THE PERSISTENCE OF HEMOPOIETIC
STEM CELLS IN VITRO

RUSSELL MEINTS and EUGENE GOLDWASSER

From the Argonne Cancer Research Hospital and Department of Biochemistry, University of Chicago, Chicago, Illinois 60637. Dr. Meints's present address is the Department of Zoology, University of Nebraska, Lincoln, Nebraska 68508.

ABSTRACT

Cells capable of forming colonies in spleens of irradiated mice (CFU) are lost temporarily when bone marrow cells from rats or mice are maintained in culture. Rat marrow CFU go through a minimum at about 3 days after which there is a slow increase in the number of CFU in culture, reaching a maximum at 9 days. Mouse marrow CFU reach a minimum at 3 days and a maximum at 7 days. Some rat marrow CFU persist in culture for as long as 28 days.

INTRODUCTION

Mammalian hemopoietic stem cells are the progenitors of most, if not all, of the mature, circulating cells of the blood (Becker et al., 1963). These cells can be readily assayed since they give rise to macroscopically visible colonies in the spleens of irradiated mice. Only a limited number of the total cells injected have the colony-forming potential; these are called colony-forming units or cells (CFU).

The assay system for CFU normally uses mouse tissues (fetal liver, adult spleen, or bone marrow) injected into the irradiated host mice. It has been known for some time, however, that transplanted rat marrow cells can also prolong the survival of irradiated mice (Congdon and Lorenz, 1954; Nowell et al., 1956), and Nowell has shown (Nowell et al., 1970) by karyotype analysis that rat cells form colonies of rat cells in the mouse spleen which have normal capacity for erythroid and immunological differentiation.

Another method of studying primitive hemopoietic cells involves the use of primary cell cultures of the mixed population of cells from adult bone marrow (Krantz et al., 1963) or fetal liver (Cole and Paul, 1966; Marks and Rifkind, 1972). These systems have been used to study the events caused by inducers, such as erythropoietin, that convert essentially undifferentiated cells into a specific differentiated lineage. The developmental events important in the action of erythropoietin have, in part, been described in earlier papers from this laboratory (Dukes et al., 1964; Gross and Goldwasser, 1969a, 1971). We have shown (Goldwasser and Gross, 1969), in addition, that adult marrow cells, in culture for as long as 20 days, still retain responsiveness to erythropoietin, and we have also found that precursors of erythropoietin-responsive cells can be found after at least 4 days in vitro (Gross and Goldwasser, 1972). These observations made it plausible to assume that CFU may persist in vitro if culture conditions were correct, even though Krantz and Fried (1968) found no significant CFU after 3–4 days in culture of mouse spleen cells. In the experiments reported in this paper, we have shown that both rat and mouse CFU are present in bone marrow cell cultures after incubation in vitro, the former reaching a maximum at 9 days and the latter at 7 days after the cultures were started. While this paper was being prepared, Chervenick
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MATERIALS AND METHODS

Culture Methods

Cultures were prepared in 15-ml plastic bottles with screw caps. Cell concentrations ranging from 10 to 20 million nucleated cells per ml in a total volume of 3 ml. The medium contained 30% fetal calf serum, 5% rat serum containing unlabeled iron at a concentration of 4 µg/ml (Gross and Goldwasser, 1969 b), and 65% NCTC 109. All sera were heat treated (56°C for 30 min) before use.

Bone marrow cell suspensions were prepared from male Sprague-Dawley rats (200-250 g). Femurs and tibias were removed aseptically and split longitudinally with heavy scissors. The marrow was scraped into culture medium and the clumps were broken up first mechanically with forceps, then by gentle pipetting. We have found that repeated vigorous pipetting has a selective damaging action on CFU. The cell suspensions were freed of connective tissue, debris, and bone chips by filtration through a stainless steel screen. Cell concentrations were determined by hemocytometer counting. The suspensions were diluted to the desired concentration, put into culture bottles, and gassed with 10% CO2 in air for 3 h. The bottles were then capped tightly and incubated at 37°C. Additional gassing for pH maintenance was done only as warranted by visual changes in the indicator color which was an indication of a change of 0.2-0.3 pH units in the cultures. The medium was not changed during the incubation periods.

The cultures were assayed at appropriate intervals in irradiated mice. During the first 5-7 days rat marrow cells remained in suspension or only loosely adherent to the surface of the flask and could be resuspended by gentle rinsing of the bottom of the flask. After about 7 days the cells attached to the surface and treatment with trypsin was necessary for removal. This was done using a 0.25% trypsin, 1% ethylenediaminetetraacetate solution at 37°C for 30 min, or longer if necessary. After trypsinization the cells were washed twice with phosphate-buffered saline (0.14 M NaCl, 0.01 M phosphate buffer, pH 7.2) and resuspended in complete medium before injection into the recipient mice.

Cell cultures of mouse bone marrow were done similarly, but removal from the culture flasks required the use of trypsin as early as 4 days, since mouse cells attach earlier than rat marrow cells.

Measurement of Spleen Colonies

Mice (CF # 1) 14-20 wk old, weighing 20-25 g., were used in all experiments as recipients. These animals were irradiated at approximately 58 rad/min measured with a Victoreen meter (Victoreen Instrument Division, V. L. N. Corp., Cleveland, Ohio), to a total of 850 rad with a 250 kV Maxitron (General Electric Co., Milwaukee, Wis.) using 1 mm Al and 0.25 mm Cu filtration and HVL of 1.05 mm Cu at a distance of 77 cm. The mice, in plastic containers, were rotated under the X-ray source during irradiation.

Groups of eight animals were injected intravenously with one or two million rat marrow cells or 2 x 10⁵ mouse marrow cells 4-5 h after irradiation. The mice were kept four to a cage and provided with food and Terramycin-supplemented water ad libitum. 8 days after irradiation they were injected with approximately 250,000 cpm of 59Fe (as ferric citrate); 24 h later the spleens were removed and fixed in Bouin's solution. Iron uptake per spleen, which is a measure of erythropoiesis (Smith, 1964), was determined immediately in an automatic gamma counter, after which the spleens were left in the fixative for at least 24 h before colonies were counted. All colony counting was done on blind samples. Results are expressed as CFU per spleen and percent uptake of 59Fe per spleen per million rat cells injected or per 2 x 10⁵ mouse cells injected. Control mice received only culture medium. The mean values for all control mice were 0.8 ± 1.7 colonies per spleen and 0.43 ± 0.18% of 59Fe taken up per spleen.

Culture dishes and flasks were bought from Falcon Plastics Division of B-D Laboratories, Inc., Los Angeles, Calif., fetal calf serum and trypsin solution from Gray Industries Inc., Fort Lauderdale, Fla., and NCTC 109 from Microbiological Associates, Inc., Bethesda, Md.

RESULTS

Assay of Rat Cells in Mice

Fig. 1 shows the effect of injecting increasing numbers of fresh rat marrow cells on spleen CFU and iron uptake. It is clear that with rat cells the colony-forming capacity is considerably less than that reported for mouse marrow cells in mice. The rat cells colonize with an efficiency of about two colonies per 10⁴ injected cells. Whether this represents a relatively smaller absolute number of stem cells in rat marrow, in contrast to mouse marrow, or in reduced efficiency of transplantation is not yet determined. In the same figure the data for iron uptake by the spleens show a parallel relationship to the CFU data, suggesting that a
constant fraction of the colonizing cells is engaged in erythropoiesis.

**Rat Marrow Cell Cultures**

The effect of time of culture of rat marrow cells on the number of spleen CFU and iron uptake is shown in Fig. 2. Cultures were analyzed daily for the first 4 days and then at 2-3-day intervals. All the data in Fig. 2 are expressed as CFU or iron uptake per spleen per 10^6 cells injected. Control values, i.e. CFU and iron uptake in the spleens of mice that received cell-free culture medium, are indicated by solid points; the mean control values are indicated by the horizontal line, and the shaded area represents 1 SDM. Experimental points are indicated by open circles with some replicate results (from an independent experiment done at another time) indicated by X. The peak values include the standard deviation of the mean.

During the first few days of culture, both CFU and iron uptake declined precipitously, reaching a minimum at 3-4 days. After the 4th day, the number of CFU rose to a maximum at the 9th day (about 90% of the original number) and then declined again to control values by the 14th day. Splenic erythropoiesis, as measured by iron uptake, increased at a slower rate than did CFU, reaching a maximum at 11 days, then slowly returned to control levels by the 21st day. The maximum iron uptake on the 11th day was about 80% of the original value at day zero.

In a separate experiment (Table I) we found that cultures of rat marrow cells assayed after 28 days of incubation contained a significant number of CFU \((P < 0.001)\), although iron uptake was probably not significantly different from that of the control mice \((0.05 < P < 0.10)\).

During the time in culture some morphological changes in the cells also occurred. By the 4th and 5th days, mature erythrocytes were no longer present and the cells began to form monolayers. Many spindle-shaped cells were visible and the absolute number had decreased sharply. By the 7th day, when the number of CFU had increased, there appeared to be a new morphological steady
FIGURE 2  Effect of length of incubation of rat marrow cells on spleen CFU and iron uptake. The upper frame shows iron uptake as percent of the injected dose; the lower frame shows CFU per million injected cells. At each time of incubation the rat marrow cells were injected into mice irradiated 4-5 h earlier. The open circles refer to incubated cells and the crosses to an experiment done independently with the same in vitro incubation time. The closed circles refer to control mice injected with cell-free medium. The horizontal lines represent the mean values for control mice and the shaded areas ±1 SD. The vertical bars are ±1 SD of the experimental group. The points are the mean values of the surviving mice (three to eight) of the eight originally injected.

TABLE I

| Colony-Forming Cells after 28 Days in Culture |
|-----------------------------------------------|
|                  | CFU |
| Fe uptake         |     |
| %                 |     |
| Medium only       | 0.34 ± 0.09  | 0.0 |
| Cultured cells    | 0.51 ± 0.21  | 5.8 ± 3.0 |
| (2 X 10^6 injected) |     |

By t test the difference in iron uptake was 0.02 < P < 0.05 and for CFU P < 0.001.

FIGURE 3  Effect of length of incubation of mouse marrow cells on spleen CFU and iron uptake. The upper frame shows the iron uptake as percent of the injected dose; the lower frame shows CFU per 2 X 10^6 injected cells. The symbols and conditions are as described in the legend to Fig. 2.

state. At the 11th day, virtually all of the cells were firmly attached to the flask surface.

Mouse Marrow Cell Cultures

Cultures of mouse bone marrow cells yielded results that were similar to those found for rat cells but with a somewhat different temporal relationship (Fig. 3). In the mouse cell cultures events appeared to be telescoped, CFU peaking at about 40% of the original level at 7 days after the initially sharp fall and subsequently returning to the control level by 10 days. The mean CFU at 7 days was 13 with a standard deviation of 13. Controls had no colonies. A t test shows that those are significantly different populations (P < 0.01). In contrast to the data from rat cell experiments, we found no significant recovery of erythropoietic activity after the reappearance of CFU. In addition, the establishment of an attached monolayer was evident by the 4th day.

DISCUSSION

The data presented in this paper show that very shortly after transfer of bone marrow cells (both
rat and mouse) from the animal to culture medium there was an eclipse of CFU. We have no ready explanation for this eclipse except to suggest that it may be the result of transference from the topographical organization characteristic of the tissue in the bone cavity to the dispersed condition in culture. It may be that dispersion into the culture medium and maintenance, for a time, in suspension acted to trigger the normally quiescent CFU (Lajtha et al., 1969) into cell cycle. Other external factors such as androgens (Byron, 1970) and cyclic adenosine monophosphate (Byron, 1971) appear to do the same thing. It may, further, be the case that cycling cells do not have the appropriate surface properties to enable them to colonize the irradiated spleen. The coincidence of establishment of monolayers of cells and reappearance of CFU lends a small amount of support to those speculations. Regardless of the mechanism involved in the eclipse phase, the observation of its existence explains why earlier studies failed to find CFU in culture.

After the eclipse there was a fairly rapid increase in number of CFU with time, suggesting a doubling time for rat cells of about 32 h in vitro. This value is considerably longer than the 8 h estimated by Lajtha et al. (1971) for CFU in vivo and, if valid, may be indicative of the suboptimal conditions for stem cell replication under the culture conditions. Alternatively, this estimate of doubling time may represent a maximal value assuming that no differentiation occurs during that period. If, as the data on iron uptake suggest, erythroid differentiation does occur, the doubling time could be much less than 32 h.

The second sharp fall in number of CFU with increasing time in culture was unexpected, but may, again, reflect conditions that are inappropriate for maintenance of the CFU population in a steady state in vitro. There is evidence, however, for some degree of maintenance of CFU since even at 28 days of culture some colony-forming ability was detected.

Despite the major problem still unsolved, that of providing the proper milieu for stem cell replication and persistence in culture, the system described here may be useful for studying some of the properties of CFU in vitro. For instance, the delay of 2 days between the peak of CFU and the peak of erythropoiesis when rat cells were used, indicates that the temporal relationship between colony formation and differentiated function was close to normal in the cultured cells. We do not, as yet, understand the failure of regeneration of erythropoiesis when mouse cells were used.

Further experiments in which this system was used to study the possible competition between CFU replication and induced differentiation in vitro will be reported in another paper.

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