Evidence for Differentiation of Human Leukemic Blood Cells in Diffusion Chamber Culture

By D. Hoelzer, E. Kurrle, H. Schmucker, and E. B. Harriss

Peripheral blood cells of 21 patients with different forms of acute leukemia were cultured in diffusion chambers (5 x 10^6 cells/chamber) implanted intraperitoneally in 650 R preirradiated host mice over a period of up to 21 days. In patients with acute myeloid leukemia (AML), acute erythroleukemia (AEL), or acute monomyelocytic leukemia (AMMoL), the total number of cells which developed during this culture period exceeded the implanted value and also the values for normal peripheral blood cells from ten controls. In acute undifferentiated leukemia (AUL), two out of six patients showed considerable growth whereas the others, and also two patients with acute lymphoid leukemia (ALL), had poor growth. Differential counts revealed that the rise in total cells was due mainly to proliferation of blast cells and formation of granulopoietic cells. The latter exceeded the numbers from normal peripheral blood cells in 9 out of 13 patients with AML, AEL, or AMMoL and in 2 out of 6 patients with AUL, but not in the 2 patients with ALL. The production of granulopoiesis was not restricted to proliferating cells, but included mature cells which were of abnormal morphology in some cases. From the amount of granulopoiesis and the time of its development it was assumed that they were at least partly derived from leukemic blast cells. Chromosome analyses to decide whether the granulopoietic cells were of leukemic or normal cell origin are in progress.

Lack of differentiation of blast cells in human acute leukemia in vivo could be due either to a cellular or to a regulatory defect. In recent years, leukemic cells have been extensively cultured in vitro, in semisolid media for colony growth, or in liquid suspension, to study the influence of regulatory factors. However, observations on the degree of differentiation of cells in the colonies and in liquid culture and their interpretation are conflicting.

Differentiation has been reported in some cases as similar to that in the granulopoietic colonies derived from normal cells.\(^1\)\(^3\) In contrast, Moore et al.\(^4\) found that, although some degree of maturation occurred in most cases, this might be abnormal or arrested at the promyelocyte-myelocyte stage. A critical appraisal by Morley and Higgs\(^5\) led to the conclusion that, whereas in a small proportion of patients the appearance of cultures could not be clearly distinguished from that of controls, in the majority only qualitatively abnormal cells developed with wide variation in the degree of differentiation. Similarly, in liquid culture, differentiation may occasionally proceed to mature granulocytes.\(^6\)\(^8\) It thus seems that there was great heterogeneity between patients but

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that, in some cases of AML, differentiation of leukemic cells in vitro is possible, in rare cases up to mature granulocytes.

It is reasonable to postulate that in AML the cells affected are as undifferentiated as pluripotent stem cells (CFU-S), and the culture system used for investigation in vitro should support the proliferation and differentiation of stem cells earlier than colony-forming cells (CFU-C). An approach to the problem is provided by the diffusion chamber (DC) technique developed for hemopoietic cells by Benestad and Bøyum and Borgström and adapted for human cells by Bøyum et al. In this system, proliferation of normal CFU-S from mouse bone marrow and of granulopoietic progenitors (CFU-C) from human peripheral blood has been demonstrated. Therefore, in the present study, the ability of peripheral blood cells from 21 patients with various forms of acute leukemia to proliferate and to differentiate in DC has been studied.

MATERIALS AND METHODS

Patients. Peripheral blood cells from 23 patients with varying forms of acute leukemia were studied in culture. All were untreated at the time of the investigation, which was at diagnosis for 19 patients and at the first relapse for 4 patients (Z.K., S.A., T.A., and C.L.). Results from two patients were not evaluated, since in one case the patient had a pseudomonas infection and the cultures became infected and in the other there was only sufficient material available to follow the DC culture up to day 9. Clinical and hematologic details of the 21 patients presented here are given in Table 1. The diagnoses of acute myeloid leukemia (AML), acute erythroleukemia (AEL), acute myelomonocytic leukemia (AMMoL), acute undifferentiated leukemia (AUL), and acute lymphoid leukemia (ALL) were based on cell morphology and cytochemistry (peroxidase, naphthol-AS-D-acetate-esterase, and PAS staining) of blood and bone marrow smears. One patient (T.R.) had a previous history of polycythemia vera. Leukocyte counts in peripheral blood ranged from 2.8 to 664 x 10^9 cells/liter and blast cells from 4% to 94%.

Cell suspension for implantation. Blood cells rather than bone marrow cells were chosen for this study in leukemic patients to avoid the problem of confusing new granulopoiesis with possible survivors of immature granulopoietic cells from the inoculum. From peripheral blood samples of 20-40 ml, mononuclear cells were separated by removal of granulocytes and erythrocytes using the Isopaque-Ficoll method of Bøyum. The cells were resuspended in TC 199 medium buffered to pH 7.2 containing 500 units penicillin and 50 μg streptomycin/ml at a cell concentration of 5 x 10^6 cells/ml. With this method of separation, the average granulocyte content of the blood for 10 normal persons was reduced from 56%, to 1.3%, and in leukemic patients from 18% to 1.9% (see Table 1). For some patients (indicated in Table 1) who required treatment upon admission, these technically time-consuming investigations could not be immediately carried out, so the cells were stored under liquid nitrogen, with dimethylsulfoxide as cryoprotective agent, and used later.

Diffusion chamber technique. The diffusion chamber technique according to Benestad, Bøyum and Borgström, and Breivik et al. was used, as described in detail previously. Diffusion chambers were constructed by gluing MF Millipore filters of pore size 0.22 μm to each side of a Millipore Plexiglas ring of 10-mm inner diameter and 2-mm thickness with Millipore MF cement. The chambers were tested for leaks and sterilized by dry heat at 80°C before being filled with 0.1 ml cell suspension (5 x 10^6 cells) and sealed.

Host animals and implantation. CBA mice, 25-30 g in weight and 2 to 3 mo old were given 650 R whole body irradiation (300 kV, Thoraeus II filter, HVL 3.6 mm Cu, 28 R/min) 4-6 hr before implantation of two chambers each into the peritoneal cavity. At weekly intervals, chambers were removed, cleaned of adhering connective tissue, and reimplanted into fresh irradiated hosts.

Harvesting. For each patient, four to six chambers were harvested at 1, 3, 6, 9, 13, 17, 21 days after implantation. The chambers were shaken for 1 hr in 0.5% pronase in buffered Hanks’ solution...
Differentialiation of leukemic cells

Table 1. Clinical and Hematologic Data on Patients and Cell Suspensions

<table>
<thead>
<tr>
<th>Patient (Age, Sex)</th>
<th>Peripheral Blood</th>
<th>Cell Suspension</th>
<th>Granulopoietic Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type of Leukemia</td>
<td>WBC (x 10⁹/liter)</td>
<td>Blast Cells</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>P.G. (31, M)</td>
<td>AML</td>
<td>45.2</td>
<td>54.5</td>
</tr>
<tr>
<td>H.J.* (27, F)</td>
<td>AEL</td>
<td>7.0</td>
<td>37.0</td>
</tr>
<tr>
<td>H.E. (17, F)</td>
<td>AEL</td>
<td>2.8</td>
<td>22.0</td>
</tr>
<tr>
<td>B.G. (53, M)</td>
<td>AML</td>
<td>167.0</td>
<td>74.8</td>
</tr>
<tr>
<td>H.T. (49, F)</td>
<td>AML</td>
<td>5.7</td>
<td>29.0</td>
</tr>
<tr>
<td>H.F. (56, M)</td>
<td>AML</td>
<td>4.0</td>
<td>14.8</td>
</tr>
<tr>
<td>Z.K. (64, M)</td>
<td>AML</td>
<td>4.5</td>
<td>41.5</td>
</tr>
<tr>
<td>B.B.* (66, F)</td>
<td>AML</td>
<td>272.5</td>
<td>82.0</td>
</tr>
<tr>
<td>B.S.* (36, F)</td>
<td>AML</td>
<td>7.5</td>
<td>65.0</td>
</tr>
<tr>
<td>K.J.* (42, M)</td>
<td>AML</td>
<td>183.0</td>
<td>90.0</td>
</tr>
<tr>
<td>T.R.* (67, M)</td>
<td>AML</td>
<td>664.0</td>
<td>84.0</td>
</tr>
<tr>
<td>R.H.* (48, M)</td>
<td>AUL</td>
<td>198.4</td>
<td>80.0</td>
</tr>
<tr>
<td>P.H.* (68, M)</td>
<td>AMML</td>
<td>109.6</td>
<td>62.5</td>
</tr>
<tr>
<td>S.A. (23, F)</td>
<td>AUL</td>
<td>38.7</td>
<td>86.0</td>
</tr>
<tr>
<td>T.A. (18, F)</td>
<td>AUL</td>
<td>29.0</td>
<td>66.2</td>
</tr>
<tr>
<td>K.A.* (29, M)</td>
<td>AUL</td>
<td>4.1</td>
<td>4.0</td>
</tr>
<tr>
<td>H.E.* (18, F)</td>
<td>AUL</td>
<td>44.1</td>
<td>90.5</td>
</tr>
<tr>
<td>C.L.* (37, F)</td>
<td>AUL</td>
<td>13.8</td>
<td>68.0</td>
</tr>
<tr>
<td>S.B.* (17, F)</td>
<td>AUL</td>
<td>524.8</td>
<td>94.0</td>
</tr>
<tr>
<td>B.J. (59, F)</td>
<td>ALL</td>
<td>19.5</td>
<td>10.5</td>
</tr>
<tr>
<td>L.R.* (19, F)</td>
<td>ALL</td>
<td>354.0</td>
<td>88.0</td>
</tr>
</tbody>
</table>

Mean for 10 normal persons 5.5 82.2 16.5 0.1 1.1

AML, acute myeloid leukemia; AUL, acute undifferentiated leukemia; AEL, acute erythroleukemia; ALL, acute lymphoid leukemia; AMML, acute myelomonocytic leukemia.

*Cells stored under liquid nitrogen.

To liquify the clot, from the resulting single cell suspension, the total nucleated cells were determined for individual chambers. Suspensions were centrifuged for 5 min at 200 g into 20% albumin, and smears for differential counts were prepared from individual chambers; good agreement between single chambers was found. Later, total nucleated cells were counted for single chambers, and smears made from the pooled cells. Smears were stained with May-Grünwald-Giemsa and in 11 investigations also for peroxidase.

Evaluation. Differential counts of nucleated cells were performed on 200 cells on each of two slides for peripheral blood smears and on 500 cells on each of two slides for cell suspensions before implantation. For cell suspensions from DC, 500 cells for each of four individual chambers and 500 cells on each of two slides for pooled chambers were counted.

Cells were classified as blast cells; proliferating granulopoietic cells (promyelocytes and myelocytes); nonproliferating granulopoietic cells (metamyelocytes, juveniles, bands, and segments); macrophages; and lymphocytes. The percentage of peroxidase-positive cells was determined from examination of 1000 nucleated cells.

RESULTS

Total Nucleated Cells

Figure 1 gives the change in total nucleated cells per chamber with duration of DC culture for the patients studied. In normal persons (shaded area), there
Fig. 1. Change in number of total nucleated cells per chamber with duration of diffusion chamber culture. The SD of four to six chambers at each interval is given as a vertical bar. (A) Patients with AML, AEL (●), or AMMoL (○). (B) Patients with AUL (●) or ALL (○). The thick line and shaded area represent the mean ± SEM for ten normal persons.
Fig. 2. Change in number of blast cells per chamber with duration of diffusion chamber culture. Mean ± SEM for ten normal persons are represented as in Fig. 1. (A) Patients with AML, AEL (●), or AMMol (○). (B) Patients with AUL (●) or ALL (○).
Fig. 3. Change in number of proliferating granulopoietic cells (promyelocytes and myelocytes) per chamber with duration of diffusion chamber culture. Mean ± SEM for ten normal persons represented as in Fig. 1. (A) Patients with AML, AEL (•), or AMMoL (○). (B) Patients with AUL (•) or ALL (○).
was an initial decrease in total nucleated cells up to day 6, followed by an increase to a maximum at day 13 of about twice the number of cells implanted.

In the leukemic patients of group I (AML, AEL, and AMMoL, Fig. 1A), the cell number decreased initially only up to day 1 or day 3 and then increased in most cases over varying periods, sometimes exponentially, with no clear-cut maximum but well above the normal level in general. In the patients with AUL (Fig. 1B) by contrast, the initial decrease in cell number was more marked and prolonged to at least day 6 with little increase thereafter, and only in two cases did growth reach or exceed the normal level. In two cases of ALL there was no growth over the period of observation.

The main types of cells which developed were blast cells, granulopoietic cells, macrophages, and lymphocytes. Megakaryocytes and erythropoietic cells were occasionally seen, but not in all cases.18

**Blast Cells.**

From Fig. 2 it is clear that the pattern of total nucleated cells in leukemic patients was largely governed by blast cell growth. In group I (Fig. 2A), blast cells increased in most cases after day 1 to values greatly exceeding normal, whereas in patients with AUL (Fig. 2B), a definite increase was seen in only three out of six cases. For the two patients with ALL, cell numbers again decreased throughout.

In normal persons, the blast cells, rising to over $2 \times 10^5$ cells per chamber at day 13, might have originated from stimulated lymphocytes that had undergone blast transformation and from blast cells that later developed into granulopoietic (or rarely erythropoietic or megakaryopoietic) precursors.

**Proliferating Granulopoietic Cells**

No remarkable numbers of proliferating granulopoietic cells were found in normal persons before day 6, when they could be identified with certainty in three out of ten persons. There followed a continuous increase up to day 21, with the mean value reaching $20,000 \pm 10,000$ (SEM) cells per chamber (Fig. 3).

In leukemic patients of group I (Fig. 3A), a rise was observed as early as 1–3 days after initiation of culture, and cell numbers exceeded normal in 9 out of 13 cases. In AUL (Fig. 3B), the more variable pattern gave an increase to values less than or equal to normal in four out of six cases, whereas the other two rose well above the normal level. It should be noted that in this group no increase was observed before 6–9 days after chamber implantation. The two patients with ALL showed a slight rise in proliferating granulopoietic cells.

**Nonproliferating Granulopoietic Cells**

In normal persons, the initial slow decrease to day 6 was probably attributable to death of those mature granulocytes initially implanted. The rise thereafter proceeded to $16,000 \pm 6,000$ (SEM) cells per chamber at day 21, exceeding the implanted number by two- to threefold (Fig. 4).

Development of granulopoietic cells proceeded beyond the myelocyte stage also in leukemic patients. In group I (Fig. 4A), numbers of nonproliferating granulopoietic cells increased above their implanted level and above the nor-
Fig. 4. Change in number of nonproliferating granulopoietic cells (metamyelocytes, juveniles, band and segmented granulocytes) per chamber with duration of diffusion chamber culture. Mean ± SEM for ten normal persons are represented as in Fig. 1. (A) Patients with AML, AEL (e), or AMMoL (o). (B) Patients with AUL (e) or ALL (o).
mal level in most cases. The increase began at different times, and in general not before day 9. From the patients with AUL (Fig. 4B), the three cases which had the most rapid increase in proliferating granulopoietic cells (C.L., K.A., and S.B.) showed also a marked rise in nonproliferating granulopoietic cells. A slight increase was observed for the two cases of ALL.

In normal persons, the ratio of nonproliferating to proliferating granulopoietic cells which developed was about 0.8. This relation indicated that, even for normal peripheral blood cells, maturation was not complete in our culture conditions. A similar finding was made for normal bone marrow cells by Fauерholdt and Jacobsen19 and by Øyum et al.,12 although in contrast Squires20 observed a ratio of ≤5:1 between nonproliferating and proliferating granulopoiesis, more like the situation in the bone marrow in vivo. In comparison with normal, the relation of proliferating to nonproliferating granulopoiesis from patients with AML, AEL, or AMMoL varied considerably, and in two cases there were even more nonproliferating than proliferating cells.

**Peroxidase-positive Cells**

For the ten normal persons, the number of peroxidase-positive cells per chamber decreased to day 3, and rose after day 9 to reach the initially implanted number by day 17. In all the 11 leukemic patients for whom peroxidase staining was done, an increase was observed after day 3. Even the two patients

![Graph showing change in number of peroxidase-positive cells per chamber with duration of diffusion chamber culture. Mean ± SEM for ten normal persons are represented as in Fig. 1. Patients with AML, AEL, or AMMoL ( ). Patients with AUL ( ).](image)
with AUL, with low initial values, showed a marked increase in peroxidase-positive cells (Fig. 5).

Cell Morphology

Blast cells harvested from chambers were identified as having fine nuclear chromatin structure, one or more nucleoli and basophilic cytoplasm. Sometimes the blast cells were very large with giant nuclei, and in some cases they showed vacuolation. If cells had granules in the cytoplasm but retained the fine nuclear structure, they were still counted as blast cells. The percentage of blast cells having such cytoplasmic granules varied from 0\(^\circ\) to 90\(^\circ\) between patients. As promyelocytes or myelocytes, only cells having signs of nuclear maturation, i.e., more condensed chromatin were counted. Differentiation up to mature myelocytes was observed in all cases except one (B.S.), and up to band and segmented granulocytes in 16 cases. Often the granulopoietic cells were abnormal with poorly developed granules in the cytoplasm, irregularly shaped

Fig. 6. Morphology of cells from patient R.H. (case 12) with AML. May-Grünwald-Giemsa, \(\times 1250\). (A) Day 0, blast cells implanted; (B) day 13, myelocytes; (C) day 13, metamyelocytes; (D) day 17, band and segmented granulocytes.
DIFFERENTIATION OF LEUKEMIC CELLS

A

B

C

D

Fig. 7. Morphology of cells from patient C.L. (case 18) with AUL. May-Grunwald-Giemsa, x 1250. (A) Day 0, blast cells and lymphocyte from cell suspension implanted; (B) day 17, group of granulopoietic cells with cytoplasmic granules; (C) day 20, segmented granulocytes; (D) day 20, abnormal segmented granulocytes with immature nuclear structure.

or hypersegmented nuclei, but these abnormalities were also observed to a certain extent in cells developing from normal blood cells. Degenerated blast cells, appearing superficially similar to polymorphonuclear cells, could in general be differentiated from granulopoietic cells. Examples of the types of cells observed are shown in Figs. 6, 7, and 8.

DISCUSSION

The present study shows that remarkable numbers of granulopoietic cells can arise from the DC culture of peripheral blood cells from leukemic patients. An important question is whether these cells are descendants of leukemic cells or of normal stem cells. One possibility of solving this problem is to undertake a cytogenetic analysis of the cells in culture. The few reports of chromosome studies of leukemic cells cultured in the agar or methyl cellulose techniques demonstrate that leukemic cells can form colonies in vitro and are capable of
Fig. 8. Morphology of cells from patient T.R. (case 11) with AML. May-Grünwald-Giemsa, x 940. (A) Day 0, blast cells implanted; (B) day 13, myelocytes and large unclassifiable cell; (C) day 17, atypical mature granulopoietic cells; (D) day 17, granulopoietic cells of various stages of differentiation including segmented granulocytes.

varying degrees of differentiation into granulopoietic cells. In two cases of acute and one of subacute myeloid leukemia investigated cytogenetically by Moore and Metcalf,21 which had abnormal karyotypes in direct bone marrow analysis, pooled colony cells had similar karyotypic abnormalities with differentiation proceeding to the myelocyte/metamyelocyte stage in AML and to predominantly mature polymorphs in subacute myeloid leukemia. Individual colonies analyzed by Aye et al.,22 having the original abnormal karyotype differentiated only to the promyelocyte stage; in another patient, differentiation and karyotype were normal in spite of an abnormality in direct bone marrow preparations. Colonies containing cells with leukemic karyotype have also been found by Duttera et al.23 for AML in remission and by Dicke et al.24 for AML cells pretreated with phytohemagglutinin.

In these investigations no further cell separations have been made in order to implant a highly enriched leukemic cell population, and chromosome analysis
 DIFFERENTIATION OF LEUKEMIC CELLS

Table 2. Relationship of Granulopoietic Cells Developing in Diffusion Chambers to the Number of Lymphocytes in the Implanted Cell Suspension

<table>
<thead>
<tr>
<th>Patient</th>
<th>Type of Leukemia</th>
<th>No. of Lymphocytes Implanted x 10^3</th>
<th>Granulopoietic Cells (Maximum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. P.G.</td>
<td>AML</td>
<td>105</td>
<td>22</td>
</tr>
<tr>
<td>2. H.J.</td>
<td>AML</td>
<td>41</td>
<td>21</td>
</tr>
<tr>
<td>3. H.E.</td>
<td>AML</td>
<td>206</td>
<td>17</td>
</tr>
<tr>
<td>4. B.G.</td>
<td>AML</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>5. H.T.</td>
<td>AML</td>
<td>226</td>
<td>13</td>
</tr>
<tr>
<td>6. H.F.</td>
<td>AML</td>
<td>358</td>
<td>17</td>
</tr>
<tr>
<td>7. Z.K.</td>
<td>AML</td>
<td>125</td>
<td>21</td>
</tr>
<tr>
<td>8. B.B.</td>
<td>AML</td>
<td>35</td>
<td>17</td>
</tr>
<tr>
<td>9. B.S.</td>
<td>AML</td>
<td>122</td>
<td>None found</td>
</tr>
<tr>
<td>10. K.J.</td>
<td>AML</td>
<td>46</td>
<td>13</td>
</tr>
<tr>
<td>11. T.R.</td>
<td>AML</td>
<td>166</td>
<td>17</td>
</tr>
<tr>
<td>12. R.H.</td>
<td>AML</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td>13. P.H.</td>
<td>AML</td>
<td>84</td>
<td>13</td>
</tr>
<tr>
<td>14. S.A.</td>
<td>AUL</td>
<td>32</td>
<td>17</td>
</tr>
<tr>
<td>15. T.A.</td>
<td>AUL</td>
<td>50</td>
<td>Decrease</td>
</tr>
<tr>
<td>16. K.A.</td>
<td>AUL</td>
<td>254</td>
<td>17</td>
</tr>
<tr>
<td>17. H.E.</td>
<td>AUL</td>
<td>278</td>
<td>17</td>
</tr>
<tr>
<td>18. C.L.</td>
<td>AUL</td>
<td>47</td>
<td>17</td>
</tr>
<tr>
<td>19. S.B.</td>
<td>AUL</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>20. B.J.</td>
<td>ALL</td>
<td>420</td>
<td>17</td>
</tr>
<tr>
<td>21. L.R.</td>
<td>ALL</td>
<td>39</td>
<td>21</td>
</tr>
</tbody>
</table>

Mean for 10 normal persons 392 17-21 36
SEM ± 17 ± 16

studies are still in progress. However, from the amount and time of appearance of granulopoietic and peroxidase-positive cells it can be deduced that they were at least partly derived from leukemic blast cells (LBC). The absolute numbers of granulopoietic cells that developed from leukemic peripheral blood cells exceeded those from normal cells by up to 40-fold. Table 2 gives a comparison of absolute numbers of lymphocytes implanted and maximum numbers of granulopoietic cells appearing during DC culture for normal and leukemic blood cells. In ten normal persons, an average of 36,000 granulopoietic cells were produced, and it must be assumed that they were derived from stem cells included in the cell population considered morphologically as lymphocytes. Since for leukemic patients, LBC formed an average of 67% of the cell suspension (Table 1), the number of lymphocytes implanted was correspondingly less than in normal persons (Table 2). If these included a similar concentration of stem cells to normal one would expect far less granulopoiesis to develop, but the contrary was found. It was therefore concluded that in most of the leukemic cases studied, LBC were able to differentiate along the granulopoietic pathway, even in some cases of so-called undifferentiated leukemia. An alternative explanation might be that the concentration of normal stem cells was increased in leukemia or that the concentration was normal, but that they were stimu-
lated to produce more granulopoiesis than normal. Both these suggestions seem unlikely, in view of the granulocytopenia found in vivo. A further argument in favor of the leukemic cell origin of granulopoietic cells in DC culture might be that, in a few cases (e.g., B.B., Z.K., H.T.), they appeared in large numbers during the first few days, whereas from normal peripheral blood cells they were not observed before day 6.

The increase of peroxidase-positive cells from patients with AML, AEL, or AMMoL to numbers much higher than normal might be another indication for differentiation of LBC. This conclusion is limited in patients with AML because the leukemic cells themselves are peroxidase positive, and their proliferation alone without further differentiation would lead to an increase, but at least in the two patients with AUL and few peroxidase-positive blast cells initially (the peroxidase-positive cells for C.L. on day 0 were granulocytes), the increase might be interpreted as differentiation of LBC.

The proliferation and differentiation of LBC might be due to the absence of an in vivo acting inhibition and/or to the stimulation provided in the DC culture system. Release from inhibition cannot be the only explanation, since in parallel studies of these patients' cells in agar culture without adding any source of colony-stimulating factor, no colony or cluster formation has been observed in most of the cases with AML, AEL, or AMMoL. Therefore stimulation provided by the irradiated mouse host seems to be largely responsible. Irradiation of the host animals increases granulopoiesis in DC culture and the effect is probably dose-dependent, but the factors which promote such growth are as yet unidentified. Gordon and Blackett have demonstrated that a factor which stimulates colony growth from CFU-C in the agar system in vitro can be found in DC, and it has been postulated that other factors must be present which may promote the growth of earlier stem cells, as indicated by the proliferation of murine CFU-S in chambers.

The fact that peripheral blood cells from most of the leukemic patients developed granulopoiesis in DC but only a few produced appreciable numbers of colonies in our parallel agar cultures even when stimulated (unpublished data), as well as the fact that Morley and Higgs failed to observe increased differentiation of leukemic cells with increased concentrations of colony stimulating factors in agar culture might mean that the leukemic cells which were stimulated in DC were of earlier ancestry than CFU-C, and responsive to some factor which stimulated CFU-S. The continuity of the stimulation in vivo and its renewal by reimplantation of DC into freshly irradiated hosts may also play an important part.

Fauerholdt and Jacobsen did not observe any marked proliferation or differentiation in DC culture of bone marrow cells from eight patients with AML. Reasons for this difference might be the relatively low radiation dose (500 R) to the host mice or the fact that bone marrow was investigated, since mature granulocytes or their extracts can inhibit granulopoiesis in DC. It might also be due to the strain of host mice used or other minor variations in the DC technique. In contrast, in the present investigations granulopoiesis developed in most cases of AML, and in some cases of AUL and, for the reason discussed above, was probably derived from LBC. It is therefore concluded that
LBC are not inherently unable to differentiate, but that they can do so under suitable culture conditions.

Addendum

Cytogenetic studies have been started in collaboration with Dr. K. P. Hellriegel (Department of Internal Medicine, University of Cologne). From three patients (cases 11–13) who originally developed appreciable numbers of granulopoietic cells in diffusion chambers, frozen cells were again cultured and chromosome analyses made at day 11 of culture. In each investigation, sufficient numbers of mitoses suitable for cytogenetic study were found. In all three cases, clonal numerical and structural chromosome aberrations, partly including marker chromosomes, were observed. In addition, a few mitoses with normal karyotypes were seen, which might be descendants from a remaining normal stem cell population. These preliminary results indicated that at least part of the diffusion chamber cells in mitosis were of leukemic origin.

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