CONCISE REPORT

Production of Leukemic Blast Growth Factor by a Human Bladder Carcinoma Cell Line

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Circulating blast cells from the peripheral blood of acute myeloblastic leukemia patients include a subpopulation capable of colony formation in the presence of phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM). We describe the complete replacement in the blast assay of PHA-LCM by conditioned medium from a human bladder carcinoma cell line, HTB9. Both conditioned media contain a stimulator of blast cell growth that elutes as a single peak from a Sephadex G100 column with an apparent molecular weight of 30,000. It is shown that this leukemic blast growth factor is distinct from erythroid-potentiating activity (EPA) and possibly granulocyte macrophage colony-stimulating factor.

CLONOGENIC blast cells from the peripheral blood of patients with acute myeloblastic leukemia (AML) form colonies in cultures made viscid with methylcellulose and in the presence of appropriate growth stimuli.[1] These include fetal calf serum (FCS) and growth factor(s) in media conditioned by phytohemagglutinin-stimulated leukocytes from normal donors (PHA-LCM). The assay for leukemic blast progenitors has proven useful in probing the cellular biology of the disease; in particular, blast colonies have been demonstrated, by replating experiments, to contain new blast progenitors.[2] This secondary plating efficiency or PE2 has proven to be a characteristic contributing to outcome variation in clinical trials of AML.[3]

The interpretation of the results has been limited because the factor(s) in PHA-LCM required for the growth of blast colonies was not identified. It was not clear whether growth stimulation was mediated by one of the known hematopoietic growth factors present in the conditioned medium; these factors include granulocyte macrophage colony-stimulating factor (GM-CSF),[4] erythroid-potentiating activity (EPA),[4,5] and interleukin 2 (IL2).[6] Further, the use of the blast colony assay has been limited because of great variation in potencies of different batches of PHA-LCM. A stable source of leukemic blast growth factor (LBGF) would be helpful in resolving these difficulties.

Others have found that supernatants of bladder carcinoma cell lines contain hematopoietic growth factors.[7,8] Accordingly, we screened different cell lines for their capacity to support the growth of blast cells in culture. The results indicated that conditioned medium from the human bladder carcinoma line HTB9 is an active source of LBGF. Preliminary evidence was obtained that LBGF may not be the same as any known hematopoietic growth factor, including GM-CSF, EPA,[9] and IL2.

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MATERIALS AND METHODS

Source of cells. Mononuclear cells were obtained from the peripheral blood of AML patients (French-American-British classification, M1 to M6) by centrifugation through a Ficoll-Hypaque density gradient (ρ = 1.077). After removal of T lymphocytes by E rosetting, the cells were either plated immediately or stored frozen in liquid nitrogen in the presence of 10% dimethyl sulfoxide.

Blast assay. The blast assay was a modification of the technique described by Buick et al.[1] Blast cells were plated in Petri dishes (35 mm, Lux, Miles Lab, Naperville, Ill) at different cell concentrations (10^5/mL to 4×10^5/mL) in the presence or absence of conditioned medium, in alpha medium [α minimum essential medium (α-MEM), Flow] containing 1% methylcellulose (Dow Chemicals, Midland, Mich) and 10% fetal calf serum (FCS: GIBCO, Grand Island, NY; or Flow Lab, MacLean, Va). After five to seven days, colonies containing more than 20 cells were counted using an inverted microscope. The values thus obtained are referred to as primary plating efficiency (PE1). The secondary plating efficiency (PE2) was determined by pooling cells from primary colonies and assaying the resulting suspension for clonogenic blast cells, as described previously.[2]

Assays for growth factors. LBGF was assayed in 96 microwell plates (Linbro Flow Lab, MacLean, Va), using 10^3 to 10^4 blast cells per well depending on the sample, in 100 μL of α-MEM supplemented with FCS (10%) and methylcellulose (1%); the relative potencies of different conditioned media were assessed by testing each at five different dilutions. Colony counts were expressed as percent maximum stimulation of a standard included in the same experiment. These were plotted on probit paper against the logarithm of the corresponding concentrations. One unit of activity is equivalent to 50% maximum stimulation of the standard.[10] GM-CSF and EPA assays were performed with nonadherent mononuclear bone marrow cells from normal donors in Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Grand Island, NY) (50,000 cells per mL) containing methylcellulose (1%), FCS (10%), deionized bovine serum albumin (Hoechst, 10 mg/mL), iron saturated transferrin (Hoechst, Calbiochem-Behring, La Jolla, Calif 360 μg/mL) and erythropoietin (Connaught, Willowdale, Ontario, Canada 1 U/mL) as detailed elsewhere.[4] Colonies were counted on day 7 and day 14 of culture.

Preparation of PHA-LCM and HTB9 CM. PHA-LCM was prepared from normal leukocytes as described previously (10^6 cells per mL in α-MEM with 10% FCS and 1% PHA-M).[1] After seven days, the supernatants were harvested, filtered, and stored at −20 °C.

The cell line HTB9 was a gift from Dr E.R. Stanley (Albert Einstein, Bronx, NY) and Dr R.A. Phillips (Ontario Cancer Institute, Toronto). The cells, originally derived from the American tissue culture collection, were grown in α-MEM in 4% FCS. They were trypsinized (Difco Lab, Detroit 2 mg/mL) at confluency and

expanded fivefold. Conditioned medium was harvested, filtered through Millipore membranes, and stored at −20 °C. For cloning, the cells were seeded at 1,000 cells in 10 mL α-MEM with 10% FCS, in 100-mm tissue culture plates (Falcon, Becton-Dickinson, Oxnard, Calif) and allowed to grow for two weeks. After removal of the culture medium, selected, well-separated clones were isolated in glass cloning rings and trypsinized. They were successively transferred to 96 microwell and 24 well tissue culture plates (Linbro) containing α-MEM supplemented with 10% FCS. Conditioned media were harvested at confluency and tested in the blast assay. Conditioned medium from another human bladder carcinoma line MGHU-1, was also tested. It was a gift from Drs R. Buick and I. Tannock, (Ontario Cancer Institute, Toronto).

Chromatography on Sephadex G100. Crude conditioned media (50 mL) were concentrated to 5 mL by ultrafiltration over a YM-10 membrane (Amicon, Lexington, Mass) without loss of activity. After extensive dialysis (36 hours, 3 × 21 of buffer), the sample was loaded on a calibrated column of Sephadex G100 (1.65 × 95 cm) and eluted with a buffer containing 160 mmol/L NaCl, 4 mmol/L HEPES pH 7.3, and 100 mg/mL polyethyleneglycol 6,000. Fractions (4 mL) were filtered, sterilized (0.22 μ), and stored at −20 °C. In subsequent experiments, it was found that the biological activity was most stable when the Sephadex fractions and HTB9-CM were stored at 4 °C.

RESULTS

Comparison of PHA-LCM and HTB9-CM. Conditioned media from a number of continuous hematopoietic cell lines were tested for their capacity to stimulate the growth of blast colonies in culture. HL60 and K562 (myelopoietic leukemia lines) and MOLT-3 and Jurkat (lymphopoietic leukemia lines) were inactive. Two carcinoma cell lines, MGHU-1 and HTB9, yielded conditioned media that stimulated blast colony formation. When assayed, these conditioned media and PHA-LCM gave maximal colony stimulation at concentrations in the cultures between 5% and 10%. Accordingly, we undertook to compare PHA-LCM and HTB9-CM at the concentration of stimulator and cell numbers in the dishes that yielded optimal colony formation without background. Under these conditions HTB9-CM was always more effective than PHA-LCM in stimulating blast cell growth (Table 1).

Since PE2 is a parameter of clinical significance, PHA-LCM and HTB9-CM were compared using the replating assay. For each sample, four independent PE2 values were obtained; after exposure to PHA-LCM or HTB9 in the primary cultures, cells were harvested, washed, and replated in presence of the same conditioned medium or its alternative (Table 1). All four values were comparable except for sample 2, which is the only example where PHA-LCM was a more effective stimulator than HTB9-CM. We concluded that HTB9-CM can be used instead of PHA-LCM for PE2 determination.

Separation on Sephadex G-100. Since PHA-LCM and HTB9-CM were both potent stimulators of blast colony formation, we asked whether similar molecules in each preparation were responsible for the activity. Both conditioned media were concentrated and fractionated on columns of Sephadex G-100. Typical results of two independent separations for each conditioned medium are shown in Fig 1.

Table 1. Comparison of PE1 and PE2 Values Obtained With HTB9 CM and PHA-LCM

	Patient	PE1	PE2	
			PHA-LCM	HTB9 CM
PHA-LCM	1	570 ± 24	41 ± 9	73 ± 6
	2	148 ± 10	108 ± 28	22 ± 9
	3	1,266 ± 102	150 ± 13	187 ± 26
	4	135 ± 35	81 ± 12	— —
	5	112 ± 11	42 ± 9	64 ± 4
	6	126 ± 37	152 ± 14	82 ± 25
HTB9 CM	3	1,544 ± 183	246 ± 16	365 ± 19
	4	657 ± 58	89 ± 13	— —
	5	302 ± 17	54 ± 9	65 ± 10
	6	390 ± 14	93 ± 13	134 ± 31

Data are mean of duplicate cultures for PE1 and quadruplicates for PE2 ± SD

Cell concentrations: 10^5 to 3 × 10^5/mL (PE1) and 10^4 per well (PE2).

The fractions were tested on the same target cells (sample 3) to obtain the profiles in Fig 1; in addition, similar results were obtained with five additional samples.

The peak of activity was consistently identified after the albumin peak, with an apparent molecular weight of 30,000. Thus, filtration through Sephadex G-100 did not disclose differences between the active molecules in PHA-LCM and HTB9-CM.

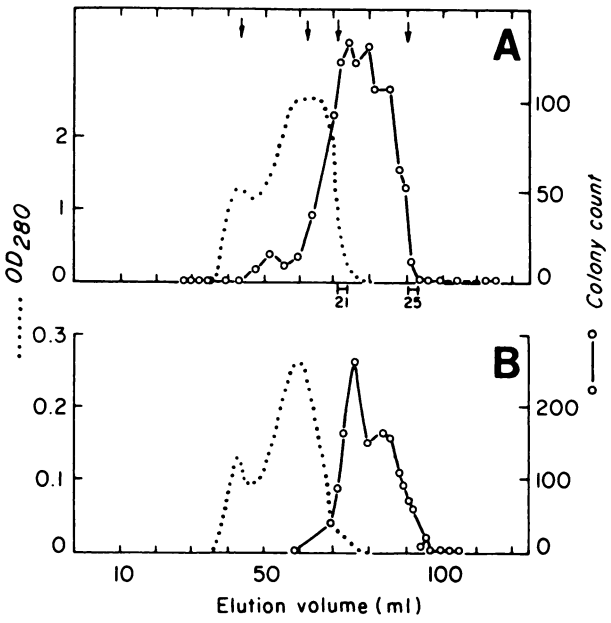

Fig 1. Elution profiles of PHA-LCM (A) and HTB9 CM (B) on Sephadex G100. Arrows show the positions of the molecular weight standards; from left to right these were: dextran blue (exclusion volume), bovine serum albumin, ovalbumin and alpha chymotrypsinogen. Fractions from both separations were tested at 10% in culture in presence of 1,500 blast cells per well. Results shown are typical of two independent fractionations for both conditioned media. The horizontal bars under the PHA-LCM profile mark the positions of the fractions (21 and 25) used for the assays presented in Table 2. Fraction 21 corresponds to the LBGF peak and fraction 25 to the GM-CSF peak.

Table 2. Nonidentity between GM-CSF, EPA, and LBGF

Stimuli	Concentration (%)	Colonies per 10^5 cells* Normal Bone Marrow G/M	Normal Bone Marrow E and Mix	AML Blood
A. 0		0	0	0
PHA-LCM	10	100	68	1,830
Fraction 21	10	6	9	1,045
25†	10	80	20	90
EPA	0.025	6	74	0
B. HTB9 control	10	104	22	1,940
HTB9 90 °C, 10 min	10	70	18	80

*Results shown are typical of two independent cultures for each target cell.

†Fractions from gel filtration of PHA-LCM on Sephadex G100. Elution volumes, 74 mL (fraction 21) and 92 mL (fraction 25).

Table 3. Cloning of the Cell Line HTB9

Clone	Activity U/mL	Subclone	Activity U/mL
A2	339	A2.2	398
B2	46	2.7	112
B4	20	2.6	100
A4	8	2.9	66
		2.5	56
		2.4	21
		A4.10	240
		4.3	63
		4.5	30
		4.7	29
		4.2	8

Activity of the conditioned medium from the starting cell line, 182 U/mL.

Comparison with other growth factors. Both PHA-LCM and HTB9-CM contain a number of hematopoietic growth factors, including GM-CSF and EPA. We asked whether LBGF was the same as any of these factors. Three approaches were taken. First, fractions obtained from the Sephadex column were tested on normal bone marrow for their capacity to stimulate granulocyte/macrophage colonies (G/M), erythroid colonies (E), and colonies of erythropoietic cells that included a second lineage (mix). Data from fractions 21 and 25 were revealing: as may be seen from Table 2, fraction 21 stimulated blast colony formation while fraction 25 was much less active. In contrast, fraction 25 was an effective stimulator of granulopoietic colonies, whereas fraction 21 had little activity. These results are evidence for a biochemical separation of LBGF from GM-CSF.

Second, highly purified EPA was obtained as a kind gift from Drs David Golde and J. Gasson. This preparation was tested for its ability to stimulate blast colony formation. Purified EPA was, as expected, a potent stimulator of erythropoietic and multilineage colony formation; in contrast, it was incapable of stimulating blast colony formation (Table 2).

Third, HTB9-CM was heated to 90 °C for ten minutes and compared with untreated controls for its capacity to stimulate normal myelopoietic colonies and blast colonies. It is apparent (Table 2) that the former activity was not markedly affected by heating whereas blast colony formation was reduced to approximately 5% of control.

Taken together, these data are consistent with the view that LBGF is not identical with either GM-CSF or EPA. Further detailed characterization will be required for the comparison of LBGF with other factors such as pluripoietin, which is also derived from a bladder carcinoma cell line.[8]

Clonal variation in LBGF production. A steady decline in stimulatory activity in the conditioned medium was observed when the cell line was passaged for several months. To investigate whether this phenomenon was due to the clonal dominance of a subpopulation of low producers, cells that were frozen at an early stage were thawed, allowed to recover for one week, and cloned at low density. Titration curves of the culture supernatants from different clones harvested at confluency showed a large interclonal variation. When the highest producer and the lowest producer were subcloned, heterogeneity was again observed; however, there was a tendency for high or low production, in concordance with the LBGF-producing efficiencies of clones of origin (Table 3). Thus, production of highly active conditioned medium can be achieved by expanding the cells shortly after cloning and assessing the supernatants of individual clones.

DISCUSSION

Evidence is presented in this study that a conditioned medium from an established cell line, HTB9, can replace PHA-LCM in clinical studies of AML in which the protocol requires the determination of PE2 as a prognostic parameter. The advantage of a permanent cell line over normal leukocytes resides in the fact that it constitutes a renewable source for production of conditioned medium. This facilitates quantitation of the blast assay for continuous clinical trials and for comparative studies between different groups.

LBGF from PHA-LCM and HTB9 CM elutes from Sephadex G100 as a single peak with an apparent molecular weight of 30,000. The result is consistent with the view that the same molecular species may be released by both cell types. This also excludes both PHA and IL2 as possible mitogenic factors for blast progenitors. PHA has a molecular weight that far exceeds the one reported here for LBGF[11]; IL2 has an apparent molecular weight of 15,000[6] and would elute after the CSF peak from the Sephadex G100 column. The heat sensitivity and the failure of EPA and CSF to support blast colony formation indicate that LBGF is a distinct entity. The target specificity of LBGF remains to be determined by purifying the growth factor and cloning the gene. The availability of a permanent cell line as a source of factor-producing cells greatly facilitates both molecular cloning and studies designed to determine the biological properties of LBGF and its relationship to other growth factors.

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