7  $\times 10^6$  cells; range,  $1.7-5.4 \times 10^6$  cells) were similar to control cultures (mean,  $1.9 \times 10^6$  cells; range,  $0.8-6.3 \times 10^6$  cells).

Progenitor kinetics. The hemopoietic progenitor content of each initial marrow sample as revealed by methylcellulose assays is given in Table III. Tables IV and V show the subsequent numbers of progenitors detected in the nonadherent and adherent fractions of long-term cultures assayed at the times shown. For 8 of the 13 patients, abnormal (blast) colonies and/or clusters were readily apparent in initial marrow assays (e.g., see Fig. 3 a). In two other cases (nos. 7 and 8), these became evident in assays of the nonadherent fraction of 1-wk-old long-term cultures, even though such colonies were not detected in assays of the original marrow sample. Thus, for 10 of the 13 patients studied, marrow cells capable of producing abnormal colonies or clusters were detected. These decreased progressively with time in long-term culture in eight cases. In one of these (no. 6), such cells were no longer found after 1 wk. However, in the other seven cultures, they persisted at detectable levels for 3 wk and occasionally for 4 wk, but not thereafter (Table IV). Adherent layers from these long-

	Differential (percent values based on 300 cells analyzed)				
Weeks of culture	Blasts	Myelocytes, metamyelocytes, and polymorphs	Others* (mainly monocytes and macrophages)		
1	52	0	48		
2	60	6	34		
4	52	12	36		
1	84	<2%	15		
2	93‡	<2%	6		
3	96‡	<2%	3		
4	94	<2%	5		
5	86	<2%	13		
1	66	20	14		
2	57	30	13		
3	74	23	3		
4	47	46	7		
5	20	68	12		
6	19	72	9		
1	38‡	44	18		
2	51‡	22‡	27		
3	60‡	21	19		
4	72	25	3		
5	37	31	32		
	Weeks of culture 1 2 4 1 2 3 4 5 1 2 3 4 5 6 1 2 3 4 5 5	Weeks of culture Blasts   1 52   2 60   4 52   1 84   2 93‡   3 96‡   4 94   5 86   1 66   2 57   3 74   4 47   5 20   6 19   1 38‡   2 51‡   3 60‡   4 72   5 37	Notices analyzed)Weeks of cultureMyelocytes, metamyelocytes, and polymorphs1 $52$ 02 $60$ 64 $52$ 121 $84$ $<2\%$ 2 $93\ddagger$ $<2\%$ 3 $96\ddagger$ $<2\%$ 4 $94$ $<2\%$ 5 $86$ $<2\%$ 1 $66$ $20$ 2 $57$ $30$ 3 $74$ $23$ 4 $47$ $46$ 5 $20$ $68$ 6 $19$ $72$ 1 $38\ddagger$ $44$ 2 $51\ddagger$ $22\ddagger$ 3 $60\ddagger$ $21$ 4 $72$ $25$ 5 $37$ $31$		

Table II. Morphological Analysis of the Nonadherent Fraction of Long-term AML Cultures as a Function of Time after Initiation

\* Including some erythroblasts for the first 2 wk only, occasional lymphocytes (<5%), and usually <10% of cells that could not be identified.

‡ Presence of Auer Rods noted.

term cultures, which were usually evaluated after 4 wk, also did not contain any detectable progenitors of abnormal (blast) colonies or clusters by this time (Table V).

A contrasting pattern was seen in long-term cultures initiated with marrow cells from the other two patients where abnormal (blast) colony or cluster progenitors were initially detected (nos. 10 and 11). In these two experiments, such progenitors were maintained in both the nonadherent and adherent fractions for at least 8 wk (Tables IV and V). In the other three experiments (nos. 9, 12, and 13), progenitors of abnormal (blast) colonies or clusters were not detectable at any time (Tables III-V).

Normal granulocyte/macrophage colonies were rarely seen in initial assays. CFU-E and BFU-E, however, were detectable in the marrow of most patients studied but usually, although not always, at reduced levels. Those patients whose marrow showed a normal concentration of erythropoietic progenitors were noted to be among those whose leukocyte counts were not elevated and whose numbers of circulating blasts were relatively low (Table III).

In nine experiments (nos. 1–9), "normal" CFU-C (yielding typical granulopoietic colonies; Fig. 3 b and 3 c) were readily detectable in assays of both nonadherent and adherent fractions of long-term cultures that had been maintained for 4 wk or more. In fact, almost as soon as such cells became detectable, they appeared as the predominant type of progenitor present

Table III.	Results of Methylcelli	ılose
Assays of	Initial Marrow Sampl	es

		No. of progenitors/2 $\times$ 10 <sup>5</sup> buffy coat cells						
Experiment no.		CFU-E	BFU-E	CFU-C	Abnormal (blast) colonies and clusters			
1		122	195	ND	+			
2		13	4	ND	+			
3		0	2	ND	+++			
4		157	11	ND	+			
5		0	0	ND	+			
6		32	52	ND	+			
7		2	0	1	_			
8		30.5	10.5	3	-			
9		3	10	0	-			
10		285	23	ND	++			
11		8	0	ND	++			
12		0.5	0.5	0	-			
13		1	0.5	0	-			
Normal	Mean	194	92	73	-			
values* $(n = 11)$	±2SD	66–568	30–284	12–412	-			

ND, not detected.

\* From reference 30.

(Tables IV and V). In two experiments (nos. 6 and 9), "normal" CFU-C were already detectable after only 1 wk of long-term culture. Thus, all experiments where progenitors of

Table IV. Results of Methylcellulose Assays of Nonadherent Cells from Long-Term AML Cultures Evaluated at Weekly Intervals

<b>_</b> .	Abnormal ( colonies and	blast) d clusters	No. of progenitors of normal granulocyte colonies (CFU-C) per long-term culture			
Experiment no.	1-3 wk	4–8 wk	1-3 wk	4–8 wk		
1	+	_	ND	0-101		
2	+	-	ND	17-65		
3	+++	-	ND	13-32		
4	+	-	ND	20-197		
5	+	-	ND	1.5		
6	_	-	160-570	18-131		
7	++	-	ND	91-120		
8	+	-	ND	4-180		
9	-	-	277	20		
10	++	++	ND	ND		
11	+	+	ND	ND		
12	-	-	ND	ND		
13	-	-	ND	ND		
Controls*	-	-	225-21,390	4–1,496		

ND, not detected

\* Range of values obtained from concurrently carried long-term normal marrow cultures (11).

Table V. Results of Methylcellulose Assays of Simultaneously
Assessed Adherent and Nonadherent Fractions of Long-Term
AML Cultures

		No. of progenitors per culture					
Weeks of culture	Experi- ment no.	BFU-E A:NA*	CFU-C A:NA	CFU-G/E A:NA	Abnormal (blast) colonies and clusters A:NA		
4	2	15:7	62:49	<7":<3	+:+		
	3	86:80	270:38	10:<40	+:+		
	4	14:4	34:60	<1:<1	-:-		
	5	<5:<1	5:2	<5:<1	-:-		
	9	13:5	87:20	<6:<1	-:-		
	11	<7:<10	<7:<10	<7:<10	+++:+		
	12	16:3	30:<3	<8:<3	-:-		
	Controls‡	340:79	1142:682	4:2			
6	2	18:ND§	77:ND	5:ND	-:ND		
	3	15:2	78:23	2:2	-:-		
	4	26:1	244:197	2:<1	-:-		
	7	23:4	364:116	<6:<2	-:-		
	11	<7:<4	<7:<4	<7:<4	++:+		
	13	<11:<17	<11:<17	<11:<17	-:-		
	Controls‡	79:2	324:93	3:<2			
8 and 9	1	6:ND	6:ND	<3:ND	-:ND		
	2	15:ND	38:ND	<7:<3	-:ND		
	3	5:ND	5:ND	<3:ND	-:ND		
	11	<6:<4	<6:<4	<6:<4	++:+		
	Controls‡	50:3	162:58	<12:<2			

\* A, adherent; NA, nonadherent

**‡** Values from four to six concurrently evaluated long-term normal marrow cultures (11, 12).

§ Not done (very few nonadherent cells).

<sup>II</sup> None detected; value shown represents the number that would correspond to the detection of one colony in the portion of the culture actually assayed.

abnormal (blast) colonies or clusters disappeared with time in culture (i.e., nos. 1–8) also showed concomitant emergence of "normal" CFU-C. However, this latter phenomenon was not limited to such cultures, as exemplified by experiment 9. Note that in this case, although the initial marrow contained 90% M1 type blasts and no detectable progenitors of any kind (i.e.,  $<1/4 \times 10^5$  cells), after 1 wk of long-term culture, the nonadherent population of  $3 \times 10^5$  cells was found to contain very few blasts and 277 CFU-C. This number of CFU-C is just within the lower limit measured for a large number of 1-wk-old normal long-term MAL cultures in which CFU-C were detected after week 4, values also fell within the normal range, although as in experiment 9, usually close to the lower limit (Table IV).

CFU-E were rarely detected in either the nonadherent or adherent fraction of long-term AML cultures that had been maintained for 4 wk or more (data not shown). The majority of BFU-E present were found in the adherent layer (Table V), a finding also characteristic of normal marrow cultures (10). Adherent layer assays from seven of the nine experiments in which nonadherent CFU-C became readily detectable after 3 wk also revealed the presence of typical normal marrow



Figure 3. Photomicrographs of colonies generated in methylcellulose assays shown as they appear without fixation or staining. (a) Numerous small clusters of cells of abnormal morphology in the 1-wk nonadherent cell assay from experiment no. 2; (b) small granulocyte colony (in the 4-wk nonadherent cell assay of experiment no. 2) typical of those detected in assays of nonadherent fractions from all

4- to 8-wk-old cultures in experiments nos. 1-9; (c) large granulocyte colony typical of those unique to the adherent layer of normal long-term marrow cultures (10) and here derived from a progenitor present in the 6-wk-old adherent layer of experiment no. 4. (This colony was subsequently shown to be cytogenetically normal, see Table VI).

adherent layer CFU-C capable of generating very large granulocyte colonies containing at least 500 cells (Fig. 3 c). In eight of these nine cultures, BFU-E were readily detectable, and in four cases, CFU-G/E were also detected. The numbers of these primitive progenitor types (per culture) were low compared with normal cultures, but higher than expected if related to calculated input values. For example, in experiment 7, assays of 6-wk-old long-term cultures revealed the presence of 27 BFU-E, although none had been detected in assays of the initial marrow. Thus, the number present after 4 wk appears to correspond to at least one-third of the maximum number of BFU-E that might be assumed to have been present initially. Similarly, in experiment 3, initial assays suggested that long-term cultures were seeded with 250 BFU-E, although after 4 wk the number detected was 166, i.e., more than two-thirds of the apparent input value. Taken together, these data suggest that direct assessment of the normal progenitor content of the initial marrow of many AML patients may significantly underestimate the true value due to inhibition of normal differentiation in the presence of sufficient numbers of leukemic blasts.

In experiments 10 and 11, where cells capable of abnormal (blast) colony and cluster formation in methylcellulose persisted, and where elevated numbers of nonadherent blast cells were maintained (Table II), no CFU-C were detected at any time, although an occasional BFU-E was found in experiment 10 (data not shown). Both cases 10 and 11 were females over 70 yr old. Both failed to achieve remission.

Long-term marrow cultures established from cases 12 and 13 demonstrated yet a different pattern. Nonadherent cell assays consistently failed to yield colonies or clusters of any kind. A few BFU-E and CFU-C were detected in the week 4 adherent layer of experiment 12. None were detected in the adherent layer of experiment 13 (Tables IV and V). Nevertheless, in spite of the absence of progenitors capable of proliferation in methylcellulose assays, a very large nonadherent cell population (>2 × 10<sup>6</sup> cells) was maintained in experiment 13 for 6 wk. As shown in Table II, this included a significant blast component and some leukemic cells that were able to undergo varying degrees of granulopoietic differentiation in culture. The adherent layer in this experiment was of average cellularity ( $2.3 \times 10^6$  cells). Both cases 12 and 13 failed to enter remission.

Cytogenetic studies. Cytogenetic studies were undertaken in two experiments (nos. 3 and 4). As shown in Table VI, all direct marrow metaphases obtained from patient no. 3 were found to belong to a 9q- population and proliferating normal cells were not detected. For patient no. 4, half of the direct

Table VI. Comparison of Cytogenetic Findings on Progenitors Maintained in Long-Term AML Cultures from Two Patients with Initial Results from Direct Marrow Preparations

Patient		Direct	Progenitors in 4–6-wk adherent layers			
	Karyotype	marrow preparation	BFU-E	CFU-C	CFU-G/E	
3	46, XY, 9q-	11	0	0	0	
	46, XY	0	7	10	2	
	Total	11	7	10	2	
4	$\begin{array}{l} 43, XY, -8, -13, \\ -14, -15, -16, \\ -21, +M_1, \\ +M_2, +M_3, \\ der(16)t(16q;?) \end{array}$	10	0	0	0	
	46, XY	10	8	7	0	
	Total	20	8	7	0	

marrow metaphases showed a complex karyotype, the remainder being chromosomally normal. Morphologically normal colonies generated in assays of 4–6-wk-old adherent layers from both experiments were plucked and analyzed individually. All proved to be chromosomally normal (Table VI).

#### Discussion

In the last decade, technical advances in a number of areas have been applied to the study of AML. Analysis of the proliferative (2–4) and differentiative (1, 20) potential of leukemic progenitor cells from patients with a clinical diagnosis of AML suggests a marked degree of heterogeneity between patients. This is further strengthened by additional evidence of heterogeneity with respect to the cell of origin (1), specific chromosomal changes observed (21, 22), and the possible involvement of particular oncogenes in leukemic cells from different individuals (23, 24).

Thus far, however, such studies have been limited either to fresh marrow (or blood) samples or to autonomous cell lines derived from such samples. In the present study, we used marrow aspirates from newly diagnosed AML patients to initiate long-term cultures and then followed the kinetics of clonogenic progenitor cells detectable in such cultures over the next 8-9 wk. Heterogeneity in the behavior of all types of progenitors was seen. However, from these studies, two basic patterns appeared. The first was characterized by the detection of significant numbers of BFU-E and CFU-C in long-term cultures maintained for more than 3 or 4 wk, even though typically none or very few of these progenitor types could be identified in initial marrow assays. Long-term marrow cultures from nine patients showed this pattern. In eight of these, progenitors of abnormal (blast) colonies or clusters were detected early on (after 0 or 1 wk in long-term culture), but their numbers declined rapidly and these could no longer be detected after 4 wk. In the second group, CFU-C and BFU-E were rarely detected at any time. Long-term marrow cultures from four patients showed this latter pattern. In two of these, progenitors of abnormal (blast) colonies or clusters were detected both initially and subsequently for the entire duration of the experiment. In the other two sets of cultures in this latter category, such progenitors were not detected at any time. However, in one of these experiments, the maintenance of a leukemic progenitor population might be inferred from the fact that a large population of nonadherent blasts was maintained for at least 6 wk in spite of the weekly removal of half of their numbers.

These findings raise a number of interesting practical and theoretical issues. First, what is the mechanism that leads to the apparent emergence of progenitors capable of normal differentiation (in excess of that predicted from input values) and the concomitant disappearance of abnormal (blast) colony progenitors with time in long-term culture? One possibility is that the production of CFU-C (and sometimes BFU-E) from normal stem cells and their subsequent differentiation may be suppressed by the presence of a large leukemic blast population. For reasons not yet clear, it appears that long-term cultures are not usually able to support the maintenance of clonogenic blast progenitors. Accordingly, as these decline in long-term cultures, the mechanism for suppression of normal hemopoiesis would be lost and the progeny of previously quiescent stem cells might become detectable. Suppression of normal hemopoiesis by leukemic cells has been suggested by others (25), although these findings remain controversial (26). Nevertheless, it is an attractive explanation for the present findings and one that has recently received additional support from our studies of long-term CML cultures. In these, the neoplastic Ph<sup>1</sup>positive population also declines rapidly, often allowing previously undetectable Ph<sup>1</sup>-negative (nonclonal) progenitors to become apparent (12, 13, 27). In the present study, a chromosomal marker for the AML population was known for two of the patients whose long-term cultures showed emergence of progenitors capable of normal differentiation. In both instances, these late-appearing progenitors were also found to be chromosomally normal.

A second and related question pertains to the "normalcy" of such progenitors. Although it seems likely that these would have derived from residual stem cells outside of the leukemic clone, this may not have been the case in every experiment. A number of lines of evidence suggest that leukemic transformation may be a multi-step process involving a series of genetic changes, the first of which might not necessarily be detectable at the chromosomal level (28). Moreover, the demonstrated ability of clonal AML progeny to execute normal differentiation programs under conditions prevailing in vivo (1, 28) as well as following experimental manipulation in vitro (28) suggests that abnormal differentiative behavior may often be due to secondary changes, possibly associated with the evolution of more frankly leukemic subpopulations. Thus, neither phenotypic nor cytogenetic normalcy can be taken as rigorous evidence that the progenitors detected in long-term AML cultures were not neoplastic. Because of the typically rapid rate of expansion of the leukemic clone in patients with untreated AML, the most aggressively proliferating subpopulations predominate and earlier stages in their evolution cannot be readily studied. It is, therefore, not surprising that very little is known about the cellular changes that precede overt disease. The present studies are relevant in this regard since they suggest that long-term culture may offer a novel method for selecting earlier stages of leukemic cell development. Further pursuit of this question will clearly require clonal markers that antedate the first leukemogenic event and analysis of such patients is currently underway.

On the other hand, it is already clear from studies of treated AML patients that in many instances hemopoiesis in remission is regenerated from coexisting nonclonal stem cells (1). In fact, this has stimulated efforts to develop a variety of methods for removing residual leukemic cells from AML marrow samples destined for autologous reinfusion following treatment of subsequent relapses. In view of current technical and practical problems facing investigators in this area, selection in favor of normal elements using the type of culture procedure described here offers an alternative that may be worthy of future consideration.

Finally, a comment is warranted regarding the clinical outcome of patients grouped according to the behavior of their marrow cells in long-term culture. Obviously, a series of 13 patients is too small to draw any general conclusions. Nevertheless, the fact that all four patients whose long-term marrow cultures failed to yield normal progenitors also failed to achieve a first remission is suggestive that biological parameters of importance may emerge from more extensive studies of the type described here. Similarly, it will be of interest to determine why some leukemic marrow cell populations appear able to proliferate under conditions set up in long-term culture but not in dilute suspension in methylcellulose and whether this has clinical significance.

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#### References

1. Fialkow, P. J. 1982. Cell lineages in hematopoietic neoplasia studied with glucose-6-phosphate dehydrogenase cell markers. J. Cell. Physiol. Suppl. 1:37-43.

2. Moore, M. A. S., N. Williams, and D. Metcalf. 1973. In vitro colony formation by normal and leukemic human hematopoietic cells: characterization of the colony-forming cells. *J. Natl. Cancer Inst.* 50: 603–623.

3. Chang, L. J. A., J. E. Till, and E. A. McCulloch. 1980. The cellular basis of self renewal in culture by human acute myeloblastic leukemia blast cell progenitors. *J. Cell. Physiol.* 102:217-222.

4. Park, C. H., M. A. Savin, B. Hoogstraten, M. Amare, and P. Hathaway. 1977. Improved growth of *in vitro* colonies in human acute leukemia with the feeding culture method. *Cancer Res.* 37:4595–4601.

5. Dexter, T. M., T. D. Allen, and L. G. Lajtha. 1977. Conditions controlling the proliferation of haemopoietic stem cells in vitro. J. Cell. Physiol. 91:335-344.

6. Mauch, P., J. S. Greenberger, L. Botnick, E. Hannon, and S. Hellman. 1980. Evidence for structured variation in self-renewal capacity within long-term bone marrow cultures. *Proc. Natl. Acad. Sci. USA*. 77:2927–2930.

7. Gartner, S., and H. S. Kaplan. 1980. Long-term culture of human bone marrow cells. Proc. Natl. Acad. Sci. USA. 77:4756-4759.

8. Kodama, H.-A., Y. Amagai, H. Koyama, and S. Kasai. 1982. A new preadipose cell line derived from newborn mouse calvaria can promote the proliferation of pluripotent hemopoietic stem cells in vitro. J. Cell. Physiol. 112:89-95.

9. Keating, A., J. W. Singer, P. D. Killen, G. E. Striker, A. C. Salo, J. Sanders, E. D. Thomas, D. Thorning, and P. J. Fialkow. 1982. Donor origin of the *in vitro* haematopoietic microenvironment after marrow transplantation in man. *Nature (Lond.).* 298:280–283.

10. Coulombel, L., A. C. Eaves, and C. J. Eaves. 1983. Enzymatic treatment of long-term human marrow cultures reveals the preferential location of primitive hemopoietic progenitors in the adherent layer. *Blood.* 62:291–297.

11. Coulombel, L., C. J. Eaves, I. D. Dubé, D. K. Kalousek, and A. C. Eaves. 1984. Variable persistence of leukemic progenitor cells in long-term CML and AML marrow cultures. *In* Long-Term Bone Marrow Culture: Past Experience and Future Possibilities. D. G. Wright and J. S. Greenberger, editors. Alan R. Liss, Inc., New York. In press.

12. Coulombel, L., D. K. Kalousek, C. J. Eaves, C. M. Gupta, and A. C. Eaves. 1983. Long-term marrow culture reveals chromosomally normal hemopoietic progenitor cells in patients with  $Ph^1$ -positive chronic myelogenous leukemia. *N. Engl. J. Med.* 308:1493–1498.

13. Dubé, I. D., D. K. Kalousek, L. Coulombel, C. M. Gupta,

C. J. Eaves, and A. C. Eaves. 1984. Cytogenetic studies of early myeloid progenitor compartments in  $Ph^1$ -positive chronic myeloid leukemia (CML). II. Long-term culture reveals the persistence of  $Ph^1$ -negative progenitors in treated as well as newly diagnosed patients. *Blood.* 63:1172–1177.

14. Bennett, J. M., D. Catovsky, M.-T. Daniel, G. Flandrin, D. A. G. Galton, H. R. Gralnick, and C. Sultan. 1976. Proposals for the classification of the acute leukaemias. *Br. J. Haematol.* 33:451-458.

15. Krystal, G., C. J. Eaves, and A. C. Eaves. 1984. CM Affi-gel blue chromatography of human urine: a simple 1-step procedure for obtaining erythropoietin suitable for in vitro erythropoietic progenitor assays. *Br. J. Haematol.* 58:533-546.

16. Eaves, C. J., and A. C. Eaves. 1978. Erythropoietin (Ep) doseresponse curves for three classes of erythroid progenitors in normal human marrow and in patients with polycythemia vera. *Blood.* 52: 1196–1210.

17. Cashman, J., D. Henkelman, K. Humphries, C. Eaves, and A. Eaves. 1983. Individual BFU-E in polycythemia vera produce both erythropoietin dependent and independent progeny. *Blood.* 61:876-884.

18. Seabright, M. 1971. A rapid banding technique for human chromosomes. *Lancet.* II:971-972.

19. Dubé, I. D., C. J. Eaves, D. K. Kalousek, and A. C. Eaves. 1981. A method for obtaining high quality chromosome preparations from single hemopoietic colonies on a routine basis. *Cancer Genet. Cytogenet.* 4:157–168.

20. Smith, L. J., J. E. Curtis, H. A. Messner, J. S. Senn, H. Furthmayr, and E. A. McCulloch. 1983. Lineage infidelity in acute leukemia. *Blood.* 61:1138-1145.

21. Rowley, J. D. 1983. Chromosome changes in leukemic cells as indicators of mutagenic exposure. *In* Chromosomes and Cancer. Academic Press, New York. 140–158.

22. Yunis, J. J. 1983. The chromosomal basis of human neoplasia. Science (Wash. DC). 221:227-236.

23. Collins, S., and M. Groudine. 1982. Amplification of endogenous *myc*-related DNA sequences in a human myeloid leukaemia cell line. *Nature (Lond.).* 298:679–681.

24. Eva, A., S. R. Tronick, R. A. Gol, J. H. Pierce, and S. A. Aaronson. 1983. Transforming genes of human hematopoietic tumors: frequent detection of *ras*-related oncogenes whose activation appears to be independent of tumor phenotype. *Proc. Natl. Acad. Sci. USA*. 80:4926–4930.

25. Morris, T. C. M., T. A. McNeill, and J. M. Bridges. 1975. Inhibition of normal human in vitro colony forming cells by cells from leukaemic patients. *Br. J. Cancer.* 31:641–648.

26. Spitzer, G., D. S. Verma, M. Beran, A. R. Zander, K. A. Dicke, K. B. McCredie, S. Siegel, and S. Tindle. 1981. Human myeloid leukaemic cell interactions in vitro with normal myeloid colonies. *Br. J. Cancer.* 43:149–156.

27. Dubé, I. D., Z. A. Arlin, D. K. Kalousek, C. J. Eaves, and A. C. Eaves. 1984. Non-clonal hemopoietic progenitor cells detected in long-term marrow cultures from a Turner Syndrome mosaic with chronic myeloid leukemia (CML). *Blood.* 64:1284–1287.

28. Jacobson, R. J., M. J. Temple, J. W. Singer, W. Raskind, J. Powell, and P. J. Fialkow. 1984. A clonal complete remission in a patient with acute nonlymphocytic leukemia originating in a multipotent stem cell. *N. Engl. J. Med.* 310:1513–1517.

29. Fibach, E., and E. A. Rachmilewitz. 1981. Tumour promoters induce macrophage differentiation in human myeloid cells from patients with acute and chronic myelogenous leukaemia. *Br. J. Haematol.* 47: 203-210.

30. Eaves, A. C., and C. J. Eaves. Erythropoiesis in culture. *In* Cell Culture Techniques: Clinics in Haematology. E. A. McCulloch, editor. W. B. Saunders, Eastborne, England. Vol. 2, No. 2.