

In Vitro Differentiation of Leukemic Progenitor Cells in Various Types of Acute Nonlymphocytic Leukemia¹

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ABSTRACT

The effects of colony-stimulating factors on differentiation of leukemic progenitor cells were investigated in various types of acute nonlymphocytic leukemia. Two different sources of colony-stimulating factors were used in this study: human placental conditioned medium; and phytohemagglutinin-stimulated leukocyte-conditioned medium. At the end of culture, colony types were determined by dual esterase staining in permanent preparations. The majority of the colonies formed in acute myeloblastic and acute promyelocytic leukemias were neutrophilic even when stimulated by phytohemagglutinin-stimulated leukocyte-conditioned medium, which contains a potent stimulator of macrophage colony growth from normal marrow cells. On the other hand, both neutrophilic and monocytic colonies were formed in acute myelomonocytic leukemia (AMMoL). The proportions of these two types of colonies were variable, depending on the nature of added colony-stimulating factor and its concentration. These findings suggest that the leukemic progenitors in acute myeloblastic and acute promyelocytic leukemias have a tendency to differentiate mainly into a neutrophilic lineage *in vitro* and that the leukemic progenitors in AMMoL differentiate into both neutrophilic and monocytic lineages *in vitro*. In addition, in two cases of esterase-negative AMMoL, both neutrophilic and monocytic colonies were detected as in the other well-defined cases of AMMoL. This study seems to be of value in understanding the nature of leukemic progenitor cells and also shows that morphological analysis of leukemic colonies may be helpful in the classification of acute nonlymphocytic leukemia.

INTRODUCTION

In vitro cloning techniques have shown that the bone marrow and peripheral blood of patients with ANLL⁴ contain progenitor cells capable of forming colonies in culture (5, 19, 22, 24, 26). These colonies were considered to be leukemic in origin for a number of reasons, including the fact that cells within the colonies remained blast-like in morphology (5, 20) and that chromosomal markers characteristic of leukemia clones were identified in some colonies (10, 13, 21). Up to the present, several important properties of leukemic progenitor cells have been revealed: (a)

leukemic progenitors are capable of self-renewal as determined by replating assay (4, 7); and (b) a high proportion of the progenitors are in the S phase of the cell cycle (20).

The *in vitro* differentiation of leukemic progenitor cells may merit investigation. Marie *et al.* (18) showed that differentiation events occurring during leukemic colony formation are abnormal. However, cytochemical studies on cells constituting colonies have not been performed sufficiently. In the present study, we have examined the effects of 2 different CSF (HPCM and PHA-LCM) on leukemic differentiation in various types of ANLL, including AML, APL, and AMMoL. Using our new methods, which made it possible to obtain permanent preparations from methylcellulose cultures (23), colony types were compared with normal myeloid colonies cultured similarly to leukemic colonies.

MATERIALS AND METHODS

Patients. Twenty-eight patients with ANLL were studied (Table 1). The type of leukemia was classified according to the clinical data and cytochemical findings obtained by dual esterase staining (17). The cells from AML and APL showed positive naphthol AS-D chloroacetate esterase reaction and little or no α -naphthylbutyrate esterase reaction. Both chloroacetate esterase-positive cells and butyrate esterase-positive cells were detected in AMMoL. In Cases 22 and 26, however, the diagnosis was based on the findings of Wright-Giemsa staining, because the leukemic cells did not show positive esterase reaction.

Leukemic Colony Formation. Peripheral blood and/or bone marrow specimens were obtained from the patients at the time of diagnosis or relapse prior to treatment. Leukemic colonies were formed according to the method of Minden *et al.* (19) with minor modifications. Mononuclear cells were isolated by Ficoll-metrizoate (Lymphoprep; Nyegaard, Oslo, Norway) density centrifugation at $400 \times g$. In order to avoid the formation of T-lymphocyte colonies, sheep E-rosette-forming cells were subsequently removed by the technique described by Minden *et al.* (19). About 2% or less of E-rosette-positive cells were left after this rosetting technique. Cells from the E-rosette-negative fraction were plated in 35-mm Petri dishes (Lux Scientific Corp., Newbury Park, Calif.) at a concentration of 1×10^5 cells/ml in 0.8% methylcellulose (Dow Chemical Co.) in α -medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 20% fetal calf serum (Flow Laboratories) with or without 10% CSF. Two different conditioned media were used as the sources of CSF in this study. PHA-LCM was obtained from the supernatants of cultured leukocytes (1×10^6 cells/ml) incubated for 7 days in α -medium with 10% fetal calf serum and 1% phytohemagglutinin (Wellcome HA-15). HPCM was prepared according to the method of Burgess *et al.* (6). Culture plates were incubated for 7 to 9 days at 37° on a fully humidified atmosphere containing 5% CO₂ in air. Colonies containing 20 or more cells were counted with an inverted microscope. Cells within a part of the colonies were pooled and tested for E-rosette formation.

Normal Myeloid Colony Formation. Bone marrow specimens were obtained from normal volunteers after written informed consent. Cell preparation (E-rosette-positive cell depletion) and culture were performed by the same method as for leukemic colony formation.

Morphological Examination. Morphological examination was performed by the membrane filtration technique, a new method for obtaining

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⁴ The abbreviations used are: ANLL, acute nonlymphocytic leukemia; CSF, colony-stimulating factor(s); HPCM, human placental conditioned medium; PHA-LCM, phytohemagglutinin-stimulated leukocyte-conditioned medium; AML, acute myeloblastic leukemia; APL, acute promyelocytic leukemia; AMMoL, acute myelomonocytic leukemia; E-rosette, erythrocyte rosette; N/M, neutrophil/macrophage; AMoL, acute monocytic leukemia.

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Table 1
Clinical data and colony-forming ability in 28 cases of ANLL

Case	Diagnosis	Clinical status	Sample	NCC ^a ($\times 10^6/\mu\text{liter}$)	Blasts (%)	No. of colonies/ 1×10^5 cells			Clinical response to chemotherapy
						(-)	HPCM	PHA-LCM	
1	AML	U	BM	41.2	79	0	169	767	U
			PB	119.6	69	0	135	1147	
2	AML	R	PB	4.5	35	ND	ND	750	F
3	AML	U	PB	30.4	27	0	627	60	CR
4	AML	U	PB	30	57	0	137	456	CR
5	AML	R	PB	13.6	94	0	0	261	CR
6	AML	U	PB	25.1	29	0	81	115	U
7	AML	U	PB	13.7	90	0	23	8	CR
8	AML	U	BM	174	84	0	83	8	PR
9	AML	U	BM		78	0	0	0	F
10	AML	U	BM	245	98	0	0	0	CR
11	AML	U	PB	131.5	80	0	0	0	CR
12	AML	R	PB	7.7	88	0	0	0	CR
13	AML	U	PB	78.1	97	0	0	0	CR
14	AML	U	PB	126.8	95	0	0	0	CR
15	APL	U	BM		92	0	265	426	F
16	APL	U	BM	294	89	0	27	78	CR
			PB	20.2	73	0	69	52	
17	APL	U	PB	1.5	70	ND	59	ND	CR
18	APL	U	PB	8.5	76	0	0	T	F
19	APL	U	BM		72	0	0	0	F
20	AMML	U	BM	293	89	196	1717	523	U
			PB	65.2	86	1011	2465	1654	
21	AMML	U	BM	840	94	370	391	652	F
			PB	117.6	87	180	1248	1068	
22	AMML	U	BM	305	42	780	1560	1860	PR
23	AMML	U	BM	651	96	0	580	344	U
			PB	308.4	94	0	453	868	
24	AMML	R	PB	6.0	75	5	84	340	F
25	AMML	R	BM	47	40	121	311	73	PR
26	AMML	U	BM		99	0	148	28	CR
27	AMML	U	PB	34.8	49	0	0	T	CR
28	AMML	U	BM		62	0	0	0	CR

^a NCC, nuclear cell count; (-), no stimulator; U, untreated case; BM, bone marrow; U, undetermined due to early death; PB, peripheral blood; R, case in relapse; ND, not done; F, failure; CR, complete remission; PR, partial remission; T, E-rosette-positive cells were observed in the pooled colonies.

permanent preparations of cells cultured in methylcellulose medium (23). The preparations obtained by this technique were stained with Wright-Giemsa or dual esterase staining according to the method of Li *et al.* (17). If most of the cells in a colony were chloroacetate esterase-positive or butyrate esterase-positive, the colony was considered a neutrophilic or monocytic (monocyte/macrophage) colony, respectively. If a colony consisted of both chloroacetate esterase-positive cells and butyrate esterase-positive cells, then the colony was considered a N/M mixed colony. Esterase reaction of leukemic colonies was often weak and sometimes deficient. The former were classified as similar to normal myeloid colonies, while the latter ones (*i.e.*, those that are deficient) seemed to be mostly blastic colonies on the basis of the results of Wright-Giemsa staining. Almost all of the esterase-negative colonies formed in normal bone marrow cultures were eosinophilic colonies as determined by Biebrich-Scarlet staining (15).

RESULTS

Twenty-eight cases of ANLL were studied for leukemic colony-forming ability using the methylcellulose culture system with or without exogenous CSF. As summarized in Table 1, colonies were formed in 8 of 14 cases of AML, in 3 of 5 cases of APL, and in 7 of 9 cases of AMML. In general, colony-forming ability was higher in AMML than in AML and APL cases. Leukemic cells in these AMML cases were relatively immature. Case 28, in which colony growth was not detected, was differentiated type AMML. In several AMML cases (Cases 20, 21, 22, 24, and 25), colony formation was observed even in the absence of exogenous CSF. These spontaneous colonies were still observed after phagocyte depletion by means of the carbonyl iron

technique in 2 (Cases 20 and 22) of 3 (Cases 20, 21, and 22) AMML cases examined (data not shown). In the AML and APL cases, colony formation required the presence of CSF. Apparent correlation was not observed between colony-forming ability and remission rate.

Morphology of Normal Myeloid Colonies. To compare normal myeloid with leukemic colonies, normal human bone marrow cells were cultured under the same conditions as were those for leukemic colony formation. Normal marrow cells yielded mainly neutrophilic colonies on Day 8 when stimulated by HPCM, and many macrophage and eosinophilic colonies besides neutrophilic ones were observed when stimulated by PHA-LCM (Table 2). At a later culture period, *e.g.*, 14 days, several macrophage and eosinophilic colonies were formed also by HPCM. Therefore, cultures were examined on Days 7 to 9, when the differences of these 2 stimulators were clear-cut.

Table 3 shows the relationship between the number and the cellular composition of colonies and the concentrations of PHA-LCM. Proportions of macrophage colonies, N/M mixed colonies, and eosinophilic colonies increased along with increasing concentrations of PHA-LCM, whereas neutrophilic colonies decreased.

Morphology of Leukemic Colonies. Cells within the colonies formed in leukemic patients were generally immature in morphology when examined in Wright-Giemsa-stained preparations compared with the cells in normal myeloid colonies. Interestingly, most of the colonies formed in APL cases consisted of immature cells with abundant coarse granules, characteristic of this type of leukemia (23). However, some degrees of maturation of cells

Table 2
Types of colonies

		HPCM					PHA-LCM						
Sample		No. of colonies/ 10 ⁵ cells	% of colonies					No. of colonies/ 10 ⁵ cells	% of colonies				
			N ^a	NM	M	Eo	E (-)		N	NM	M	Eo	E (-)
Normal	BM ^b	1	344	99	1	0	0	682	36	10	12	42	
Normal	BM	2	317	98	0	1	1	537	71	2	13	14	
Normal	BM	3	264	99	0	0	1	406	59	3	14	24	
Normal	BM	4	220	93	0	4	3	325	42	8	18	32	
1	AML		169	100	0	0	0	767	99	0	0	1	
2	AML		ND			ND		750	96	2	2	0	
3	AML		627	100	0	0	0	60	98	0	2	0	
4	AML		137	100	0	0	0	456	91	7	1	1	
6	AML		81	100	0	0	0	115	61	2	1	36	
7	AML		23	100	0	0	0	8	100	0	0	0	
8	AML		83	100	0	0	0	8	0	0	100	0	
15	APL		265	100	0	0	0	426	100	0	0	0	
20	AMML		2465	93	3	2	2	1654	17	9	62	12	
21	AMML		1248	77	12	11	0	1068	38	44	13	5	
22	AMML		1560	9	0	3	88	1860	1	0	14	85	
23	AMML		453	98	0	0	2	868	19	3	6	72	
24	AMML		84			ND		340	46	1	11	42	
26	AMML		148	37	3	43	17	28	2	2	39	57	

^a N, neutrophilic colonies; NM, N/M mixed colonies; M, monocyte/macrophage colonies; Eo, eosinophilic colonies; E (-), esterase-negative colonies; ND, not done.

^b E-rosette-negative mononuclear cells obtained from normal bone marrow.

Table 3
Relationship between the number and the cellular composition of colonies and the concentrations of PHA-LCM

Sample		Stimulator	%	No. of colonies/ 1 × 10 ⁵ cells	% of colonies				
					N ^a	NM	M	Eo	E (-)
Normal	BM ^b	1	PHA-LCM	5	446	50	7	12	31
				10	682	36	10	12	42
				20	602	24	11	16	49
Normal	BM	4	PHA-LCM	5	299	61	5	12	22
				10	325	42	8	18	32
				20	351	19	17	27	37
20	AMML		PHA-LCM	5	1705	29	9	53	9
				10	1654	17	9	62	12
				20	1637	6	2	80	12
21	AMML		PHA-LCM	5	1200	57	33	5	5
				10	1068	38	44	13	5
				20	924	26	53	15	6

^a N, neutrophilic colonies; NM, N/M mixed colonies; M, monocyte/macrophage colonies; Eo, eosinophilic colonies; E (-), esterase-negative colonies.

^b E-rosette-negative mononuclear cells obtained from normal bone marrow.

were often observed after culture in many cases. In some AML cases, most of the colonies consisted of maturing cells such as myelocytes and metamyelocytes. Mature macrophage colonies as well as immature monocytoic colonies were observed in some AMMoL cases. The extent of *in vitro* leukemic cell maturation was different from patient to patient. Cytochemical reactivity of cells was also stronger after culture than that seen upon direct marrow examination. Therefore, colony types were further confirmed by dual esterase staining (Table 2). In several cases (Cases 6, 20, 22, 23, 24, and 26), a considerable number of esterase-negative blastic colonies were observed. The growth of these blastic colonies was more prominent in cultures of AMMoL cells stimulated by PHA-LCM than that in AML. Furthermore, neutrophilic colonies stimulated by PHA-LCM showed weaker chloroacetate esterase reaction than did those stimulated by HPCM in most cases of both AML and AMMoL, although this finding was also observed in normal myeloid colonies (data

not shown).

In contrast to normal bone marrow cultures, leukemic progenitors in AML and APL formed mostly immature neutrophilic colonies after 7 to 9 days of culture even when stimulated by PHA-LCM, except for Case 8. In some AML cases (especially differentiated type), however, many scattered macrophages were observed in cultures as background cells. On the other hand, leukemic progenitors in AMMoL formed neutrophilic, monocytic, and N/M mixed colonies in the presence of PHA-LCM. In Cases 21 and 26, a significant number of monocytic colonies were formed even when stimulated by HPCM. The percentages of monocytic colonies were much higher when stimulated by PHA-LCM than by HPCM, and increased with increasing concentrations of PHA-LCM as observed in normal bone marrow cultures (Table 3).

In Cases 22 and 26, the leukemic cells prior to culture did not show positive esterase reaction, but some colonies obtained

from these cases were positive for esterases. These colonies showed weaker esterase reaction than did those in the other cases, or only a part of the cells in the colonies were esterase positive.

DISCUSSION

The ability of several different CSFs to stimulate leukemic colony growth has been reported by several authors. These CSFs include PHA-LCM (5, 16, 19, 24), conditioned media from a T-cell hairy cell leukemia cell line (16, 19), HPCM (12, 27), conditioned media from a giant-cell tumor cell line (3, 16), and leukocyte feeder layers (12, 22, 26). However, comparisons of the type of leukemic colonies formed by different stimulators have not been reported. We examined the cellular composition of colonies using our new technique for morphological examination of cells cultured in methylcellulose medium (23).

In the analysis of the leukemic colonies by dual esterase staining, many esterase-positive colonies were observed in our culture system unlike those in the reports of Marie *et al.* (18). The reason for this difference is uncertain but may be due in part to different culture conditions. The observation that PHA-LCM tends to stimulate more immature (with weaker esterase reaction) colony formation than does HPCM may be important, because only PHA-LCM was used by Marie *et al.* as the stimulator of leukemic colony growth.

Another difference in the 2 conditioned media used by us was shown in the normal bone marrow cultures. While HPCM stimulated mainly neutrophilic colony formation, PHA-LCM stimulated the formation of many macrophage and eosinophilic colonies besides neutrophilic ones after 7 to 9 days of culture of normal marrow cells.

It is of interest that leukemic progenitors in AML and APL formed mostly immature neutrophilic colonies even when stimulated by PHA-LCM which contains a potent stimulator of macrophage colony growth from normal marrow cells. This may mean that AML and APL leukemic progenitors have a tendency to differentiate into a neutrophilic lineage *in vitro* regardless of the nature of added CSF.

In AMMoL, both types of colonies were formed when stimulated by PHA-LCM and, in some cases, even when stimulated by HPCM. The proportion of these 2 types of colonies was variable, depending on the nature of added CSF and its concentration. These findings suggest that both leukemic progenitors of neutrophilic and monocytic lineages may exist in AMMoL and that some of the leukemic progenitors in AMMoL may be bipotent and differentiate into either neutrophilic or monocytic lineages depending on the culture conditions.

AMoL was not included in our study. Taetle and Ivor (28) reported that colonies obtained from 2 cases of AMoL consisted of monoblasts and immature macrophages. Their study suggests that AMoL leukemic progenitors have a tendency to differentiate into a monocyte/macrophage lineage *in vitro*. It should be of interest whether monocytic colonies would be also predominant even when stimulated by HPCM which is a potent stimulator of neutrophilic colony growth from normal marrow cells.

In 2 cases of esterase-negative AMMoL, the diagnosis was confirmed after culture, since both neutrophilic and monocytic colonies positive for esterases were detected as in the other well-defined cases of AMMoL. Therefore, analysis of leukemic

colonies may be helpful in the classification of ANLL.

In contrast to our present data, human acute promyelocytic leukemia cell line (HL-60) has been reported to differentiate into macrophage lineage not only by 12-*O*-tetradecanoylphorbol-13-acetate, but also by the T-lymphocyte-conditioned media (8, 11). However, the nature of HL-60 cells seems to be different from that of fresh cases of APL, since not only chloroacetate esterase-positive cells but also a significant number of butyrate esterase-positive cells were detected even in the uninduced HL-60 cell line.⁵

In our present study, the *in vitro* cloning and morphology data were not correlated with subsequent clinical response such as remission rate. However, the radiosensitivity and cell cycle status of leukemic progenitor cells seemed to be related to the degree of cyto-reduction after chemotherapy.⁶

Recently, several chemical compounds have been reported to affect the differentiation of normal and abnormal myeloid cells *in vitro* (1, 2, 9, 14, 25). Studies are in progress to investigate further the effect of these chemical compounds on the differentiation of leukemic progenitor cells as well as CSF presented in this paper.

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