Cultivation of Leukemic Human Bone Marrow Cells in Diffusion Chambers Implanted Into Normal and Irradiated Mice

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In order to elucidate the question of whether the maturation defect in vivo in acute leukemia is due to environmental or cellular factors, we have cultured human leukemic cells in a nonleukemic milieu, i.e., diffusion chambers implanted into the abdominal cavity of normal and irradiated mice. For each harvest, the cell count was measured and differential counts and the number of peroxidase-positive cells determined. The cell number decreased with time, without significant difference between culture in irradiated (500 rads) and normal mice. The blast cells succeeded only in developing distorted promyelocytes and myelocytes. There was a general pattern of increase in the number of peroxidase-positive cells. The study supports the concept that acute myeloid leukemia (AML) is a disturbance of cellular maturation due to cellular rather than environmental defects.

ONE OF THE classic questions about acute leukemia is whether the maturation defect in vivo is due to environmental or cellular factors. Recently, methods for growing mouse bone marrow cell colonies in agar-gel medium have been described by Pluznik and Sachs1 and Bradley and Metcalf.2 Later, the technique was applied to human bone marrow cells, first by Senn, McCulloch, and Till,3 and subsequently by Pike and Robinson.4 Also, leukemic human bone marrow cells and peripheral blood cells have been cultured.3-9 Of particular interest has been the use of this system to study the colony-forming and differentiating potential of leukemic cells.

The results of culturing leukemic human bone marrow cells and peripheral blood in soft agar varies considerably, both with respect to numbers and maturation. Robinson and Pike5 and Robinson et al.6 found decreased numbers of colonies but maturation similar to that observed by cultivating normal bone marrow and blood. Greenberg et al.7 found either no colonies or formation of excessive numbers of small abortive colonies and normal cellular maturation. Moore et al.8 made the same observation concerning the ratio of colonies to clusters (cluster, 3-40 cells); with regard to the morphology, they found that cellular differentiation occurred, but it was usually abnormal. On the other hand, Paran, Sachs et al.9 found the cloning efficiency 30 times higher than the average cloning efficiency from nonleukemic patients, and furthermore, the colonies were larger. The degree of maturation in these colonies was the same as in the colonies from normal persons. However, the results of Paran et al.6

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are based on only one AML bone marrow and two peripheral blood cultures. Paran et al. were inclined to conclude from these few data that the apparent differentiation in vitro is due to a colony-stimulating factor (CSF), and proposed CSF as a way to treat leukemia.

Golde and Cline have cultured normal and leukemic bone marrow and blood cells using an in vitro diffusion system; in one culture, initiated with peripheral blood myeloblasts, mature neutrophils constituted the majority of cells in culture at day 25. The other three cases were not morphologically described.

The last culture method applied in the hematologic field is the in vivo diffusion chamber method. The technique was introduced by Algire et al. and was first specially used for hematopoietic cells by Berman and Kaplan, who cultured mouse bone marrow cells for up to 30 days. Later the method was elaborated by Benestad and Bøyum and Borgstrøm, who cultured mouse bone marrow and peripheral blood cells. Normal human bone marrow cells in diffusion chambers have been grown by Bøyum et al. In the present study, we used the in vivo diffusion chamber technique for culturing human leukemic bone marrow cells in order to elucidate further the question of the in vivo maturation defect of leukemic cells.

**MATERIALS AND METHODS**

*Nonleukemic Bone Marrow*

To illustrate the applicability of the method for the study of normal human bone marrow cell growth and differentiation, the results of one typical normal case are included in this paper (Fig. 1).
Leukemic Bone Marrow

Bone marrow was obtained from 12 untreated patients with acute myeloid leukemia (AML). Four patients were excluded because of technically poor slides. The total material thus comprises that of eight patients. Bone marrow was aspirated from the posterior iliac spine or the sternum into a syringe containing EDTA (1 part 1% Na₂-EDTA in 0.7% NaCl to 4 parts bone marrow cell suspension). The bone marrow particles were broken down by repeated aspiration through a needle, size 80 x 0.8 mm. The suspension was then separated according to Byum et al. The plasma layer containing the majority of nucleated bone marrow cells was pipetted off and washed two times in Hanks’ solution containing 20% human serum, type AB, tested and found negative for cytotoxic antibodies. The cells were counted in a Neubauer hemocytometer, diluted with Hanks’ solution to the desired concentration, and injected into diffusion chambers; the final serum concentration was less than 0.1%. Trypan blue staining showed less than 3% stained cells.

Diffusion Chamber Culture

The chambers were made according to Benestad but with a tube glued (Intramedic polyethylene tubing PE 60; interior diameter, 0.030 inches) to a radially located hole in the ring. The chambers were sterilized by radiation (4.5 Mrads at the Danish Atomic Energy Establishment, Ris). The chambers were filled with marrow suspension through the tube and subsequently closed by melting the plastic tube and squeezing it with a pair of tweezers. The number of injected cells varied from \(4 \times 10^6\) to \(10^8\), in a volume of 150 \(\mu\)l. After filling, the chambers were placed in antibiotic Hanks’ solution (TC Penicillin- Streptomycin, Difco) at room temperature until implantation.

Mice and Implantation Procedure

Eight to ten-week-old outbred female mice of the NMRI strain, weighing 20-25 g, were used as chamber recipients (mice were provided by Dr. Mølgaard-Hansen, Ll. Skensved, Denmark). This is a type with a very small incidence of spontaneous leukemia. The same strain was used as chamber recipient when we cultured normal human bone marrow. In those cases, leukemia did not develop in the implanted cells during culture. All mice received 500 rads whole-body irradiation from a 200-kVp x-ray machine, at a dose rate of 46 rads/min, 3 or 24 hr before chamber implantation. The chambers were placed in the peritoneal cavity during nembutal anesthesia. Controls showed that cell growth was independent of whether one or two chambers were present in the peritoneal cavity. On day 0, all the chambers were implanted into irradiated mice. On day 7, they were retransplanted, half the chambers to irradiated mice, the remainder to unirradiated mice. The cells in the chambers were cultured for 13-40 days, in most cases around 19 days. For each culture, between two and seven harvests were done (average, five). Each harvest consisted of at least four chambers from each group.

Chamber Cell Harvest

The mice were killed by neck luxation. The connective tissue formed around the chambers after the first days of culture was removed, and the chambers, still closed, were then shaken for 70 min in 3 ml of 0.5% Pronase solution (Pronase P, Serva) with 5% Ficoll (Pharmacia, Uppsal, Sweden) dissolved in Hanks’ solution. Thereafter, the content was removed. Initially this was done by aspiration through the tube glued to the plastic ring, later through a hole made in the Millipore filter. The chambers were then washed by Hanks’ solution. The content was weighed and samples taken for cell counts. Some smears were stained with May-Grünwald-Giemsa, others were stained with peroxidase and counterstained with Giemsa. A differential count of at least 100 cells was performed on each smear. As in the initial inoculum, all cells present were comprised by the differential count. The membranes freed from the plastic ring were attached to slides and stained with hematoxylin-eosin. None or very few cells were found on the inside of the filters.

Statistical Method

The non-parametric Mann-Whitney’s test was used.
RESULTS

Normal Bone Marrow

The variation of cell counts as a function of time in one typical case is illustrated in Fig. 1. After retransplantation on day 7, half the chambers to irradiated mice and the remainder to normal mice, the number of cells was significantly ($p < 0.05$) higher on days 14, 17, and 19 in the irradiated recipient.

The differential counts showed the following distribution until approximately day 20 in the chambers retransplanted to irradiated mice: segmented neutrophils, 20%; metamyelocytes, 18%; myelocytes, 30%; promyelocytes, 4%; myeloblasts, 2%; macrophages, 12%; and lymphocytes, 14%. After this time, the number of macrophages starts to increase and the proliferative part of the myeloid compartment decreases. The differential count on day 28 shows 24% segmented neutrophils and 76% macrophages. In the chambers retransplanted to normal mice the percentage of macrophages was higher: about 20 at day 12; otherwise the proportional distribution was the same. The morphology of the myeloid cells was normal, with no signs of distorted cells or nucleocytoplasmic dysdifferentiation.

Leukemic Bone Marrow

Proliferation in vivo. The variation of cell counts as a function of duration of culture is illustrated in Fig. 2. A common growth pattern was a fall in cell number until approximately day 6, followed by a rise and then usually by a second decline. One experiment showed a second rise between days 33 and 40; the MGG-stained slides on the corresponding days were morphologically poor, but on day 33 most of the cells were macrophages, while on day 40 there was unmistakable myelopoiesis.

On day 7, half the chambers were transplanted to irradiated mice and half to normal mice. The cell number was, in almost all cases, higher in the irradiated
mice than in the normal mice, but did not reach a significant level ($p > 0.05$). Otherwise the curves were parallel. The percentage of lymphocytes and macrophages was 10% or less and almost constant at all time points.

*Maturity in vivo.* Patterns of cell maturation in culture are shown in Fig. 3. Cells were divided in nongranulated blast cells and in granulated cells. Some cells succeeded in becoming promyelocytes: large cells with azurophilic granules and with a loose nucleus with nucleoli; some cells appeared slightly more mature with a slightly more condensed nucleus without nucleoli or with a more oval nucleus than promyelocytes. However, this type of differentiation was always distorted, and normal-looking myelocytes were never observed. Also, cells differentiated further than the myelocyte stage were not present. There was an absolute increase in promyelocytes in all eight cases (Fig. 3); this,
however, took place at different days. The absolute number of blast cells decreased with time in culture. Again, no difference was observed between cells cultured in irradiated or in normal mice.

**Peroxidase-stained cells.** The patterns of the peroxidase reaction are shown in Fig. 4. The number of peroxidase-positive cells varied greatly. However, most cases showed, at variable time points, a rise in the absolute number of peroxidase-positive cells, as an expression of the ability of the cells to form azurophilic granules in the diffusion chambers.

With respect to change in absolute numbers of peroxidase-positive cells, there was no difference between cells cultured in irradiated and in normal mice. The absolute number of granulated cells (May-Grünewald-Giemsa stain) was consistently lower than the absolute number of peroxidase-positive cells. However, the difference varied considerably from one culture to another. The explanation probably is that before the azurophilic granules are visible in the microscope, the peroxidase reaction becomes positive.17

**DISCUSSION**

In this study, the in vivo diffusion chamber culture technique has been used to elucidate the question whether leukemic bone marrow cells can differentiate when transplanted to a nonleukemic milieu. The cellular proliferation, as measured by cell counts, was not found to be substantially different from that of normal bone marrow cells.16 Böyum et al.16 found that preirradiation (500–600 rads) of the host animals enhanced the growth of normal human cells in the granulocytic series, as measured by the number of cells. Moreover, the cellular composition changed, so that the percentage of cells in the proliferating granulocytic pool increased in the irradiated group. This difference became apparent after a culture period of 9 days. A similar enhancing effect was found in the present study of normal marrow (Fig. 1); in contrast, no significant effect on the number of cells was found when leukemic cells were cultured in irradiated mice.

Definite in vivo maturation of leukemic cells, as is seen when normal human bone marrow cells are cultured was not observed in this study. In this respect, there was no difference between chambers implanted into irradiated and normal mice. Some blast cells made attempts to differentiate to promyelocytes and myelocytes, but this differentiation was always distorted. Normal-looking myelocytes and fully differentiated neutrophils were never observed. There was an absolute increase in the number of promyelocytes, so the result cannot be explained only by a preferential death of blast cells.

As mentioned in the introduction, reported observations concerning the ability of human leukemic cells to differentiate in vitro vary from completely normal maturation5–7 to dysdifferentiation with signs of nucleo-cytoplasmic dissociation.8 These culture systems depend on CSF. Whether CSF plays a role in the diffusion chamber technique is not known. On the other hand, it must be emphasized that, to our knowledge, there is no information that leukemic cells with abnormal karyotypes have been converted into normally differentiating cells with normal karyotypes by the presence of CSF. In the diffusion chamber assay, the leukemic cells were transferred to a xenogenic, but nonleukemic, environment, and only pseudodifferentiation was observed; at least the cells could not pass the myelocyte stage.
The number of peroxidase-positive stained cells showed a general pattern of increase with time in culture. This may illustrate a certain, although limited, ability of the cells to differentiate in the chambers.

In conclusion, under the conditions of the experiment, the data reported here provide evidence supporting the concept of AML as a disturbance of cellular maturation which is due to cellular and not to environmental factors.

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L Fauerholdt and N Jacobsen