Interacting Populations Affecting Proliferation of Leukemic Cells in Culture

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

Peripheral blood cells from three patients with acute leukemia have been studied using a suspension culture method previously described. Cytogenetic studies in two of the patients permitted the identification of the proliferating cells in the cultures as being derived from a leukemic population. Cell separation studies using velocity sedimentation supported the concept that growth of the leukemic cells in culture is dependent on an interaction between two populations of leukemic cells.

RECENTLY, WE REPORTED studies of the growth in culture of peripheral blood leukocytes from nine patients with acute leukemia. The properties of these cell populations varied greatly from patient to patient, both in respect to growth kinetics and response to factors in media conditioned by leukocytes (LCM) or to phytohemagglutinin (PHA). We proposed that this patient-to-patient variation might result from varying concentrations of two cell populations, one capable of producing growth-promoting factors, either spontaneously or in response to PHA, and the second capable of responding to such factors by cellular proliferation. The studies leading to this hypothesis were greatly facilitated by the finding that peripheral blood cells from patients with leukemia retained growth potential after freezing at -70°C with 5% dimethylsulfoxide; using such frozen cells, it was possible to perform and repeat a variety of experiments on cells obtained at the same bleeding.

In the present paper, we extend the study to three additional patients. Peripheral blood cells from two of these patients contained chromosomal abnormalities, permitting the identification of proliferating populations. Cell separation studies using velocity sedimentation at unit gravity supported the concept of two interacting populations as determinants of the growth of leukemic peripheral blood cells in culture.

MATERIALS AND METHODS

Patients With Leukemia

Table 1 contains the clinical diagnosis and hematologic findings for the three patients included in the study. In each instance, blood was obtained by venipuncture; after separation of the buffy coat, some of the cells were used for initial characterization. The remainder were frozen at a concentration of 2-4 x 10^7 cells/ml in α-medium (Flow Laboratories), containing 10% fetal calf serum (FCS) and 5% dimethyl sulfoxide (Fisher Scientific Company, Toronto). Cells were frozen in aliquots of 3-4 ml in 5-ml Falcon plastic test tubes (No. 2005) by placing them...
Table 1. Hematologic Data on Leukemia Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Hemoglobin (g/100 ml)</th>
<th>Leucocyte Differentiation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Platelet (10^3) per cu mm</td>
<td>WBC (10^3) per cu mm</td>
</tr>
<tr>
<td>HIL</td>
<td>AML(t)</td>
<td>7.2</td>
<td>70</td>
</tr>
<tr>
<td>MCK</td>
<td>AMML(t)</td>
<td>10.2</td>
<td>22</td>
</tr>
<tr>
<td>FEI</td>
<td>AML</td>
<td>5.6</td>
<td>15</td>
</tr>
</tbody>
</table>

*PMN denotes polymorphonuclear neutrophils.
†AML denotes acute myelogenous leukemia.
‡AMML denotes acute myelomonocytic leukemia.

Directly into a freezer (Kelvinator series 100) at -70°C. When required, tubes were removed and the contents thawed at 37°C. The cells were washed in a medium containing 20% FCS and maintained at 4°C until placed in suspension culture. This procedure usually yielded a recovery of both nucleated cells and capacity for growth of 60%–80% of values obtained before cells were frozen.1

Leukocyte-conditioned Medium (LCM) and Phytohemagglutinin (PHA)

Leukocyte-conditioned medium (LCM) was prepared from cultures of peripheral blood cells obtained from normal volunteer donors using the method described by Iscove et al.3; in this procedure, culture medium with 20% FCS was layered over peripheral blood leukocytes immobilized in agar. After incubation for 7 days, the conditioned medium was harvested, filtered through a Nalgene 0.45-μ filter and stored at 4°C.

Phytohemagglutinin (PHA; HA 15) was obtained from Wellcome Research Laboratories, Beckenham, England.

Suspension Cultures

Cells were cultured in suspension as previously described.1 Leukocytes were suspended in 3 ml of a medium (without nucleosides) containing 20% FCS. Where appropriate, either LCM at a concentration of 20% or PHA at a concentration of 1% v/v were added to the culture. Cells were usually cultured at a concentration of 2 × 10⁵ cells/ml. However, in separation experiments, the cell concentration varied from 5 × 10⁴ to 2 × 10⁵ cells/ml; previous experiments indicate that the response in culture is linear over this range.1 Duplicate or triplicate cultures were used, and the values for each determination are shown where appropriate.

Growth was assessed by pulse-labeling the cultures with tritiated thymidine \(^3\)HTdR for 2 hr and measuring the radioactivity in trichloroacetic acid-insoluble material. The conditions for pulse-labeling have been given in detail elsewhere.1 In addition, radioautographic evidence has been presented1 that increasing incorporation of \(^3\)HTdR as a function of time in culture is a reflection of increasing numbers of labeled nuclei; the increase has a radiation sensitivity in the range usually associated with cellular proliferation. Taken together, these data support the view that \(^3\)HTdR incorporation, determined as a function of time in culture, provides a measurement of cellular proliferation.1

Chromosome Preparations

Chromosomes were prepared by the method of Tjio and Whang.4 The slides were stained with Giemsa and karyotypes prepared from photographs of appropriately spread plates.

Cell Separation by Velocity Sedimentation

The velocity sedimentation procedure (Staput) of Miller and Phillips5 was used to fractionate cell suspensions. In this procedure, nucleated peripheral blood cells were layered on a shallow gradient of fetal calf serum and allowed to sediment for approximately 4 hr at 4°C. Fractions were collected from the bottom of the sedimentation vessel and Coulter counts measured. Constant
volumes of cells were cultured from each fraction to determine the profiles of \(^{3}\)HTdR incorporation with or without LCM or PHA.

**Cell Counts**

Except for Staput fractions, nucleated cells were counted using a hemocytometer.

**Irradiation Procedures**

Aerated cells were irradiated at cell concentrations up to \(10^7/\text{ml}\) in a \(^{137}\)cesium irradiator designed by Cunningham, Bruce, and Webb,\(^6\) at a dose rate of 96 rads/min. During irradiation the tube of cells was kept surrounded by crushed ice.

**RESULTS**

**Growth Curves**

In order to obtain a preliminary characterization of the peripheral leukocytes from the three patients, their responses to LCM and PHA were measured. Frozen cells were thawed and cultured in suspension with growth medium alone (control) and with the addition of either LCM (20\%) or PHA (1\% v/v). At intervals cultures were pulse-labeled with \(^{3}\)HTdR and incorporation of the isotope into acid-insoluble material determined. The results are displayed in Fig. 1.

In agreement with our previous findings,\(^1\) great variation was observed, ranging from dependence on added stimulator for continuing \(^{3}\)HTdR incorporation (Fig. 1A, HIL) to apparent independence (Fig. 1B, MCK), with patient FEI (Fig. 1C) providing an intermediate pattern.

**Chromosomal Analysis**

Diploid cells, whether normal or leukemic, have not been observed to develop chromosome abnormalities in suspension culture for up to 10 days. However, cells in culture derived from the peripheral blood of both HIL and MCK showed chromosomal abnormalities. For HIL, the cells were pseudodiploid with a missing chromosome in group C and an extra chromosome in either group F or G. For MCK, the major line was aneuploid with a chromosome number of 47, the extra chromosome being in group C. In this patient, the abnormality was observed in peripheral blood cells before culture, and the same abnormality was seen after 7 days of culture in the presence of either LCM or PHA. For HIL, chromosome studies were not done before culture; however, cytogenetic abnormalities were present in the majority of cells cultured for 7 days with either LCM or PHA. The chromosome data are summarized in Table 2 and provide evidence that leukemic cells were proliferating in cultures of both HIL and MCK peripheral blood leukocytes. Further, the same cytogenetic abnormality was present in cultures containing either LCM or PHA. This finding is particularly significant for cells from HIL, since increased \(^{3}\)HTdR incorporation was not observed in cultures of these cells in the absence of either LCM or PHA on day 7 (Fig. 1, top panel).

**Analysis by Velocity Sedimentation**

Velocity sedimentation at unit gravity was performed on cells from patient HIL and FEI in an attempt to determine whether cells responding to PHA
could be separated from cells responding to LCM. The results are given in Fig. 2 (HIL) and Fig. 3 (FEI). The nucleated cell profile for cells from patient HIL showed a single peak with a sedimentation velocity of 4.5 mm/hr (Fig. 2, top panel). The $^3$HTdR uptake profile after 9 days in culture (Fig. 2, bottom panel) indicates that the peak response to both LCM and PHA was at approximately 3.7 mm/hr. However, the two profiles did not coincide; in the area from 5.5 to 9 mm/hr there was a relative enrichment of LCM-responsive cells compared to
Table 2. Cytogenetic Data on Leukemic Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Before Culture</th>
<th>Seven-Day Culture With LCM*</th>
<th>Seven-Day Culture With PHA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIL</td>
<td>Not done</td>
<td>n = 73</td>
<td>n = 24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>44 (5)†</td>
<td>44 (4)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45 (18)†</td>
<td>45 (2)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46 (42)†</td>
<td>46 (17)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47 (7)§</td>
<td>47 (1)§</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48 (1)†</td>
<td></td>
</tr>
<tr>
<td>MCK</td>
<td>n = 71</td>
<td>n = 82</td>
<td>n = 106</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45 (4)†</td>
<td>45 (9)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46 (19) I</td>
<td>46 (18) I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47 (48)**</td>
<td>48 (1)†</td>
</tr>
</tbody>
</table>

*The figure outside the bracket denotes the number of chromosomes per metaphase, and the figure inside the bracket represents the number of metaphases with this chromosome count.
†There was no consistent pattern of chromosome loss from any of these karyotypes.
‡Pseudodiploidy line (see text and Fig. 3)
§These karyotypes had an extra chromosome in either group F or G.
¶Chromosomes counted from spread but not karyotyped.
IThese karyotypes were diploid.
**Aneuploid line with extra chromosome in group C.
††Karyotype with two extra chromosomes in group C.

PHA-responsive cells. Incorporation of \(^3\)HTdR in cultures without PHA or LCM was very low (less than 150 cpm/culture) for all fractions.

The profile of Coulter counts for Staphyl fractions from patient FEI (Fig. 3) showed two peaks; the first of these is close to the origin, the usual position of cellular debris, and, in one experiment, examination of these fractions revealed
no intact cells. The second peak, with a sedimentation velocity of 3.5 mm/hr, represents the major nucleated cell population. The lower panel of Fig. 3 contains the results obtained when equal volumes from each fraction were cultured in growth medium alone or with either PHA or LCM for 9 days and then pulse-labeled with ³HTdR. It is evident that a good separation of the two activities was observed. A major peak of PHA-responsive cells was seen at 3 mm/hr and a minor peak at approximately 6–8 mm/hr. In contrast, the major peak of cells responding to LCM was seen in fractions sedimenting between 4 and 5.5 mm/hr. ³HTdR incorporation was also observed in control cultures of fractions with sedimentation velocities of 3–6.5 mm/hr, the peak activity being at 4.5 mm/hr. This sedimentation velocity is similar to the peak sedimentation velocity in LCM-stimulated cultures. This activity in control cultures of Staput fractions differs from results obtained when unseparated cells are used, where ³HTdR incorporation decreased after 5 days in the absence of LCM or PHA (see Fig. 1C). The reason for the difference is unknown, but might result either from enrichment of a minority population capable of ³HTdR incorporation in growth medium alone, or from the removal of inhibitor populations. Thus, in both FEI and HIL, evidence was obtained to indicate that PHA-responsive cells could be separated, at least partially, from LCM-responsive cells.

Mixing Experiments

The velocity sedimentation profiles of Figs. 2 and 3 were found to be highly reproducible. It was possible, therefore, to use the nucleated cell profiles and sedimentation velocities as the basis for pooling fractions in order to test for interactions between cell populations differing in sedimentation velocities. Two
pools of cells were prepared, pool A consisting of slowly sedimenting cells and pool B of rapidly sedimenting cells. The fractions contributing to each pool are shown in Figs. 2 and 3. Based on the sedimentation analysis, pool B should contain LCM-responsive cells with some contamination by PHA-responsive cells, while pool A should contain most of the PHA-responsive cells, although in the case of HIL, pool A would also contain a major LCM-responsive component.

These pools were used in mixing experiments designed on the following principles. In order to act as stimulator without contributing to $^3$HdR incorporation, cells of pool A are irradiated. This irradiated population incorporated very little $^3$HdR regardless of the addition or absence of PHA (Table 3, rows 5 and 6). The responding population consisted of cells of pool B. Cultures were made of these cells, with and without the addition of irradiated pool-A cells, and $^3$HdR incorporation was measured after 9 days in culture. Mirror-image experiments, using intact cells of pool A mixed with irradiated pool-B cells, were not done; these were omitted because insufficient cells were available in each Statpot fraction to permit all permutations to be tested within a single experiment, and the purpose of the present study was to investigate pool-B (LCM-responsive) cells. Separate experiments on the PHA-responsive cells of pool A were not done in order to conserve the material for other purposes.

The data from two experiments on HIL and FEI are given in Table 3. For HIL, control cultures consisted of $2 \times 10^5$ pool-B cells per ml and were made up to cell numbers equivalent to the other groups by the addition of $2 \times 10^5$ irradiated pool-B cells per ml. These cultures yielded very little $^3$HdR incorporation (Table 3, row 1). The addition of irradiated pool-A cells alone did not increase $^3$HdR incorporation (Table 3, row 2). When PHA was added to either cell mixture, significant stimulation of incorporation was observed; however, PHA and irradiated pool-A cells were five times more effective in stimu-

<table>
<thead>
<tr>
<th>Cells With or Without PHA</th>
<th>Patient HIL</th>
<th>Patient FEI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>B + B (irr)†</td>
<td>101</td>
<td>94</td>
</tr>
<tr>
<td>B + A (irr)</td>
<td>154</td>
<td>102</td>
</tr>
<tr>
<td>B + B (irr) + PHA</td>
<td>9378†</td>
<td>7235</td>
</tr>
<tr>
<td>B + A (irr) + PHA</td>
<td>39467</td>
<td>36587</td>
</tr>
<tr>
<td>A (irr)</td>
<td>159</td>
<td>201</td>
</tr>
<tr>
<td>A (irr) + PHA</td>
<td>276</td>
<td>103</td>
</tr>
</tbody>
</table>

*Both intact and irradiated cells were cultured at $2 \times 10^5$/ml to give a total of $4 \times 10^5$ cells/ml and 3 ml/culture; the results are expressed as the average of duplicate cultures. Variations between duplicates were no more than 5%.

†A (irr) and B (irr) denote cells irradiated with 1000 rads.

‡Cells of pool B were not cultivated directly in the presence of PHA only. However, the fractions contributing to the pool were cultivated with the lectin in order to construct the Statpot profile of Figs. 2 and 3. The number of cells and the $^3$HdR incorporation per fraction are known, and make possible a calculation of $^3$HdR incorporation in pool-B cells with PHA. The calculated value for expt. 1 (HIL) is 5287 cpm/culture, and expt. 1 (FEI), 7853 cpm/culture. These values make it unlikely that pool-B cells with PHA in the absence of irradiated pool-A cells would yield values of the order given in line 4 of the table.
lating \(^3\)H\text{dR} incorporation than was the addition of PHA to irradiated plus unirradiated pool-B cells (compare row 3 to row 4 of Table 3). The effect of PHA alone on pool-B cells may be attributed, at least in part, to contamination of this pool with PHA-responsive cells (see Fig. 2).

The results obtained using cells from FE! differ only in that pool-B cells with or without PHA (Table 3, rows 1 and 3) showed significant \(^3\)H\text{dR} incorporation. This finding corresponds to the incorporation in control cultures observed in Staut fractions contributing to pool B. However, as in the case of HIL, the greatest stimulation occurred in cultures of pool-B cells mixed with irradiated pool-A cells and PHA (Table 3, row 4). We conclude that an interaction occurs between cells of pool B and cells of pool A; this interaction is facilitated by PHA and leads to increased \(^3\)H\text{dR} incorporation by pool-B cells.

DISCUSSION

We have proposed\(^1\) that cells in the peripheral blood of patients with acute leukemia consist of at least three functionally distinct populations as analyzed in suspension culture. First, a minority population responds to stimulator molecules in LCM by proliferation. Second, other cells are able to respond to PHA by (1) proliferation and (2) the production of factors capable of stimulating the first population. Finally, the majority of the cells do not proliferate in culture under any of our experimental conditions.

The data presented in this paper provide support for this hypothesis. The finding that cells in mitosis after 7 days of culture have karyotypic abnormalities in two patients provides strong support that leukemic, rather than normal, cells were proliferating in these cultures. The large numbers of aneuploid metaphases in cultures with PHA in patient HIL make it unlikely that contaminating normal lymphocytes were responding to this mitogen. Rather, the PHA-responsive population appears to belong to the same leukemic clone as the LCM-responsive cells. This finding is in contrast with observations in chronic myelogenous leukemia, where PHA stimulation of peripheral blood yields cells with diploid karyotypes.\(^7\)

Fractionation of the population by velocity sedimentation supports the view that at least two, and probably three, distinct subpopulations are present. Neither PHA nor the LCM-responsive profiles coincides exactly with the major nucleated cell peak in the two patients reported in this paper. Further, in cells from the peripheral blood of patient FE!, a clear separation was obtained between the PHA-responsive and the LCM-responsive subpopulations. In HIL, some separation was obtained, although it was not as marked.

The mixing experiments (Table 3) provide evidence of a functional interaction between PHA-responsive and LCM-responsive cells. One thousand rads of irradiation obliterated the proliferative response to PHA in pooled fractions known to contain PHA-responsive cells. Irradiated cells from this pool failed to stimulate \(^3\)H\text{dR} incorporation in cells from fractions pooled on the basis of their capacity to respond to LCM. However, when PHA was added to the mixture (Table 3, row 4), a markedly increased stimulation of incorporation was observed. These results are consistent with the hypothesis that leukemic peripheral blood contains cells that can respond to PHA by proliferation and
cells that respond by stimulating the proliferation of cells in a separate population.

The mechanism of this interaction has not been demonstrated by the experiments reported in this paper. However, we have previously shown that PHA stimulation of leukemic peripheral blood leukocytes results in the production of factors capable of stimulating other leukemic cells, themselves unresponsive to PHA (see reference 1 and Table 6).

This model of interacting cell populations in leukemia is similar in some respects to that for normal granulopoiesis. Normal human marrow contains both committed granulopoietic progenitors and cells responsible for the production of factors required for the proliferation and differentiation of these progenitor cells in culture.8 The coexistence of cells capable of proliferation with cells capable of stimulating such proliferation in leukemic populations is consistent with the view that leukemic cell proliferation in culture is regulated by cellular interactions in a manner analogous to that of normal granulopoiesis.

Finally, the data in this paper contribute to the evidence for heterogeneity among patients with the clinical diagnosis of AML, observed when cells from such patients are examined in culture. This heterogeneity has been documented extensively by Moore et al. using an assay for granulocytic colony formation in culture9; for growth of leukemic cells in suspension we now add variations in the sedimentation velocities of leukemic cell populations to the variation in growth rate and requirements for exogenous stimulation reported previously.1

REFERENCES

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MT Aye, JE Till and EA McCulloch