Feeder Cell Requirements for Leukemia Cell Colony Formation in Cultures Supplemented with Phytohemagglutinin

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ABSTRACT

The use of phytohemagglutinin-supplemented colony cultures has offered new opportunities recently for studying acute myeloid leukemia (AML) cell growth in vitro. The active stimulator cells for AML colony-forming cells have not been identified, although this could be important for optimal application of the technique and for elucidating differences in growth between normal and leukemic progenitor cells. In this study, feeder layers were prepared from subpopulations of normal peripheral blood leukocytes which were obtained by centrifugation through Ficoll-Isopaque, erythrocyte rosette sedimentation, and adherence separation. Underlayers containing lymphocytes (B, T, or B plus T) or adherent monocytes failed to stimulate AML colony formation. The colony stimulation capacity of total mononuclear cells was decreased significantly following depletion of T-lymphocytes. The highest AML colony numbers were obtained when adherent monocytes and T-lymphocytes in combination were added to phytohemagglutinin-containing cultures. Stimulation of AML colony formation depended on the quantitative interrelationship of monocytes and T-lymphocytes in the cultures. Thus, AML colony-forming cells, unlike normal marrow granulocyte-monocyte colony-forming cells, do not respond to monocyte stimulation alone and require for their proliferation an inducing factor derived from phytohemagglutinin-exposed T-lymphocytes and monocytes.

INTRODUCTION

In vitro colony formation by AML cells in PHA-containing cultures has become an accepted procedure for studying neoplastic cell proliferation in patients with AML. These cultures are of potential value in the analysis of the leukemia progenitor cell compartment, and interesting results on the features of proliferation and differentiation of leukemia progenitor cells are accumulating; e.g., cell cycle characteristics (20), self-replication capacities (1), buoyant density properties (30), and cytogenetic abnormalities (15, 30) of leukemia progenitor cells have been studied, and recent investigations have demonstrated specific growth abilities of karyotypically distinct subpopulations (15) and induction of cellular differentiation in culture (18). The colony formation techniques utilized by different groups vary in their detailed design. As stimulators, PHA (6) or PHA and leukocyte feeder cells (14) have been directly incorporated into the cultures, or PHA-LCM has been supplemented (2, 22).

Proliferation of AML cells usually does not occur or is limited to cluster formation (10, 13, 25, 27) in a leukocyte feeder colony assay without PHA (24). This indicates a positive role of the lectin in leukemia colony formation. It is unclear whether the effect of PHA is direct or whether it is mediated through an indirect mechanism on the leukemic colony-forming cells, e.g., by stimulating normal leukocytes to release essential factors into the medium. The findings, that the stimulatory activity of PHA-LCM for leukemia colony growth exceeds significantly that of conventionally prepared LCM plus an appropriate amount of PHA (Ref. 19; Footnote 4), are in favor of an indirect function of the lectin.

Elucidation of the stimulatory role of the cellular components incorporated into a feeder layer or used as the source of the conditioned medium would seem to be important for understanding differences in stimulation of normal and leukemic cells, for clarifying differences between the activities of separate batches of stimulating materials, and for standardizing the preparation of these materials. The experiments reported here were carried out to identify the active subset of feeder layer cells in leukemia colony formation. Peripheral blood leukocytes were separated by centrifugation through Ficoll-Isopaque, E-rosette sedimentation, and adherence to tissue culture plastic. Each fraction of cells was tested for stimulation of the formation of AML colonies in culture in the presence of PHA.

MATERIALS AND METHODS

Bone marrow and blood cells were obtained from patients with AML at diagnosis or in relapse. Techniques described previously were used for cell collection and harvesting of nucleated cells (16). Pure leukemia blast cells, which had been obtained by density gradient separation combined with E-rosette depletion as described previously (28, 29), were cultured. All leukemia cell preparations used in this study yielded leukemia cell colony growth without normal cell contamination in culture as evidenced by E-rosette tests and cytogenetic analyses of colony cells. Details of these analyses have been published elsewhere (30). The leukemia blast cells were used as fresh preparations or following cryopreservation in 7.5% dimethyl sulfoxide and 20% fetal calf serum using a controlled freezing apparatus and storage in liquid nitrogen (26).

Colony Assay. Leukemic colonies were grown in the PHA-leukocyte feeder assay as reported (16, 28). Cells were plated in 35-mm Petri dishes in 0.4 ml liquid medium supplemented with 0.01 ml PHA (HA 15; Wellcome, Beckenham, Great Britain) at a concentration of 0.1 x 10⁵ to 1.0 x 10⁵/dish. During the course of this study, cell titration experiments were done repeatedly and confirmed the linearity of the dose-response curves for purified leukemia cells. Triplicate cultures were incubated at 37°C in a humidified 7.5% CO₂ atmosphere. At day 7, colonies of 50 cells or more growing at the surface of the underlayer were counted by use of an inverted microscope.

Feeder layers containing various sources of cells were prepared. The following cell concentrations of peripheral blood leukocytes or their

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3 The abbreviations used are: AML, acute myeloid leukemia; PHA, phytohemagglutinin; PHA-LCM, medium conditioned by leukocytes exposed to phytohemagglutinin; LCM, leukocyte-conditioned medium; E-RFC, erythrocyte rosette-forming cells; E-rosette(s), erythrocyte rosette(s); GM-CSA, granulocyte-monocyte colony-stimulating activity; GM-CFC, granulocyte-monocyte colony-forming cells.

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fractions were used: (a) mononuclear cells, 1 x 10^6/dish; (b) E-RFC-depleted mononuclear cells, 0.2 x 10^6 to 0.4 x 10^6/dish; (c) E-RFC-enriched mononuclear cells, 0.4 x 10^6 to 0.8 x 10^6/dish; (d) nonadherent mononuclear cells, 0.5 x 10^6 to 1.0 x 10^6/dish; (e) adherent mononuclear cells, adherent cells from 1 x 10^6 mononuclear cells/dish or from 0.4 x 10^6 E-RFC-depleted mononuclear cells/dish. These concentrations of fractionated cells were based on the yield of the fractions in the separation procedures and matched to the numbers of the same cell type in the unfractionated mononuclear cell suspension. Feeder layers with 1 x 10^6 mononuclear cells/plateau of dose-response curve were taken as the 100% stimulation value. Agar underlayers without feeder cells were used in control cultures. These gave negligible numbers (usually zero) of colonies.

**RESULTS**

Cell Separation Procedure for Feeder Layers. To prepare feeder layers of varying fractions of peripheral blood leukocytes, the following separation procedures were performed. Mononuclear cells were obtained by centrifugation (30 min, 400 x g) through Lymphoprep (Nyggaard, Oslo, Norway) with a density of 1.077 g/ml. Rosette-forming and nonrosette-forming cells were separated by a second centrifugation on Ficoll-Isoopaque after the cells (10 x 10^6) had been incubated with neuraminidase-treated (29), or S-adenosyl-L-ethionine-treated (12) sheep RBC to form E-rosettes. The E-RFC-depleted (<2% E-rosette-positive cells) and E-RFC-enriched (>95% E-rosette-positive cells) fractions were collected separately and washed 2 times. Sheep RBC in the E-RFC-enriched fraction were lysed with autologous plasma (15 min) (17), and the remaining nucleated cells were washed 2 times. Mononuclear cells (1 x 10^6) and E-RFC-depleted mononuclear cells (0.4 x 10^6) were incubated in medium (Hanks' balanced salt solution) with 2% fetal calf serum in plastic tissue culture Petri dishes (35 mm) for 1.5 hr at 37° in a 7.5% CO2 atmosphere to collect adherent and nonadherent cells. The nonadherent cells were harvested for use. The number of monocytes in this cell fraction did not exceed 2%. The dishes were then washed twice with the same medium-serum mixture, and agar containing Dulbecco's modified Eagle's medium (Hanks' balanced salt solution) with 2% fetal calf serum in plastic tissue culture Petri dishes (35 mm) for 1.5 hr at 37° in a 7.5% CO2 atmosphere to collect adherent and nonadherent cells. The nonadherent cells were harvested for use. The number of monocytes in this cell fraction did not exceed 2%. The dishes were then washed twice with the same medium-serum mixture, and agar containing Dulbecco's modified Eagle's medium was poured into these dishes to prepare underlayers of the adherent cells. Duplicate dishes with adherent cells were stained with nonspecific esterase, and the percentage of monocytes was always more than 95%.

**RESULTS**

Pure AML blast cells were plated in cultures which, besides PHA, contained different fractions of mononuclear cells as stimulators. These cell fractions were evaluated for their capacity to support leukemia colony growth. The comparative AML colony numbers are given in Table 1. The various fractions all had reduced capacities for stimulating leukemia colony formation as compared with the unfractionated mononuclear cells. To exclude the possibility of loss of stimulation capacities of monocytes due to the adherence procedure, reconstitution experiments in which nonadherent and adherent cells were recombined in feeder layers were carried out. In these cultures, colony formation was restored to 80 to 100% of control values (2 experiments).

In another series of experiments, feeder layers of adherent cells were supplemented with increasing numbers of T-lymphocytes (Chart 1). Stimulation of leukemia colony formation was restored following the addition of T-lymphocytes, which (as a single population) were unable to stimulate colony growth (see Table 1). Full recovery of stimulation was dependent on the number of T-lymphocytes, and the highest number of T-lymphocytes stimulated colony formation to above reference values.

**DISCUSSION**

Leukemic blast cell proliferation in leukocyte feeder or LCM cultures in the absence of PHA is restricted to cluster formation, and only rarely is normal-sized colony growth apparent from AML bone marrow or blood in these cultures. When AML cells are cultured in the presence of PHA-LCM or PHA and leukocyte feeder cells, the colony-forming efficiency is increased significantly. This higher efficiency can be explained by: (a) the release of a stimulating factor different from GM-CSA in the absence of PHA to which the leukemic cells respond by proliferation; (b) a shift in the dose response to normal GM-CSA due to a cofactor which is produced following PHA stimulation; or (c) a direct stimulating effect of PHA on leukemic cells. It is unlikely that this enhanced colony formation is due to mainly a direct effect of PHA, because cultures with only PHA as stimulator and LCM-stimulated cultures supplemented with PHA gave poor colony growth (Ref. 19; Footnote 4). The fact that others have used PHA-LCM in leukemia colony assays (2, 22) which contained only minimal concentrations of PHA in the final cultures is also evidence in favor of an indirect effect of PHA. Whether this effect depends on a cofactor of GM-CSA or on a separate stimulating factor cannot be concluded with certainty. The substance re-

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Cell fraction</th>
<th>Predominant cell type</th>
<th>Stimulation capacity (%)</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll</td>
<td>Interphase</td>
<td>T- + B-cells + monocytes</td>
<td>100%</td>
<td>7</td>
</tr>
<tr>
<td>Ficoll + adherence</td>
<td>Nonadherent</td>
<td>T- + B-cells</td>
<td>15 ± 22%</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Adherent</td>
<td>Monocytes</td>
<td>10 ± 12%</td>
<td>5</td>
</tr>
<tr>
<td>E-rosette Ficoll</td>
<td>Interphase</td>
<td>B-cells + monocytes</td>
<td>60 ± 36%</td>
<td>7</td>
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<td>Sediment</td>
<td>T-cells</td>
<td>16 ± 16%</td>
<td>7</td>
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<tr>
<td>E-rosette Ficoll + adherence</td>
<td>Interphase nonadherent</td>
<td>B-cells</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Interphase adherent</td>
<td>Monocytes</td>
<td>1 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

* AML colony growth in the PHA assay with different leukocyte fraction supplements; only pure leukemia blast cells (see "Materials and Methods") were plated.
* Colony numbers set at 100% (actual colony numbers varied between 54 and 140/10^6 cells plated); the other data are expressed as relative values.
* Mean ± S.D.
* Statistically significant at p = 0.001 (2-tailed, paired t test).
* Statistically significant at p = 0.01 (2-tailed, paired t test).
* ND, not done.
Acute Leukemia Colony-stimulating Cells

100-150, (25). These values are distinct from those known for GM-CSA of 44,000 with an active subunit with molecular weight of 27,000. This leukemia colony-stimulating factor has a molecular weight responsible for leukemia colony-stimulating or -enhancing activity cultures (no cells in feeder layer) were always run parallel and gave no colonies. Unstimulated 0.4 x 10^6 T-depleted mononuclear cells; d, c + 0.2 x 10^6 T-cells; e, c + 0.4 x 10^6 T-lymphocytes or their combination, as feeder cells (compared to unfractionated blood mononuclear cells). Actual colony numbers per 10^6 cells plated were 95 and 66, respectively. b, T-depleted mononuclear cells, 0.4 x 10^6; c, adherent cells from 0.4 x 10^6 T-depleted mononuclear cells; d, c + 0.2 x 10^6 T-cells; e, c + 0.4 x 10^6 T-cells; and f, c + 0.8 x 10^6 T-cells. Ordinate, number of colonies relative to the control cultures (set at 100%). C) and III, two separate experiments. Unstimulated cultures (no cells in feeder layer) were always run parallel and gave no colonies.

sensitive for leukemia colony-stimulating or -enhancing activity in PHA-LCM cultures has been purified by Price and McCulloch (25) and Fauser and Messner (8). Preliminary data suggest that this leukemia colony-stimulating factor has a molecular weight of 44,000 with an active subunit with molecular weight of 27,000 (25). These values are distinct from those known for GM-CSA preparations. Also, by isolectric focusing, the activity for leukemia blast-cell progenitors appeared to be partially separable from factors active on normal hematopoietic precursors (8). Therefore, it has been suggested that a distinct stimulating factor is involved in the formation of leukemia colonies. The experiments reported here support this suggestion. In vitro growth of AML blast-cell progenitors requires feeder cells which differ from those needed for proliferation of normal GM-CFC. Peripheral blood mononuclear cells are active sources of colony-stimulating activity for normal bone marrow GM-CFC (4, 9). When incorporated into the feeder layer of the PHA colony system, however, purified adherent monocytes were unable to stimulate leukemia colony growth. Similarly, T-lymphocytes failed to induce the formation of leukemia colonies following appropriate incubation. The combined addition of these 2 cellular components, on the other hand, provided significant stimulation. This suggests that a combination of monocytes and T-lymphocytes as well as PHA is required for the production of a factor by which leukemic progenitor cells are induced to proliferate into colonies. This effect was shown to be dependent on the actual numbers of stimulating cells in the cultures.

Various groups have indicated that a wide spectrum of stimulating activities is produced in cultures supplemented with PHA and leukocytes, e.g., GM-CSA (5, 23), erythroid burst-promoting activity (11, 31), an activity that stimulates colony-forming units-spleen (3, 32), and a factor required for the proliferation of mixed granuloerythropoietic colony-forming cells (7). It is unknown whether the growth factor required for leukemia colony formation is identical to one of these. It appears of interest to define the leukemia growth factor in relation to the series of regulators for normal hematopoietic progenitors. The results of the experiments described in this paper may be useful for optimization of the cellular conditions for cultures of human leukemic colony formation.

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