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).

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 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 38-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 10^5 to 1.0 x 10^6/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% CO_2 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

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.

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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.
4 K. Swart and B. Löwenberg, unpublished results.

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fractions were used: (a) mononuclear cells, $1 \times 10^6$/dish; (b) E-RFC-depleted mononuclear cells, $0.2 \times 10^6$ to $0.4 \times 10^6$/dish; (c) E-RFC-enriched mononuclear cells, $0.4 \times 10^6$ to $0.8 \times 10^6$/dish; (d) nonadherent mononuclear cells, $0.5 \times 10^6$ to $1.0 \times 10^6$/dish; (e) adherent mononuclear cells, adherent cells from $1 \times 10^6$ mononuclear cells/dish or from $0.4 \times 10^6$ 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 $1 \times 10^6$ 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.

**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, $400 \times g$) through Lymphoprep (Nyegaard, Oslo, Norway) with a density of 1.077 g/ml. Rosette-forming and non-rosette-forming cells were separated by a second centrifugation on Ficoll-Isoopaque after the cells ($10 \times 10^6$) 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 ($1 \times 10^6$) and E-RFC-depleted mononuclear cells ($0.4 \times 10^6$) 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°C 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 mononuclear cells did not exceed 2%. The dishes were then washed twice.

**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 presence 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 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-

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**Table 1**

<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&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td>E-rosette Ficoll</td>
<td>Interphase</td>
<td>B-cells + monocytes</td>
<td>60 ± 33&lt;sup&gt;e&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td>E-rosette Ficoll + adherence</td>
<td>Interphase nonadherent</td>
<td>B-cells</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> AML colony growth in the PHA assay with different leukocyte fraction supplements; only pure leukemia blast cells (see "Materials and Methods") were plated.

<sup>b</sup> Colony numbers set at 100% (actual colony numbers varied between 54 and 140/10<sup>6</sup> cells plated); the other data are expressed as relative values.

<sup>c</sup> Mean ± S.D.

<sup>d</sup> Statistically significant at $p = 0.001$ (2-tailed, paired t test).

<sup>e</sup> Statistically significant at $p = 0.01$ (2-tailed, paired t test).

<sup>f</sup> ND, not done.
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