Unregulated Growth of Murine Leukemic Cells
and Suppression of Normal Granulocyte Growth
in Diffusion Chamber Cultures

By Allan M. Miller, Jane B. Marmor, Peter L. Page,
James L. Russell, and Stephen H. Robinson

The patterns of proliferation of C1498 mouse acute leukemic cells have been studied using the diffusion chamber technique of cell culture. These malignant cells grow to the same maximal cell concentration irrespective of initial cell input. Leukemic cells proliferate equally well with or without the stimulus of prior host irradiation. When cells cultured for several days are diluted to the original input concentration and recultured, they rapidly proliferate back to maximal cell number. All of these findings are in sharp contrast to the behavior of granulocytes from normal mouse marrow grown in the same culture system. Co-culture of equal numbers of normal marrow cells and leukemic cells results in virtually complete inhibition of normal granulocyte growth. These experiments provide a means of studying malignant growth of leukemic cells, as compared to the controlled growth and differentiation of normal granulocytes, and mechanisms by which leukemic cells suppress normal granulocyte development.

GROWTH of normal murine bone marrow in diffusion chamber (DC) cultures has been carefully investigated by several authors. Granulocytes and macrophages grow preferentially in this system. Efforts have been directed towards evaluating the kinetics of granulocyte proliferation and elucidating factors which stimulate or inhibit cell growth. In recent studies examining the long-term patterns of growth, we have found evidence that granulocyte proliferation in DC cultures is subject to fine regulation as in the normal bone marrow. We therefore thought it of interest to study the proliferation of murine leukemic cells under the same conditions. As with normal marrow cells, the patterns of growth and the stimuli for growth were investigated in long-term culture. Distinct differences were observed between normal granulocytes and acute leukemic cells, reflecting uncontrolled proliferation of the leukemic cells.

METHODS

Virgin female CD-1 mice (Charles River Laboratories, Wilmington, Mass.) weighing 20-25 g were used as host animals for the diffusion chamber cultures throughout these experiments. C57 BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were host animals for the C1498 acute myeloid leukemic cells (also obtained from Jackson Laboratories). The tumor grew as hemorrhagic...
tissue adherent to skin and was maintained by serial subcutaneous inoculation into new host animals every 6-8 days. The cells morphologically resembled myeloblasts, were peroxidase negative, and demonstrated no differentiation throughout the time in culture. Diffusion chambers, with an internal volume of 0.15 ml and filters of 0.45-μm pore size (Millipore Corp., Bedford, Mass.), were prepared as described by Benestad. As reported by others, we found that empty chambers with filters of this pore size permitted ingress of only negligible numbers of cells.

The tumor was removed from the C57 BL/6J hosts 7-14 days after inoculation, passed through a stainless steel wire mesh (0.23-mm diameter) and suspended in cold Medium 199 containing 15%, fetal calf serum, 100 U of penicillin, and 100 μg streptomycin per ml. The cell suspensions were then forced through a number 21-gauge hypodermic needle and filtered through 35-μm mesh Nitex cloth (Ernst, Tobler & Traber, Elsmford, N.Y.). The cells were counted in duplicate in a hematocytometer, and the cell suspension was diluted with medium to obtain the desired cell concentrations. Chambers were loaded with 0.1 ml of the final cell suspensions, and the holes were sealed with nylon plugs and MF-I cement (Millipore Corp., Bedford, Mass.). The chambers were stored in ice-cold medium until implantation. Two chambers were implanted surgically into the peritoneal cavity of each recipient mouse under light ether and pentobarbital anesthesia. All procedures were performed with sterile technique.

As a stimulus for granulocyte proliferation in the DCs, some recipient mice were given 500 rads whole-body irradiation with a 250 KV x-ray apparatus at a dose rate of approximately 120 rads/min 24 hr before the chambers were implanted. Chambers were removed 1-28 days after implantation and cells harvested according to the methods described by Benestad, using Ficoll pronase to dissolve clots. Cells from the two chambers in each host mouse were analyzed collectively. Four to sixteen animals were used for each experimental point. Slides were prepared with a Shandon cytocentrifuge and stained with Wright-Giemsa stain. Occasional chambers showing evidence of contamination or leaks were excluded from analysis.

RESULTS

Patterns of Leukemic Cell Proliferation in DCs

Thirteen experiments at four initial cell concentrations, ranging from 2 × 10⁴ to 1 × 10⁶ tumor cells per chamber, were performed with irradiated host mice. Growth curves for cells from different tumor hosts measured at different times were highly reproducible, and Fig. 1 shows the mean values for all points at each input level. A similar pattern of proliferation was observed for each input concentration, with cell number falling for 1-2 days, increasing logarithmically from day 2 until day 7-9 when a stable plateau was reached, and then persisting until the experiments were terminated at 28 days. A similar plateau level of 2.4 × 10⁶ cells per chamber was observed for all four inputs studied. The shift to the left in the logarithmic phase seen with the higher tumor inputs was presumably due to the higher number of cells undergoing cell division at the beginning of the growth phase. In all of these cultures, cells were invariably leukemic blasts similar to those of the original tumor.

Host Irradiation as a Stimulus to Growth

As illustrated in Fig. 2, experiments were performed with nonirradiated host mice at three different levels of cell input, 1, 7.5, and 15 × 10⁴ cells per chamber. The patterns of proliferation were similar to those observed in irradiated hosts (Fig. 1). Figure 3 shows the findings at a representative input, 1 × 10⁶ cells per chamber, comparing growth with and without prior irradiation of host mice. The pattern of proliferation was similar under both conditions, reaching the same limiting plateau level after 7-9 days in culture.
Fig. 1. Growth curves for murine C1498 acute leukemic cells cultured in Millipore diffusion chambers in host mice given 500 rads 24 hr before culture. Curves for four different initial cell concentrations are shown. Each point represents the mean ± SE of values from 8-32 diffusion chambers.

Fig. 2. Growth curves for murine C1498 acute leukemic cells cultured in Millipore diffusion chambers in nonirradiated host mice. Curves for three different initial cell concentrations are shown. Each point represents the mean ± SE of values from 8-24 diffusion chambers.
Retransplantation of Tumor Cells

Figure 4 illustrates the effects of retransplantation of tumor cells from the plateau phase. Cells were harvested from diffusion chambers at day 9, diluted back to the original cell concentration, and reimplanted into fresh diffusion chambers and fresh, newly irradiated host mice. The growth curve was similar to that observed initially, and the transplanted cells rapidly grew back to the original plateau level. A second reculture of the transplanted cells was performed after an additional 11 days in culture. Again, the third generation transplanted cells proliferated in a manner analogous to that of the original tumor cell culture.

Interaction of Leukemic and Normal Marrow Cells

Leukemic cells and normal marrow cells were cultured together, each at initial concentrations of $2.5 \times 10^6$ cells per chamber. Host animals had been pretreated with irradiation. The growth pattern of this mixture is illustrated in Fig. 5 and is similar to that observed for leukemic cells alone. Virtually all cells in these cultures were leukemic once growth began, with no evidence of the maturing granulocytes that would have been anticipated for culture of the normal marrow cells. However, $1\% - 3\%$ macrophages were found, and these were typically absent from pure cultures of leukemic cells. The initial lag in growth of the mixed culture as compared to the pure culture of leukemic cells could be explained by the fact that only half the number of leukemic cells was present in the mixed culture inputs, as compared to the pure tumor cell controls.
Fig. 4. Growth of transplanted murine C1498 acute leukemic cells. Cells were removed from diffusion chambers after 9 days, dispersed, resuspended in fresh medium, and reimplanted at the original cell concentration in new diffusion chambers in new host mice. Cells were transplanted again after an additional 11 days of culture. Dashed line shows the growth of the original cultures that were left unperturbed. All host mice received 500 rads total body irradiation 24 hr prior to implantation of diffusion chambers. Each point represents mean ± SE for 8-32 chambers.

DISCUSSION

The findings on culture of leukemic mouse cells differ in a major fashion from those observed for normal cells and reflect uncontrolled growth of these malignant cells. As illustrated in Fig. 1, the height of the plateau is similar at all inputs studied so that cell growth always proceeds to the same limiting capacity of the culture system. By contrast, normal murine granulocytes proliferate to a

Fig. 5. Co-culture of $2.5 \times 10^4$ leukemic cells and $2.4 \times 10^4$ normal marrow cells per chamber. Growth curve for these leukemic cells alone, plated at $5 \times 10^4$ cells per chamber, is shown for comparison. Both normal and leukemic cells were from inbred C57 BL/6J mice. All host mice received 500 rads total body irradiation 24 hr prior to implantation of diffusion chambers. Each point represents mean ± SE for 8-16 chambers. The same results were obtained in two additional experiments.
plateau level dependent on cell input; with most input levels the plateau achieved is far less than the maximum (Fig. 6), but the level of the plateau becomes progressively higher with increasing cell concentration until the saturation capacity of the system is reached. Thus, in this culture system the number of leukemic cells is limited by cell density and the physical dimensions of the chamber, whereas growth of normal marrow cells is modulated in large part by factors intrinsic to the cultured cell population.

Irradiation has been shown to be a stimulus to the growth of normal granulocytic cells. In contrast, growth of leukemic cells proceeds to the same limiting concentrations with or without prior irradiation (Figs. 2, 3). Under both conditions, there is no alteration in cellular morphology, and no granulocyte differentiation occurs. Similar experiments have been performed by Fauenholdt and Jacobson with human leukemic cells grown in diffusion chambers. They have observed a fall in cell number and defective maturation, irrespective of prior host irradiation.

The transplantation experiments further reflect the malignant nature of the leukemic cell growth. The repeated growth of the leukemic cells to the limiting cell concentration (Fig. 4) is in sharp contrast to the findings for normal leukocytes. Reculture of normal marrow cells that have been grown in DCs for 7 days and are then diluted back to the original input concentration and transplanted into fresh DCs and newly irradiated mice leads to a very limited increase in cell number. This finding is not due to the reculturing of nonproliferating granulocytes; experiments with ³H-thymidine have shown that normal mouse granulocytes are in a proliferative phase throughout the plateau period.

Fig. 6. Comparison of growth curves for murine C1498 acute leukemic cells and normal mouse marrow cells cultured at similar input concentrations. Host mice received 500 rads 24 hr before cultures were implanted. Each point represents mean ± SE for 8–24 chambers.
When normal marrow elements were co-cultured with leukemic cells, there was no apparent growth of the normal marrow cells. On the basis of previous experiments, easily identified numbers of maturing normal granulocytes should have been apparent in cultures of $2.5 \times 10^4$ normal marrow cells by day 3, but none were found. Instead, the growth curve was precisely that expected for pure cultures of leukemic cells plated at this input level. After 72 hr in culture, virtually all of the cells were leukemic, and the only difference from pure cultures of leukemic cells was the presence of occasional macrophages in the mixed cultures. This experiment suggested that leukemic cells have a suppressive effect on the growth of normal marrow cells well before the limiting capacity of the culture system has been reached.

Table 1 briefly summarizes and contrasts the salient findings for normal and leukemic cells grown in diffusion chamber cultures. Earlier work has demonstrated evidence for the "modulation" of normal granulocyte growth during the plateau phase of culture in diffusion chambers. There is a steady-state of granulocyte renewal maintaining constant numbers of differentiating leukocytes. Recently completed studies have documented the constancy in number of both pluripotent (CFU-S) and committed stem cells (CFU-C) throughout this steady-state period, suggesting that this experimental system is analogous to granulopoiesis in the normal marrow, with an equilibrium between stem cell differentiation and renewal. The mechanisms of control appear to be both extrinsic factors and intrinsic properties of the cultured cell population. The intrinsic properties may be mediated via cell–cell interactions. External control may be exerted by molecular substance(s) analogous to erythropoietin, e.g., "diffusible granulocytopoietic stimulator," produced in response to irradiation of the host animals.

On the other hand, in this same culture system C1498 leukemic cells behave like populations of stem cells undergoing continued self-renewal without differentiation. These malignant cells function in an unregulated fashion and proliferae to a concentration limited only by the physical characteristics of the culture system.

It is important to point out certain differences between the C1498 mouse leukemia and acute myelogenous leukemia as encountered in man. The characteristics of C1498 cells have been studied by Goldie et al. The cytology of the tumor cells in peripheral blood has been considered to be that of an acute myelogenous leukemia, although specific characteristics such as granules and

<table>
<thead>
<tr>
<th>Table 1. Comparison of Growth of C1498 Leukemic Cells and Granulocytic Cells From Normal Mouse Marrow in Diffusion Chamber Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal Granulocytes</strong></td>
</tr>
<tr>
<td>(1) Grow to a &quot;plateau&quot; level unique to each input cell concentration</td>
</tr>
<tr>
<td>(2) Irradiation is a stimulus to growth</td>
</tr>
<tr>
<td>(3) Retransplantation into fresh environmental conditions does not significantly enhance growth</td>
</tr>
<tr>
<td>(4) Leukemic cells suppress growth of co-cultured normal marrow cells</td>
</tr>
</tbody>
</table>
Auer rods are lacking. The murine tumor grows as soft hemorrhagic tissue adherent to skin, ultimately invading peripheral blood and hematopoietic organs. This pattern is in contrast to the bone marrow origin of human leukemic cells, although chloromas are well described in man.

Despite the foregoing qualifications, the behavior of the murine leukemic cells is analogous to the growth of leukemic cells in human marrow in several respects. The leukemic cells in both cases ultimately proliferate to saturate the hemopoietic environment. Co-culturing of normal mouse granulocytes with leukemic cells suppresses normal granulocytes and the leukemic cells eventually occupy the entire chamber. Similarly, Bull et al. have reported that human leukemic cells suppress formation of normal white cell colonies in culture in vitro, although this was apparently due in part to histocompatibility differences between the normal and leukemic cell donors. Finally, extrinsic stimuli are not required for enhanced growth of mouse leukemic cells, which proliferate irrespective of prior host irradiation. The role of stimulators of granulopoiesis in human leukemia remains unclear. Some leukemic cells apparently may respond to such stimuli in vitro. Increased levels of CSF have been observed in some patients with acute myelogenous leukemia, although consistent elevations have not been demonstrated.

These experiments provide a model system for studying the unregulated proliferation of leukemic cells as compared to controlled growth of normal granulocytes, and the interactions between malignant and normal hematopoietic elements.

REFERENCES

14. Goldie H, Putler CH, Anderson MM,


Unregulated growth of murine leukemic cells and suppression of normal granulocyte growth in diffusion chamber cultures

AM Miller, JB Marmor, PL Page, JL Russell and SH Robinson