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Stimulatory Activity of PHA-LCM for Normal Human Hemopoietic Progenitors and Leukemic Blast Cell Precursors: Separation by Isoelectric Focusing

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Medium conditioned by leukocytes in the presence of phytohemagglutinin (PHA-LCM) promotes growth of human hemopoietic progenitors (CFU-GEMM, BFU-E, CFU-C) and precursors of leukemic blast cells. PHA-LCM was separated by isoelectric focusing and each fraction tested with nonadherent cells of normal individuals as well as blast cells from two patients with acute myelogenous leukemia. Activity profiles for CFU-GEMM, BFU-E and CFU-C ranged from pH 5.0–6.5. The profile for activity stimulatory for leukemic blast cells was broader and ranged from pH 5.5–7.5. Although some overlap was observed, the main peaks of stimulatory activity for normally differentiating progenitors and precursors of leukemic blast cells were separable with respect to their isoelectric point.

Key words: hemopoiesis, leukemia, hemopoietic progenitors, cell culture, stimulatory molecules, isoelectric focusing

The addition of plant lectins [1–7] such as phytohemagglutinin (PHA), pokeweed mitogen (PWM), or concanavalin A (Con A) to short-term cultures of hemopoietic subpopulations facilitates the release of growth-promoting stimulators for early hemopoietic progenitors. These molecules were instrumental in the development of culture assays for murine [8, 9] and human pluripotent hemopoietic progenitors (CFU-GEMM) [10, 11], and for precursors of human leukemic blast cells [12]. Murine pluripotent hemopoietic progenitors form large mixed colonies of granulocytes, erythroblasts, megakaryocytes, and macrophages when cultured with media conditioned by spleen cells in the presence of PWM [8, 9]. A similar type of mixed colony can be observed in cultures of human bone marrow or peripheral blood when media conditioned by peripheral leukocytes under the influence of phytohemagglutinin (PHA-LCM) are added [10, 11]. This material usually also enhances growth of committed erythroid (BFU-E) [13] and granulocytic precursors (CFU-C). Furthermore,

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it was observed that the same crude preparation of PHA-LCM stimulates the formation of colonies with blast cell properties in cultures of peripheral blood specimens derived from patients with acute myelogenous leukemia (AML) [12]. Similar growth requirements for leukemic blast cells have been reported by other authors [14, 15].

We have attempted to separate activities in PHA-LCM that stimulate CFU-GEMM, BFU-E, CFU-C, and cells that form leukemic blast colonies. A crude PHA-LCM preparation was fractionated by isoelectric focusing, and the resulting fractions were tested on bone marrow cells of normal individuals and leukemic blast cell populations from peripheral blood samples of patients with AML.

MATERIALS AND METHODS

Patient Material

Bone marrow samples were obtained from 2 normal bone marrow transplant donors, 1 bone marrow transplant recipient after stable engraftment, and 1 patient after successful treatment for pure red cell aplasia. All patients had normal hemopoietic parameters at the time of study. Specimens were aspirated into heparinized syringes, and the buffy coat was prepared by centrifugation at 150g for 10–15 min. Subsequently, adherent cells were removed as previously described [16] to minimize the endogenous production of stimulatory activities [16, 17]. The nonadherent fraction was further depleted of red blood cells and mature granulocytes by centrifugation in LSM (Litton Bionetics) at a density of 1.077 gm/ml. The resulting nonadherent, mononuclear cells were used to test the stimulatory activity of crude and fractionated PHA-LCM.

Peripheral blood specimens from two patients with acute myeloid leukemia in relapse were drawn into heparinized syringes, and mononuclear cells of density less than 1.077 gm/ml were prepared as described above. The cell population was further depleted from E-rosette-forming cells by incubation with sheep red blood cells (SRBC) at 4°C and subsequent centrifugation in LSM [18, 19]. Cells were stored at −70°C (Kelvinator) in 10% DMSO, 20% fetal calf serum (FCS), and thawed immediately prior to plating [20].

Preparation of PHA-LCM

Active PHA-LCM is released by peripheral blood cells of normal individuals. In addition, two patients with hemochromatosis who regularly undergo therapeutic phlebotomies have provided cells for the production of PHA-LCM. Material for this study was exclusively prepared using peripheral blood cells from 1 of these 2 patients. Cells were incubated in modified Dulbecco's minimum essential medium (DMEM) [21] with 10% FCS and 1% PHA [5]. The supernatant was harvested after 7 days of culture.

Separation of PHA-LCM

PHA-LCM was separated by column isoelectric focusing in a density gradient [22]. Briefly, the material was admixed with 1% LKB Ampholine carrier of pH 5–8 and loaded into the isoelectric focusing column (LKB 8101, volume 110 ml) incorporated into a sucrose density gradient (5–50% w/v). Current was applied for 16 h at 4°C. Four milliliter fractions were collected and dialysed over 48 h with 2 exchanges of modified DMEM. After dialysis, the pH of each fraction was adjusted to 7.4.

Each fraction was tested for its growth-promoting activity for CFU-GEMM, BFU-E, CFU-C, and leukemic blast cell progenitors.

Colony Assays for Hemopoietic Progenitors

Mixed hemopoietic colonies, erythroid bursts, and granulocytic colonies were grown as previously described [10, 11]. Experiments were usually performed with 2×10^5 nonadherent mononuclear target cells per culture plate. In some control experiments, unseparated buffy coat cells were used. Cells were admixed with modified DMEM, 30% FCS, 0.9 methylcellulose, and PHA-LCM preparations as indicated below. Aliquots of 0.9 ml were placed in 35 mm Petri dishes and incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2. One unit of erythropoietin (EPO) (Step III, Connaught Laboratories, Willowdale, Ontario) was added on day 4 of culture. Each dish was examined after a total incubation of 14 days for the presence of mixed colonies, erythroid bursts, and granulocytic colonies.

Crude PHA-LCM was added to control plates at a previously established optimal concentration of 5% [23]. In experimental groups, the crude preparation of PHA-LCM was replaced by material of individual fractions obtained by isoelectric focusing. Each fraction was routinely tested at a concentration of 0.5%. This concentration was established for the peak fraction (pH 5.9) as being equivalent to the stimulatory activity in 5% crude PHA-LCM.

Leukemic blast cell colonies were grown from E-rosete-forming cell-depleted preparations [20] immobilized in 0.9% methylcellulose with modified DMEM, 30% fetal calf serum, and 5% crude PHA-LCM or 0.5% of material derived from each fraction. EPO was not added. Colonies were counted after 5 to 7 days of culture. Their blast-like phenotype was verified by subjecting randomly removed colonies to further analysis by Wright stain, peroxidase reaction, and rosette formation with SRBC. Blast colonies contained E-rosette-negative cells with blast morphology and negative or slightly positive peroxidase reaction.

RESULTS

Growth Requirements of Normal and Leukemic Target Cell Populations

The growth of human hemopoietic colonies is dependent upon appropriate culture conditions. Some of the required stimulatory activities are released into the culture by adherent cells. We attempted to eliminate the endogenous production of stimulators for CFU-GEMM, BFU-E, and CFU-C by removing adherent cells. The quality of the depletion was tested in 3 experiments by examining the influence of PHA-LCM and EPO on colony growth. Colony formation was found to be dependent upon the addition of exogenous stimulators. While granulocytic colonies required only PHA-LCM, it was necessary to add PHA-LCM and EPO to promote growth of mixed hemopoietic colonies and erythroid bursts (Table I). It is of note that erythroid bursts developed only occasionally in cultures that contained EPO but no PHA-LCM. Thus, nonadherent, mononuclear bone marrow cells are appropriate target cells to analyze the stimulatory effects of PHA-LCM and its fractions in cultures that also contain EPO. No colonies were observed without PHA-LCM.

Frozen cells from both patients with AML formed E-rosette-negative, peroxidase-negative, or weakly positive colonies of blast cell morphology in the presence of 5% PHA-LCM.

Stimulatory Activity Profiles for Normal Hemopoietic Progenitors

The fractions of PHA-LCM obtained by isoelectric focusing were analyzed for their ability to promote growth of CFU-GEMM, BFU-E, and CFU-C by performing 3 types of experiments: first, to establish the activity profiles using a constant concentration of each

TABLE I. Effect of EPO and PHA-LCM on NA Cells in Culture

Patient	Culture conditions	CFU-C[a]	BFU-E[a]	CFU-GEMM[a]
M.R.	–	0	0	0
	EPO	0	1	0
	PHA-LCM	27	0	0
	PHA-LCM + EPO	29	32	2
C.C.	–	0	0	0
	EPO	0	1	0
	PHA-LCM	81	0	0
	PHA-LCM + EPO	74	57	2
G.H.	–	0	0	0
	EPO	1	0	0
	PHA-LCM	28	0	0
	PHA-LCM + EPO	24	34	1

[a]Number of colonies by 2×10^5 NA-cells/plate.

fraction; second, to examine the presence of inhibitors; third, to study the influence of increasing concentrations of the fraction with highest activity and a fraction that did not promote colony growth at 0.5%.

Nonadherent, mononuclear bone marrow cells were plated with one unit of EPO and material from each fraction at a concentration of 0.5%. Representative profiles from one of four experiments are depicted in the top panel of Figure 1. Stimulatory activities for CFU-GEMM, BFU-E, and CFU-C were present in fractions of pH 5–6.5. The plating efficiency observed for the peak fraction was comparable to that of 5% crude PHA-LCM. Although some differences became apparent in the shape of the activity profiles for different hemopoietic progenitors, it was not feasible to separate molecules with restricted specificity.

In order to examine the fractions for putative inhibitors, unseparated buffy coat cells were cultured with material of each fraction and one unit of EPO to assess the plating efficiency of BFU-E. Buffy coat cells were utilized since the addition of EPO permitted the formation of some erythroid bursts without PHA-LCM. As indicated in Figure 2, burst formation in the presence of material from each fraction was at least comparable to that of the control group (49 erythroid bursts per 2×10^5 target cells). None of the fractions led to reduction of the plating efficiency below the control value. A consistent increase was documented in 3 experiments for fractions with an isoelectric point of pH 5.9. The peak was identical to that identified with nonadherent, mononuclear target cells. In addition, a second peak was demonstrated in fractions with material of acid isoelectric point. This peak was regularly absent in cultures of nonadherent, mononuclear target cells.

Material of the peak fraction (pH 5.9) and of one of the inactive fractions (pH 7.6) was added in concentrations increasing from 0.125% to 5% (Fig. 3). Nonadherent, mononuclear cells and unseparated buffy coat cells served as target cells and were assessed for their ability to form erythroid bursts with 1 unit of EPO. The plating efficiency increased from 1 to 34 bursts for 2×10^5 nonadherent cells and from a background level of 18 to 49 bursts for 2×10^5 buffy coat cells with increasing concentrations of material with an isoelectric point at pH 5.9. The highest observed frequency was considerably above the control values of 18 and 29 bursts, respectively. The fraction with isoelectric point of pH 7.6 did not contain any stimulators when tested up to concentrations of 5%. No indication of inhibitory activities was obtained for unseparated buffy coat cell preparations.

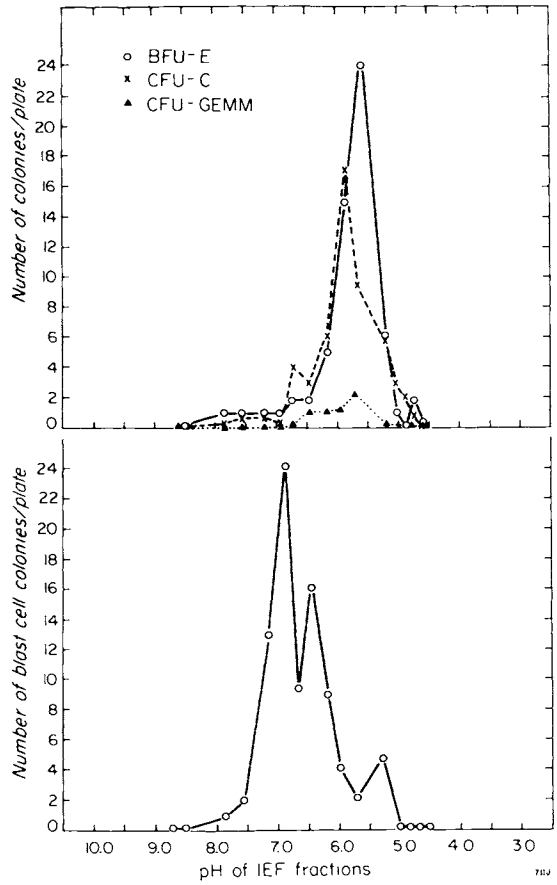

Fig. 1. Top panel. Fractions of PHA-LCM separated by isoelectric focusing were assessed for activities that promote growth of CFU-GEMM, BFU-E, and CFU-C by culturing nonadherent, mononuclear cells in the presence of 1 unit of EPO. No colonies were observed in the absence of crude or fractionated PHA-LCM. Addition of 5% crude PHA-LCM to controls yielded 2 mixed colonies, 27 erythroid bursts, and 20 granulocytic colonies. Bottom panel. A cryopreserved, E-rosette forming, cell depleted blast cell population of a patient with AML in relapse was used to identify activities that stimulate blast colony formation. Controls without crude or fractionated PHA-LCM did not yield any colonies.

Stimulatory Activity Profiles for Leukemic Blast Colony-Forming Cells

Peripheral blood cells from 2 patients with AML in relapse were depleted of E-rosette-forming cells. The resulting mononuclear, E-rosette-depleted population was used to test the fractions for activities that promote growth of leukemic blast cell progenitors. Each fraction was examined in cultures without EPO at a concentration of 0.5%. Growth of leukemic blast cell colonies was supported by material derived from fractions of pH 5.5 to 7.5 as depicted for one patient in the bottom panel of Figure 1.

The profile differed from those observed for normal progenitors and included mainly material with a more alkaline isoelectric point. It overlapped with activities for normal hemopoietic progenitors and appeared more heterogeneous, with 3 subcomponents. A bio-

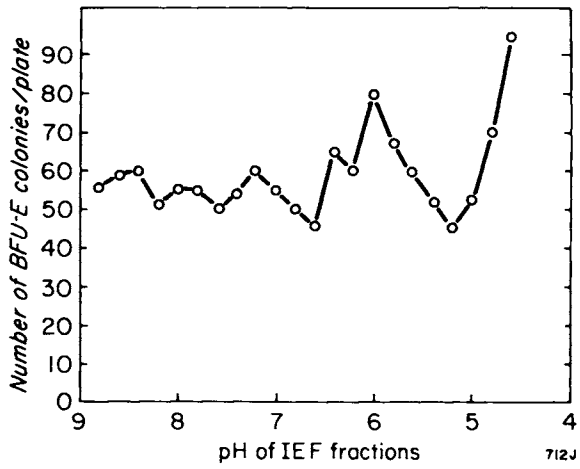

Fig. 2. Influence of PHA-LCM fractions obtained by isoelectric focusing on erythroid burst formation using 2 X 10^5 buffy coat cells as targets. All cultures contained 1 unit of EPO. Controls without crude or fractionated PHA-LCM gave rise to 49 colonies in the presence of 1 unit of EPO.

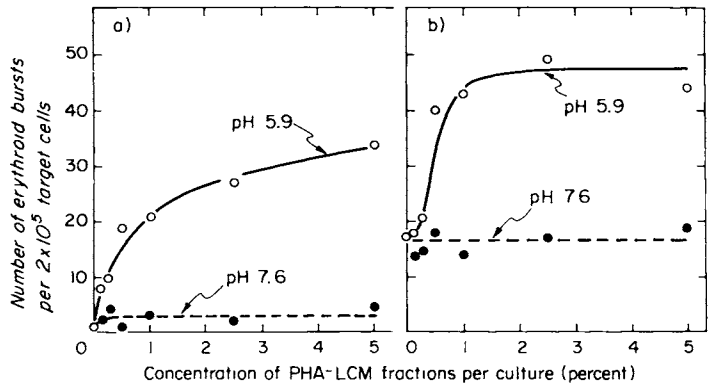

Fig. 3. Influence of increasing concentrations of 2 fractions of PHA-LCM obtained by isoelectric focusing. Both fractions were tested on nonadherent, mononuclear cells and unseparated buffy coat cells. Controls with 5% crude PHA-LCM yielded 18 bursts for nonadherent cells and 44 bursts for buffy coat cells.

logical difference was not observed since colonies grown in the presence of material with pH 5.9 and 7.2 were composed of cells with similar primitive blast-like appearance.

DISCUSSION

Stimulatory activities in PHA-LCM for CFU-GEMM, BFU-E, and CFU-C can be separated partially by isoelectric focusing from activities that promote growth of leukemic blast cell precursors. Nonadherent, mononuclear bone marrow populations of normal individuals and mononuclear, E-rosette-depleted peripheral blood cells from patients with AML were found to be appropriate target cells for the different stimulatory activities since colony formation of these populations was dependent upon the addition of PHA-LCM.

The activity profiles for CFU-GEMM, BFU-E, and CFU-C are very similar with respect to their isoelectric point, indicating that this method does not permit separation of molecules with specificity for cells of a restricted hemopoietic lineage. These data are consistent with observations by Metcalf [personal communication] for growth-promoting molecules in murine hemopoiesis. Stimulators that were recovered in the peak fraction yielded, at a much lower concentration (0.5%), a plating efficiency of CFU-GEMM, BFU-E, and CFU-C similar to that obtained with optimal concentrations of 5% crude PHA-LCM. As demonstrated for BFU-E, the plating efficiency increased above control values with addition of higher concentrations of the peak material. This observation could be explained by a number of alternatives. The removal of inhibitory activities by isoelectric focusing was not substantiated since the formation of endogenous bursts by unseparated buffy coat cells was not reduced by any of the fractions. The fractionation procedure may lead to enrichment of stimulators and then to general improvement of the culture system. Alternatively, additional subpopulations of BFU-E may be recruited. Supporting evidence for the latter may be gathered by examining the resulting bursts for such markers as fetal hemoglobin [13].

The additional peak of stimulatory activity consistently observed with unseparated buffy coat cells suggests that colony formation may be controlled by more than 1 type of stimulator in PHA-LCM. Some stimulators may interact directly with appropriate target cells; others may influence adherent cells directly or indirectly and induce the production and release of a second mediator. PHA could be considered as a candidate. Further depletion and reconstitution experiments are required to assess these postulated interacting populations and their mediator molecules more completely.

The most promising prospect of our data is related to the possibility of separating stimulatory activities for normal hemopoietic progenitors from those of leukemic blast precursors. The availability of material with specificity for leukemic blast cells would facilitate selective cloning of leukemic populations and may help to explore the relationship between normal hemopoietic progenitors and leukemic blast-forming cells.

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