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Studies of Leukemic Cell Populations in Culture

By M. T. Aye, Y. Niho, J. E. Till, and E. A. McCulloch

A suspension culture technique for the study of peripheral blood cells from patients with leukemia is described. Cells from patients in relapse were preserved by freezing with dimethylsulfoxide at -70°C to ensure the availability of the same population of cells for repeated experiments of varying designs. Cells from one AML patient proliferated only in the presence of phytohemagglutinin (PHA) or leukocyte-conditioned medium (LCM). Cells taken at a later date, after commencement of chemotherapy, failed to proliferate in response to PHA, although

the responsiveness to LCM was retained. Proliferation of the cells obtained after chemotherapy could also be stimulated by culture supernatants from the original PHA-responsive cells. Eight other patients studied showed varying responses to LCM and PHA. It is proposed that cells from leukemic patients are made up of two interacting populations, one which produces stimulatory factors and another which responds to these factors by proliferation. Delineation of these two populations and the factors produced may provide a better understanding of leukemic cell proliferation.

PREVIOUSLY WE REPORTED that the growth of leukemic cells in suspension culture is dependent on the presence of either feeder layers of leukemic cells or conditioned media derived from them.¹ The study was limited to three patients with unusual features, and detailed analysis of these was impossible because in each patient peripheral blood ceased to be available during the course of the disease. In the present paper, we describe an extension of the method to blast cells from nine additional patients with acute leukemia; we found that these cells retained their growth potential after freezing at -70°C in the presence of 5% dimethylsulfoxide. Thus, large populations of cells were available for repeated experiments of varying designs, and it was feasible to compare cells from different patients under identical conditions. Detailed studies were made of peripheral blood cells from a single patient with acute myeloblastic leukemia; these served as a guide for investigation of peripheral blood leukocytes from eight other patients. Considerable patient-to-patient variation was found, both in kinetics of growth and requirement for exogenous stimulation.

MATERIALS AND METHODS

Patients With Leukemia

Table 1 contains the clinical diagnosis and hematologic findings for nine patients with leukemia included in the study. In each instance, blood was obtained either by venipuncture or following leukopheresis using an IBM cell separator.² Cells from one patient, designated TEG, were

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Table 1. Hematologic Data on Leukemic Patients

Patient	Diag- nosis	Hemo- globin (g/100 ml)	WBC × 10 ⁻³	Peripheral Leukocyte Differential (%)										Bone Marrow Differential (%)							
				Blasts	Promyelocytes	Myelocytes	Bands	PMN†	Lymphocytes	Erythroblasts	Monocytes	Eosinophils and Basophils	Blasts	Promyelocytes	Myelocytes	Bands	PMN	Erythroblasts	Lymphocytes	Eosinophils and Basophils	
TEG-1†	AML	5.5	27.7	81	—	1	—	2	14	1	—	1	80	11	—	—	2	1	5	2	
TEG-2	AML	7.5	60	90	2	—	—	1	7	—	—	—	—	—	—	—	—	—	—	—	
BAR§	CML-B¶	12	51	6	1	20	59	3	7	—	3	4	>90	—	occ	occ	occ	—	—	—	
CLA	AML	7.3	156	91	—	—	—	3	6	—	—	—	97	—	—	—	—	3	—	—	
BID	AML	6.8	151	91	—	—	—	—	8	—	1	—	—	—	—	—	—	—	—	—	
MON	AML	10.3	85.5	99	—	—	—	—	1	—	—	—	95	—	—	—	—	4	1	—	
ING	CML-B¶	8.4	246	72	16	—	—	4	2	—	2	4	76	16	7	—	—	—	1	—	
SCO§	AML	9.4	21	91	—	—	—	—	—	—	—	9	91	4	2	—	—	2	1	—	
FER	AMML	9	72	30	—	17	30	10	10	—	11	2	66	7	17	2	6	2	—	—	
MAL	AML	11.2	43.4	67	7	11	6	9	9	—	—	—	94	5	—	1	—	—	—	—	

* 200 cells were counted.

† PMN denotes polymorphonuclear leukocytes.

‡ Bone marrow aspiration was done on admission only.

§ Samples obtained by leukapheresis.

¶ Blast crisis of chronic myeloid leukemia.

|| Bone marrow aspiration unsuccessful and only histologic section available which showed the marrow packed with blasts.

available in quantity and studied extensively. TEG was a 48-yr-old man who presented with a 6-wk history of sore throat, daily fever, shortness of breath, and easy bruising. Examination of peripheral blood and bone marrow confirmed the diagnosis of acute myeloblastic leukemia. Cells were obtained from TEG on two occasions, separated by an interval of 9 days. During this period he received treatment with rubidomycin, cytosine arabinoside, 6-mercaptopurine, and prednisone. Cells taken before treatment were designated as TEG-1 and those taken after treatment as TEG-2. Cells obtained at both bleedings were stored at -70°C (see below).

Preparation of Conditioned Medium From Peripheral Blood Leukocytes (LCM)

Leukocyte-conditioned medium (LCM) was prepared from cultures of peripheral blood cells obtained either from volunteer donors or from patients with leukemia. Two preparation methods were used. The first (method 1) was that described by Iscove et al.,³ in which culture medium with 20% fetal calf serum (FCS) was layered over peripheral leukocytes immobilized in agar and incubated for 7 days. A second (method 2) was devised to compare LCM preparations made in the presence or absence of PHA; peripheral leukocytes in suspension at 10^6 cells/ml with 20% FCS and with α medium (Flow Laboratories),⁴ with or without 1% (v/v) phytohemagglutinin (PHA [HA15], Wellcome Research Labs., Beckenham, England), were incubated in 250-ml Falcon tissue culture flasks (3024) at 37°C in an incubator flushed with 5% CO_2 and air. Supernatants from these cultures were harvested by centrifugation after 7 days of incubation. LCM prepared by either method was sterilized by filtration through Nalgene 0.45- μ filters.

Granulocytic Colony Formation in Culture by Marrow Cells

Marrow was obtained from patients in the course of investigation for hematologic conditions other than leukemia. These specimens were used as a source of granulopoietic progenitors recognized by their capacity to form colonies in culture in the presence of LCM containing colony-stimulating activity (CSA).³ Cells obtained from buffy-coat preparations were plated in methylcellulose with 20% FCS and α medium, with or without 20% LCM. After 12–14 days of incubation, colonies containing in excess of 20 cells were scored using an inverted microscope. Neutrophilic and eosinophilic colonies were enumerated. The counting criteria and the reasons for excluding macrophage colonies have been discussed in detail elsewhere.⁵

Preparation of Nonadherent (NA) Marrow Cells

Nonadherent (NA) marrow cells were prepared from marrow cell suspensions by the technique described by Messner et al.⁵ Marrow suspensions were incubated in glass Petri dishes for $\frac{1}{2}$ hr, cells not adhering removed and incubated for a further $2\frac{1}{2}$ hr at 37°C . Cells decanted from this second step of adherence were considered to be NA cells. Such cell populations respond to LCM containing CSA by producing granulocyte colonies but usually have little or no colony-forming capacity in the absence of CSA. Further, colony formation is dependent on concentration of CSA in the cultures, and accordingly NA cells are useful in assessing the CSA in various preparations of LCM.

Suspension Culture of Peripheral Blood Cells From Patients With Leukemia

The suspension culture technique as originally described¹ depended on cell counts performed at the initiation of cultures and after 10 days of incubation. In the present study, the method was modified by substituting incorporation of tritiated thymidine into trichloroacetic acid-insoluble material for cell counts. Leukocytes at concentrations from $5 \times 10^4/\text{ml}$ to $2 \times 10^5/\text{ml}$ were suspended in 3 ml of α medium (minus nucleosides) and 20% FCS, with or without LCM. Duplicate or triplicate cultures were always used, and the values for each determination are shown in the figures, where appropriate.

Preliminary experiments established the conditions for pulse labeling of the cultures with tritiated thymidine ($^3\text{HTdR}$). The concentration of thymidine that yielded maximum incorporation of $^3\text{HTdR}$ at a constant specific activity was found to be $2.5 \times 10^{-6} M$ and was achieved by adding 0.2 ml of $^3\text{HTdR}$ at specific activity of 5 Ci/mmol (and 0.2 mCi/ml) to each 3-ml culture. A pulse-labeling period of 2 hr was found to be on the linear portion of the

curve relating counts per minute in acid-insoluble material to the duration of the pulse. Accordingly, these conditions were used in subsequent experiments. After the pulse, the cells were centrifuged at 150 *g* for 15 min at 4°C, and the cell pellet was resuspended in saline and washed through a Whatman GF/c glass fiber filter paper (Canadian Laboratories, Toronto). The filter was washed twice with 5% cold trichloroacetic acid (TCA) and then twice with cold absolute methanol. The filter papers were transferred into glass scintillation vials, dried in an oven, and counted in a Unilux T.M. II liquid scintillation counter (Nuclear-Chicago) using a commercially available toluene-based scintillation fluid (Omnifluor, New England Nuclear, Boston, Mass.). The results are expressed as counts per minute per culture.

Stimulation With Phytohemagglutinin (PHA)

The effect of phytohemagglutinin (PHA) on suspension cultures of peripheral leukocytes from patients with leukemia was determined by adding the lectin (obtained from Wellcome Research Labs., Beckenham, England) at various concentrations. The suspension contained between 5×10^4 and 2×10^5 cells/ml in 20% FCS with α medium. The cultures were incubated for varying lengths of time and $^3\text{HTdR}$ incorporation determined using the pulse-labeling technique described above.

Cell-freezing Procedure

Peripheral blood with 5 U of heparin per milliliter as anticoagulant was sedimented at unit gravity for 40 min, and the supernatant plasma, containing leukocytes, was spun and washed once in α medium. The nucleated cells were resuspended at a concentration of $2-4 \times 10^7$ /ml in α medium containing 10% FCS and 5% dimethylsulfoxide (Fisher Scientific Co., Toronto). This cell suspension was then aliquoted in 3-4-ml amounts in 5-ml Falcon plastic test tubes (2005) and placed directly into a freezer (Kelvinator Series 100) at -70°C. When required, tubes were removed and thawed at 37°C in a water bath. The cell suspension from one tube was mixed with 40 ml of 20% FCS in α medium in a 50-ml centrifuge tube at 4°C. After centrifugation at 150 *g* at 4°C for 15 min, the supernatant was removed and the cell pellet resuspended in α medium. The cells were maintained at 4°C until placed in suspension culture. This procedure usually yielded a recovery of both nucleated cells and capacity for thymidine incorporation of 60%-80% of values before freezing.

Fractionation of LCM on Sephadex (G-75)

LCM from cultures containing cells of nonleukemic individuals was concentrated ten times using an Amicon stirred ultrafiltration cell with a UM 20E diaflo ultrafilter. An aliquot of this concentrated material was loaded on a Sephadex G-75 column (Pharmacia K 16/40) and eluted using a 0.015 *M* potassium phosphate buffer, pH 7.2, containing 0.1 *M* NaCl. Elution was done at a flow rate of 15 ml/hr, and fractions of 3 ml were collected.

Cell Separation by Velocity Sedimentation

The velocity sedimentation procedure (Staput) described by Miller and Phillips⁶ was used to fractionate populations of peripheral blood cells from patients with leukemia. In brief, 10^8 nucleated cells were layered on top of a shallow gradient of human serum albumin and allowed to sediment at unit gravity. After approximately 4 hr, fractions were collected from the bottom of the sedimentation vessel. Constant volumes were cultured from each fraction and the results of pulse labeling expressed as cpm per culture.

Radioautography

Cells were pulsed with $^3\text{HTdR}$ in the usual way, and then applied to glass slides using a cyto-centrifuge (Shandon). After fixation in methanol, the slides were washed for 30 min each in (1) 5% TCA containing 1% sodium pyrophosphate, (2) 5% TCA, and (3) running tap water. After coating with Kodak NTB2 emulsion, the slides were stored in a light-proof container at 4°C for 7 days. They were then processed at 17°C in Kodak Dektol developer for 2 min, Kodak fixer

for 5 min, water for 5 min, and stained with May-Grünwald-Giemsa. Labeling indices were calculated from counts made on 500 cells.

Irradiation

Aerated cells were irradiated at concentrations between 10^5 and 5×10^5 cells/ml in a ^{137}Cs irradiator designed by Cunningham, Bruce, and Webb.⁷ The dose rate was 96 rads/min. During irradiation the tube of cells was kept in a vessel containing crushed ice.

RESULTS

Response of TEG Cells to Conditioned Medium

Peripheral blood cells from patient TEG, obtained at two separate bleedings separated by treatment with chemotherapy (TEG-1 and TEG-2), were studied extensively. These cell suspensions were incapable of granulocytic colony formation in culture. Accordingly, a suspension culture method was used. All experiments employed cells preserved by freezing (see Materials and Methods).

First, experiments were carried out to test for an effect of leukocyte-conditioned medium (LCM) derived from cultures of normal leukocytes on TEG cells in suspension culture. The cells were cultivated with and without 20% LCM as described in Materials and Methods. At daily intervals cells were pulse labeled with $^3\text{HTdR}$ and incorporation assessed. The results of typical experiments with TEG-1 and TEG-2 cells are shown in Fig. 1. In the presence of LCM, $^3\text{HTdR}$ incorporation increased for 2 days and then declined slowly; in the absence of LCM, $^3\text{HTdR}$ incorporation decreased. The kinetics of response were similar for TEG-1 and TEG-2, and on this basis $^3\text{HTdR}$ incorporation by TEG cells was assessed on either day 2 or day 3 in subsequent experiments.

As described in Materials and Methods, the α medium used in these experiments was without nucleosides. As a control, kinetics were also measured in the presence of increasing concentrations of nucleosides; while this decreased

Fig. 1. The kinetics of response of $^3\text{HTdR}$ incorporation by TEG-1 (circles) and TEG-2 (crosses) to 20% normal leukocyte-conditioned medium (LCM). Responses in the absence of LCM are also shown. The value for each replicate culture is shown individually.

Fig. 2. TEG-2 cells cultured at 1×10^5 /ml with 20% normal LCM. Kinetics of $^3\text{HTdR}$ uptake and number of labeled cells per culture. The mean values for replicate cultures are shown.

the specific activity of the $^3\text{HTdR}$ in the pulse, and hence the total uptake, the rate of change in incorporation was not affected. Therefore, factors other than incorporation into DNA (for example, pools of nucleosides) were not responsible for the observed effects.

As a further control, $^3\text{HTdR}$ radioautographs were prepared at various times during the culture of TEG-2 cells. The results are given in Fig. 2, and it is evident that the number of labeled cells increased in parallel with $^3\text{HTdR}$ incorporation, while no increase in total nucleated cell counts was observed. In the initial radioautographs (time 0) the labeled cells had the appearance of leukemic blasts, with large nuclei and pale-blue cytoplasm. After 2 days in culture, the morphology was variable; the nuclei were round or oval, and cytoplasmic pseudopods were seen often. However, within the limits of morphologic criteria, no evidence was obtained that the cells labeled at the end of the culture were different from those labeled at the onset. No evidence of cellular differentiation was observed in the cultures.

Figure 3 shows the results of an experiment in which various concentrations of TEG-1 cells were exposed to two different preparations of LCM. For each of the concentrations, the kinetics of response to LCM were similar to those of Fig. 1 (data not shown), but $^3\text{HTdR}$ incorporation was cell-concentration dependent. The rate of incorporation of $^3\text{HTdR}$ increased in nearly linear fashion with increasing cell concentrations up to 10^5 cells/ml; further increases in cell concentration yielded only modest increases in $^3\text{HTdR}$ incorporation. Accordingly, cell concentrations close to 10^5 cells/ml were used in subsequent experiments.

The concentration of LCM in the cultures also affected $^3\text{HTdR}$ incorporation by TEG cells. Figure 4 contains the results of an experiment showing the effect of varying concentrations of three different preparations of LCM on $^3\text{HTdR}$ incorporation by TEG-1 cells. It is apparent that the form of the dose-

Fig. 3. Effect of increasing numbers of TEG-1 cells cultured in the presence of 20% LCM from two normal volunteers. $^3\text{HTdR}$ incorporation assessed on day 3. The value for each replicate culture is shown individually.

response curve depended on the preparation of normal LCM under study; six of eight preparations studied at different dilutions yielded curves similar to those for preparations 437B and 255A of Fig. 4, reaching plateau values at a concentration near 20%. The other two preparations, like 439A, continued to yield increasing $^3\text{HTdR}$ incorporation with increasing concentration. However, measurements at 20% LCM provided an approximate ranking of the potency of the preparations, and this concentration was used in subsequent experiments.

Radiation Sensitivity of $^3\text{HTdR}$ Incorporation by TEG Cells

In an attempt to assess the contribution of cellular proliferation to $^3\text{HTdR}$ incorporation by TEG cells, radiation survival curves were constructed. Cells were irradiated at time 0, cultivated in the presence of LCM for 3 days, and

Fig. 4. TEG-2 cells cultured at 1×10^5 /ml in the presence of increasing concentrations of three different preparations of normal LCM. $^3\text{HTdR}$ incorporation assessed on day 3. The value for each replicate culture is shown individually.

Fig. 5. Radiation survival curves for capacity of TEG cells to incorporate $^3\text{HTdR}$. Open symbols are from three experiments in which cells were irradiated on the day cultures were initiated. Closed squares are from an experiment performed simultaneously with that indicated by open squares, except that the cells were irradiated on day 3, 2 hr before a pulse of $^3\text{HTdR}$. Values for the per cent survival were calculated for each of two replicate irradiated and unirradiated cultures. The largest and smallest of the four possible ratios are shown in the figure.

then pulse labeled with $^3\text{HTdR}$ as described in Materials and Methods. The results of three experiments of this design are presented in Fig. 5; in addition, the figure contains the results of a control experiment in which the cells were irradiated on day 3, 2 hr before the pulse of $^3\text{HTdR}$.

For irradiation at time 0, the survival curve consisted of an initial rapid decrease in survival up to doses of 200 rads; the D_0 for this portion of the curve was approximately 50 rads. For doses in excess of 200 rads, survival decreased much more gradually; the D_0 for this portion of the curve was approximately 800 rads. For irradiation on day 3, a gradual exponential decrease in survival was obtained; the D_0 was similar to the less sensitive portion of the time 0 survival curve.

The survival curve obtained for irradiation of day 3, 2 hr before pulse labeling with $^3\text{HTdR}$, represents the radiosensitivity of thymidine incorporation by cells already in the DNA synthesis phase at the time of irradiation. The less sensitive component of the survival curve for irradiation at time 0 presumably has a similar basis. The initial portion of the same survival curve shows a higher radiosensitivity of a magnitude characteristic of cellular proliferation.⁸ If this interpretation is correct, a major part of the increase in $^3\text{HTdR}$ -incorporation stimulation by LCM resulted from cellular proliferation. These data, taken with the radioautographic results of Fig. 2, are consistent with the view that LCM stimulated proliferation in a specific subpopulation of leukemic cells.

Response of TEG Cells to PHA

The response of TEG cells to LCM is analogous to the response of peripheral blood leukocytes to phytohemagglutinin (PHA). To explore this analogy, the

Table 2. Response to PHA by TEG Cells at 1×10^5 Cells/ml

	Initial cpm per culture*	PHA Concentration Per cent (v/v)	cpm per culture†
TEG-1	1166	1	35,697
TEG-2	1467	3	128
		2	137
		1	197
		0.5	253
		0.1	228
		0	173

*³HTdR incorporation assessed on the day cultures were started.†³HTdR incorporation assessed on day 3.

response of TEG cells to PHA was assessed as described in Materials and Methods. The results obtained (Table 2) show that, although TEG-1 and TEG-2 respond similarly to LCM (Fig. 1), they respond differently to PHA. TEG-1 cells showed enhanced ³HTdR incorporation in the presence of 1% PHA. In contrast, TEG-2 cells failed to respond to PHA in doses from 0.1%–3%. A direct kinetic comparison of the effect of LCM and PHA on TEG-2 cells is given in Table 3; again it is evident that TEG-2 cells responded to LCM but not to PHA at all three times tested.

To test more rigorously for their ability to respond to PHA, TEG-2 cells were fractionated by velocity sedimentation (see Materials and Methods). Each fraction was assessed for its content of nucleated cells and its response in suspension culture to LCM and PHA. The results are shown in Fig. 6; it may be seen from the figure that a population able to respond to PHA was revealed by the cell-separation procedure. However, the response to PHA was still much less than to LCM. These data demonstrate that the PHA-response difference between TEG-1 and TEG-2 is probably quantitative; TEG-2 cell populations contain either fewer cells able to respond to PHA or cells less sensitive to stimulation by that lectin.

Conditioned Medium From PHA-treated Cell Populations Tested on TEG-2 Cells

Although TEG-1 cells responded well to both LCM and PHA (Fig. 1 and Table 2), it seemed possible that the response to PHA could have been, at least in part, an indirect result of the production of stimulatory factors by PHA-responsive cells in the TEG-1 population. Such factors could then behave like those in LCM and cause a stimulation of ³HTdR incorporation. As a test of this possibility, TEG-1 cells were exposed to 1% PHA for 7 days in suspension culture and the supernatants of these cultures tested for their ability to stimulate

Table 3. Response to PHA and LCM by TEG-2 Cells at 1×10^5 Cells/ml (cpm per culture)

Day	3	5	7
Control*	125	145	59
20% LCM†	10,077	9650	2737
1% PHA (v/v)	142	98	42

*Cultures without LCM or PHA.

†LCM prepared by method 1.

Fig. 6. Velocity sedimentation profiles for TEG-2 cells. Each fraction was assessed for concentration of nucleated cells and responsiveness to 20% LCM and 1% PHA. The value for each replicate culture is shown individually.

$^3\text{HTdR}$ incorporation by TEG-2 cells. The latter were used for this purpose since, in the absence of cell fractionation, they did not exhibit PHA-stimulated increased $^3\text{HTdR}$ incorporation. The results, given in Table 4, show that TEG-2 cells responded to LCM prepared from PHA-treated TEG-1 cells but not to PHA alone. As a control, LCM prepared from TEG-2 cells in the presence or absence of PHA was compared with PHA in respect to capacity to stimulate $^3\text{HTdR}$ incorporation by TEG-2 cells. The results, also presented in Table 4, show that PHA neither stimulated TEG-2 directly nor was able to induce the production of stimulator substances in medium conditioned by TEG-2 in the presence of the lectin.

A similar test was carried out using LCM prepared from normal peripheral leukocytes in the absence of agar as described in Materials and Methods. The results are presented in Table 5; it is evident that, for each of the four suspensions of normal leukocytes tested, the presence of PHA increased the activity, and in two instances LCM prepared in the absence of PHA was inactive.

Thus, both normal peripheral leukocytes and TEG-1 cells can respond to

Table 4. Autologous LCM and PHA Tested on TEG-2 Cells at 1×10^5 Cells/ml*

Source of LCM†	Test Material	Concentration Per cent (v/v)	cpm per culture
TEG-1	PHA	1	130
	LCM-1	20	125
TEG-1 and PHA	LCM-2	20	1256
	PHA	1	242
TEG-2	LCM-3	20	257
	LCM-4	20	289

* $^3\text{HTdR}$ incorporation assessed on day 3.

†LCM prepared by method 2.

Table 5. LCM Production* by Normal Cells in Response to PHA Tested on TEG-2 Cells†

	LCM Prepared With PHA	LCM Prepared Without PHA
Normal (1)	26,720	6,983
(2)	13,254	198
(3)	25,750	12,487
(4)	46,924	198

*LCM prepared by method 2.

†TEG-2 cells at 1×10^5 /ml were cultured with 20% LCM and $^3\text{HTdR}$ incorporation assessed on day 3.

PHA stimulation by producing factors that enhance $^3\text{HTdR}$ incorporation by unfractionated TEG-2 cells, although these cells do not respond to PHA alone.

Studies on Eight Other Patients With Leukemia

Cells from eight other patients with high peripheral blood blast concentrations were stored at -70°C and cultured after thawing. Table 6 contains the results obtained by culturing these populations with LCM or PHA and assessing growth by increased $^3\text{HTdR}$ incorporation. Considerable heterogeneity in response to LCM and PHA was seen. Cells from one patient (BAR) showed a pattern of response similar to that of TEG-1, that is, a decline in $^3\text{HTdR}$ incorporation in control cultures and a marked stimulation in cultures containing either LCM or PHA. Cells from five patients (CLA, BID, MON, SCO, and FER) showed increased $^3\text{HTdR}$ incorporation whether or not LCM or PHA were present; the addition of LCM yielded increases in uptake in cells from these patients and from ING, although always less marked than those observed for TEG-1 or BAR cells. Cells from three patients (SCO, FER, and MAL) responded well to PHA but less well to LCM. None of the other cell populations tested showed the response pattern characteristic of TEG-2, that is, marked stimulation by LCM but no stimulation by PHA.

Cells from each of these patients were used to prepare LCM in suspension

Table 6. Response of Peripheral Leukocytes* From Patients With Leukemia to LCM and PHA

Patient†	Initial $^3\text{HTdR}$ Uptake cpm per culture $\times 10^{-3}$	Peak Day	LCM		Peak Day	PHA	
			– LCM	+ LCM‡		– PHA	+ PHA§
			cpm per culture $\times 10^{-3}$	cpm per culture $\times 10^{-3}$		cpm per culture $\times 10^{-3}$	cpm per culture $\times 10^{-3}$
TEG-1	1.1	3	0.2	41.2	3	0.2	35.6
TEG-2	1.4	2	0.1	43.5	2	0.1	0.1
BAR	0.6	10	0.4	19.6	7	0.4	9.5
CLA	3.8	3	11.1	15.4	3	11.1	14.5
BID	0.8	7	2.7	5.4	2	2.2	1.9
MON	0.2	3	12.0	17.5	8	1.8	15.8
ING	1.1	7	1.2	4.4	7	1.2	2.3
SCO	1.0	3	4.7	6.4	7	1.6	67.5
FER	2.1	7	4.6	12.8	7	4.6	125.9
MAL	2.2	8	0.2	2.4	8	0.2	21.0

*Cells were cultured at 1×10^5 /ml.

†For clinical information see Table 1.

‡Normal LCM prepared by method 1 and used at a concentration of 20%.

§PHA was used at a concentration of 1%.

Table 7. LCM From Leukocytes of Patients With Leukemia Tested on TEG-2 Cells and NA Marrow Cells

Sources of LCM*	TEG-2 Cells† cpm per culture $\times 10^{-3}$	Colonies per 2×10^5 NA Marrow Cells‡
TEG-1	0.2	1
TEG-1 and PHA	2.5	14
BAR	0.4	2
BAR and PHA	33.8	37
CLA	1.5	12
CLA and PHA	24.2	43
BID	0.6	1
BID and PHA	48.4	25
MON	0.3	1
MON and PHA	48.8	68
ING	0.8	7
ING and PHA	46.6	40
SCO	0.3	0
SCO and PHA	46.8	60
FER	1.2	6
FER and PHA	39.1	15
MAL	0.5	0
MAL and PHA	45.4	42

*LCM prepared by method 2.

†TEG-2 cells were cultured at 1×10^5 /ml and $^3\text{HTdR}$ uptake assessed on day 3. TEG-2 cells cultured in the absence of LCM but with 1% PHA yielded 0.1×10^3 cpm per culture on day 3.

‡For method and assessment of colony growth see Materials and Methods. This NA marrow preparation yielded 0 colonies in the absence of LCM.

culture with or without PHA by the second LCM preparation procedure described in Materials and Methods. These LCM preparations were tested both for their capacity to stimulate $^3\text{HTdR}$ incorporation by TEG-2 cells and for their ability to enhance granulocytic colony formation by nonadherent cells from marrow obtained from patients without leukemia. It is evident from Table 7 that highly active preparations of LCM were obtained only from PHA-treated cultures; however, cells from all nine untreated patients responded to PHA by the production of stimulatory factors active on TEG-2 cells and nonadherent marrow cells. Neither TEG-2 nor NA marrow cells responded to PHA alone.

Fractionation of LCM

LCM preparations able to stimulate $^3\text{HTdR}$ incorporation by TEG cells were also found to contain colony-stimulating activity (CSA), detected by an enhanced frequency of colony formation by nonadherent cells from nonleukemic marrow (Table 7). A tendency for these two types of stimulatory activity to occur together in different preparations of LCM was observed repeatedly; it seemed possible that similar factors were responsible for both stimulatory activities. As an initial test of this possibility, LCM was fractionated by gel filtration on Sephadex G-75; each fraction was assessed both by $^3\text{HTdR}$ incorporation in suspension cultures of TEG cells and colony formation by nonadherent marrow cells. The results of an experiment, representative of four replicates, is shown in Fig. 7; similar elution profiles were obtained for the stimulatory activity detected by either assay procedure.

Fig. 7. Sephadex G-75 elution profiles for an LCM preparation. Fractions at a concentration of 20% (v/v) were assessed for their capacity to stimulate ^3H TdR incorporation by TEG cells and capacity to promote granulocytic colony formation by NA marrow cells. The value for each replicate culture is shown individually. (Representative of four separate experiments.)

DISCUSSION

The objective of the studies reported in this paper was to study factors influencing the proliferation of cell populations obtained from leukemic patients. The problem was approached using cell-culture methods since *in vivo* techniques have limited application in man. Unfortunately, cells from marrow or peripheral blood of many patients with acute leukemia in relapse have little or no capacity for colony formation in culture.⁹⁻¹⁵ Accordingly, a suspension-culture method was adopted for studies of leukemia in relapse. In a previous study,¹ chromosome marker data were obtained which indicated that leukemic cells proliferate in such cultures, and this finding formed the basis for the assumption that the suspension-culture method was detecting events occurring in leukemic populations. Morphologic criteria, in the absence of chromosome markers, are unsatisfactory for the identification of leukemic cells. However, morphologic studies were compatible with the view that leukemic cells were proliferating in the cultures. This hypothesis is strengthened by the data of Fig. 2; in this experiment TEG-2 cells were used, and, since these do not respond to PHA, the observed increase in labeled cells is unlikely to reflect blast transformation of normal lymphocytes.

Detailed studies were made of peripheral leukocytes obtained on two occasions from a single patient, TEG, with acute myeloblastic leukemia. The work concentrated on two properties of TEG cells. First, these cells were capable of DNA synthesis as assessed by ^3H -thymidine incorporation. DNA synthesis increased as a function of time in suspension culture provided the cultures were supplemented with medium conditioned by peripheral leukocytes (LCM). Studies of the radiation sensitivity of the increased ^3H -thymidine incorporation induced by LCM were compatible with the view that cellular proliferation was the major mechanism underlying the increase. If this was indeed the case, the peripheral blood of TEG contained a cell population incapable of

colony formation under standard conditions but able to proliferate when exposed to substances obtained from normal leukocytes. The method used to obtain LCM was identical to that used for the production of factors capable of stimulating granulocytic colony formation by cells from normal marrow (CSA).³ Indeed, LCM preparations active in stimulating ³H-thymidine incorporation by TEG cells were also found to contain potent CSA, and fractionation of the LCM by gel filtration through Sephadex G-75 failed to separate the two activities. It seems possible, therefore, that a class of cells in leukemic blood may be induced to proliferate by factors similar to those that stimulate colony formation in culture by normal granulopoietic progenitors. Extensively purified materials would be required for an experimental test of this hypothesis.

The second property of TEG cells that was examined was their capacity to condition medium, rendering it capable of stimulating both ³H-thymidine incorporation by TEG cells and granulocytic colony formation by nonadherent cells from nonleukemic marrow. The capacity to condition medium was found to be dependent on stimulation by phytohemagglutinin (PHA); thus, TEG peripheral blood contained a population of PHA-sensitive cells that responded to the lectin by producing stimulatory factors. This PHA-sensitive property was markedly decreased in TEG-2 cells, obtained after chemotherapy, compared with TEG-1 cells. The intervening chemotherapy may provide an explanation of the difference, since chemotherapy is known to suppress PHA responsiveness.¹⁶

PHA responsiveness by TEG cells was also assessed by observing increased ³H-thymidine incorporation in lectin-containing cultures. For TEG-1 cells, the response to PHA was of similar magnitude to the response to LCM; in contrast, TEG-2 cells responded well to LCM but not to PHA (Fig. 1, Tables 2, 3, 4) as measured either by increased ³H-thymidine incorporation or by capacity to condition medium. These results are compatible with the view that PHA-stimulated ³H-thymidine incorporation is indirect, resulting from increased production of stimulatory factors by cells in the TEG-1 population.

The data obtained from TEG cells were used as a guide for studies of the peripheral blood of eight additional patients, chosen because they had high blast cell concentrations in the peripheral blood. The results (Tables 6 and 7) showed great patient-to-patient variation. Indeed, the only consistent finding was that leukemic cell populations could respond to PHA by producing factors capable of stimulating cellular proliferation.

From the studies of TEG cells it was suggested that, for TEG-1 cells, ³H-thymidine incorporation after PHA might be an indirect consequence of PHA-stimulated "conditioning" of the medium. It is not known whether or not the production of stimulatory factors in conditioned medium is a property of the cells that respond by proliferation or of a second cell population. However, a precedent from studies of normal marrow⁵ and peripheral blood¹⁷ makes the latter possibility attractive. Both sources yield two separable populations that contribute to granulocytic colony formation in culture; one population consists of the cells of origin of the colonies, while the other contains cells that produce stimulatory factors. On the basis of this analogy, we advance the hypothesis that cells from patients with leukemia also consist of two interacting popula-

tions. From this point of view, the patient-to-patient variation found in our studies might represent variations in the numbers of cells in each population and the activity of the "factor-producing" population. If this two-population hypothesis proves valid, a meaningful description of the behavior of cells in leukemia may be achieved by assessing the numbers and activities of each population independently.

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