Two Classes of Murine Granulocyte Progenitor Cells Expressed in Plasma Clot Diffusion Chamber Cultures

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Two distinct classes of granulocyte progenitor cells present in normal mouse bone marrow are expressed sequentially in the vivo plasma clot diffusion chamber culture system. By several criteria, progenitor cells giving rise to granulocyte colonies on day 4 of culture (CFU-D4) are different from those giving rise to colonies on day 7 (CFU-D7). These differences include: cell cycle activity as measured by in vitro incubation with cytosine arabinoside, residual concentration in the bone marrow after in vivo treatment of donor mice with cytosine arabinoside or methotrexate, resistance to osmotic lysis, size as determined by velocity sedimentation, and the morphology of the granulocyte colonies to which these cells give rise. The CFU-D7 appears to represent an earlier progenitor cell than the CFU-D4 in the differentiation pathway of the granulocyte and is analogous in many respects to the BFU-E in the erythroid pathway.

MATERIALS AND METHODS

Female CD-1 mice (Charles River Laboratories, Wilmington, Mass.), approximately 4 wk of age and weighing 18–24 g, were used as bone marrow donors and as host mice for PCDC cultures. In some experiments mice pretreated with cytosine arabinoside (Ara-C) or methotrexate (MTX) served as marrow donors. Mice treated with Ara-C were given three intraperitoneal doses of 2 mg each at 7 hr intervals. Bone marrow was harvested 2 hr after the third injection. Bone marrow from mice treated with methotrexate was harvested 65 hr after a single injection of 1.25 mg of this agent.

Bone marrow cells were obtained by flushing both tibias with 0.5 ml of McCoy’s 5A medium (Gibco, Long Island, New York) supplemented with 20% fetal calf serum (Reheis Chemical, Phoenix, Arizona) which had been heat inactivated for 30 min at 56°C. To obtain a single cell suspension, cells were passed through a 21 gauge needle and then through 35 μm nylon mesh (Ernst, Tobler and Trabor, Elmsford, New York). All cell suspensions were counted in hemocytometers. Slides were prepared with a Shandon cytocentrifuge and stained with Wright-Giemsa stain.

Separation of marrow cells by velocity sedimentation was performed as follows. Approximately 1.3 x 10^6 cells were separated in a Stupat apparatus (O.H. Johns Scientific Co., Toronto, Canada) as described by Miller and Phillips. Cells were allowed to sediment for 4 to 5 hr at 4°C through a gradient of 0.4–2.0% BSA in phosphate buffered saline. Thirty ml fractions were collected, resuspended in McCoy’s 5A medium, counted and prepared for culture.

Measurement of cell cycle activity was performed as follows: 5.0 x 10^6 normal marrow cells/ml were incubated in vitro for 1 hr with 40 μg/ml Ara-C (Upjohn Co., Kalamazoo, Mich.), final concentration, or with McCoy’s 5A medium. Following incubation at 37°C in 5% CO₂, the Ara-C was diluted out with 49 ml of fresh medium. After centrifugation, cells were resuspended in medium, counted, and prepared for culture. In this laboratory Ara-C yields results similar to those found with 3H-thymidine, but the findings are much more reproducible with the former agent.

Osmotic lysis was measured according to the method of Niskanen and Cline. Marrow cell suspensions were pelleted and then resuspended in sterile double distilled water at room temperature for periods of 1–3 min. Osmotic lysis was terminated by the addition of 2X McCoy’s medium supplemented with 30% fetal calf serum. Following centrifugation, cells were resuspended in fresh 1X medium, counted and prepared for culture.

In all experiments, treated and control cell suspensions were cultured in spleen colony, agar colony, and PCDC assay systems. CFU-S were measured in the spleens of lethally irradiated mice as described by Till andMcCulloch. Between 5 and 10 spleens per experimental point were assayed 8 days after injection of the cells.

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Spleens were fixed in Bouin’s solution and preserved in 4% formaldehyde. CFU-C were assayed in a single layer of agar using L-cell conditioned medium as a source colony stimulating activity. Cultures were incubated for 7 days at 37°C and 5% CO2. Between 3 and 10 plates were scored per point under an inverted microscope and aggregates of 50 cells or more were considered to be colonies. The methods used for PCDC cultures have been previously described in detail. Briefly, DCs constructed with GS-type Millipore filters (Millipore Corp., Bedford, MA) or Nuclepore filters (Nuclepore Corp., Pleasanton, CA) of 0.22 μm pore size were inoculated with 1.0–3.5 x 10^6 bone marrow cells in 100 μl of medium followed by the addition of 20 μl citrated bovine plasma (Pel Freeze Corp., Rogers, Arkansas). To aid clot formation, 10 μl of a 1:5 dilution of beef embryo extract (Gibco, Long Island, New York) were added to each culture. Each chamber was immediately heat sealed, shaken to insure uniform distribution of cells and set aside for 10 min until clot formation occurred. The chambers were then transferred to ice cold medium pending surgical implantation into the abdominal cavities of normal, non-irradiated host mice. Two diffusion chambers were implanted per mouse. Each experimental group consisted of 15 such mice. Cultures were harvested at different intervals and the total number of colonies determined as previously described.7

RESULTS

As shown in Fig. 1, the development of granulocyte colonies from mouse marrow cells in PCDC cultures was similar to that previously described for rat marrow cells.7 By morphologic observation white cell colonies were granulocytic and no macrophage or monocyte colonies were observed. Peak colony numbers were observed on day 4 of culture. This was followed by a decline until day 7 and then a leveling off in the number of granulocyte colonies through day 9. Early studies showed ten to twelve day 7 colonies per 10^6 cells implanted. With improvements in technique, specifically the use of Nuclepore as opposed to Millipore filters on the DCs and the routine seeding of lower numbers of bone marrow cells per culture (1 x 10^5), this increased to 50 colonies per 10^5 cells implanted.

Improved growth of bone marrow cells in diffusion chambers constructed with Nuclepore filters has previously been reported.11 All of these experiments had simultaneous controls. Similar increases in day 4 colonies were not observed since optimal colony densities had already been achieved. Colonies on day 4 varied in size and degree of cellular maturation. Colonies were typically discrete from one another and contained several hundred cells in the terminal stages of differentiation (Fig. 2A). While some colonies of this type seemed to persist through day 7 of culture, most appeared to disintegrate after reaching the mature neutrophil stage of differentiation. Most of the colonies observed on day 7 differed from day 4 colonies in their spatial organization and the level of maturity of the constituent cells. Many day 7 colonies consisted of clusters of 3 to 6 individual colonies (Fig. 2B), analogous to “burst” configurations in the erythroid pathway of development, as compared to the discrete colonies observed on day 4. Other day 7 colonies did not have this multicentric appearance but were single colonies often containing several hundred cells. It is difficult to tell whether these large colonies were persistent day 4 colonies or colonies from an earlier
progenitor cell, similar to the large unicentric BFU-E-derived colonies observed in methylcellulose cultures. The cells within most of the day 7 colonies were in relatively early stages of granulocyte development, whereas the cells within day 4 colonies were usually mature granulocytes.

The morphologic findings suggested that day 4 and day 7 PCDC colonies may be derived from different progenitor cells. This hypothesis was examined in studies designed to investigate differences in the cycling characteristics, survival after treatment of marrow donors with Ara-C or MTX, resistance to osmotic lysis, and sedimentation velocity of these progenitor cells.

The cycling characteristics of PCDC progenitor cells, as studied by in vitro incubation of normal mouse bone marrow with Ara-C prior to culture, are shown in Fig. 3. While PCDC colony development on day 4 was markedly reduced after the incubation with Ara-C, there was virtually no decrease in the number of colonies observed on days 6 or 7. Kill rates for day 4 PCDC colony forming cells (CFU-D4) and for CFU-C, as assayed by agar culture, were comparable, 55.6% and 48% respectively. Kill rates for day 7 PCDC colony forming cells (CFU-D7) and for CFU-S, as assayed by spleen colony formation, were also similar, 2.6% and 8.5% respectively.

Changes in the concentration of CFU-D4, CFU-D7, CFU-C and CFU-S were measured in the bone...
maraows of donor mice after treatment in vivo with either Ara−C or MTX. As shown in Figs. 4 and 5, the concentration of CFU−D4 was reduced to 58% of control values (p < .02 by Student t test) in the bone marrow of Ara−C-treated mice and elevated to 160% of normal (p < .01) in the marrows of MTX-treated mice. By contrast, CFU−D7 remained virtually unaltered in both groups of animals. These changes in CFU−D4 and CFU−D7 are roughly similar to those reported by this laboratory for marrow CFU−C and CFU−S under the same experimental conditions.13 The concentration of CFU−C was reduced to 33% of normal in the bone marrows of Ara−C-treated mice and enhanced to 280% of normal in MTX-injected mice. The number of CFU−S remained close to control levels in both groups of drug-treated mice,13 as was the case for CFU−D7 in the present study.

Figure 6A shows that CFU−D4 are more resistant to osmotic lysis than CFU−D7. After 3 min of treatment with distilled water, 25.4% of CFU−D4 survive as opposed to only 7.8% of CFU−D7. Similarly (Figure 6B), CFU−C are more resistant to osmotic lysis than CFU−S. However, in contrast to the findings in the other experiments, the changes observed for CFU−D4 differ from those for CFU−C and the changes observed for CFU−D7 differ from those for CFU−S. In particular, the kinetics of CFU−C inactivation are markedly different from those of the other three cell types.

Figures 7A and 7B show a representative example of three velocity sedimentation profiles of normal bone marrow cells that were subsequently cultured in PCDC assays. As compared to the more homogeneous patterns observed for CFU−S and CFU−C, the curves obtained for CFU−D4 and CFU−D7 in PCDC cultures were relatively complex. Two closely related peaks with sedimentation velocities of 4.8 mm/hr and 5.3 mm/hr, with a trough of 5.0 mm/hr, were observed for CFU−D4; however, this bimodal configuration is probably antifactual since the two other velocity sedimentation profiles showed only a single sharp peak at either 4.8 or 5.0 mm/hr. The curve in Figure 7A is presented because CFU−S and CFU−C were assayed simultaneously in this experiment. There was also a smaller peak of CFU−D4 at 4.0 mm/hr in this study. The major peaks for CFU−D4 in these different experiments were all comparable to the peak at 4.8 mm/hr found for CFU−C. Two quite distinct peaks were observed for CFU−D7. These were found in all three experiments. The slower sedimenting peak at 3.6 mm/hr was similar to the peak at 3.4 mm/hr observed for CFU−S. The faster sedimenting peak at 4.8 mm/hr coincided with the peaks observed for CFU−D4, suggesting that some of the day 7 colonies may have been derived from precursors of the CFU−D4 type. This was further substantiated by morphologic examination of the day 7 colonies formed from the cells in these two peaks. More colonies derived from the 3.6 mm/hr as compared to the 4.8 mm/hr peak were large or multi-

Fig. 6. Effect of osmotic lysis on colony forming cells. Mouse marrow cells were incubated with distilled water for periods of 1–3 minutes prior to cell culture. (A) PCDC colony formation at days 4 and 7. (B) Ager colonies (CFU−C) and spleen colonies (CFU−S). Each point represents the mean of 2 experiments.

Fig. 7. Velocity sedimentation profiles of colony forming cells in normal mouse bone marrow. (A) PCDC colony forming cells (CFU−D4, CFU−D7). (B) Spleen colony forming (CFU−S) and agar colony forming cells (CFU−C).
centric. However, diverse colony types could be observed in both sets of cultures. Although an absolute separation of CFU-D4 and CFU-D7 cannot be made by morphologic criteria, it appears from the velocity sedimentation profiles that about one-third of the colonies observed on day 7 of culture are derived from the more mature CFU-D4 progenitor cell.

**DISCUSSION**

The existence of at least three classes of erythroid stem cells at different levels of maturity has been well shown. In vitro studies have provided evidence as well for heterogeneity of progenitor cells committed to the granulocytic pathway of differentiation. However, discrete granulocytic stem cells at different levels of maturity have not been well characterized. In vitro diffusion chamber studies have pointed to the existence of early granulocytic progenitor cells more immature than the CFU-C. Breivik, using a limiting dilution technique to assay the diffusion chamber progenitor cell (DCPC or CFU-D), found similarities between this cell and the CFU-S but demonstrated a distinction between these two cell types with regard to their responses to vinblastine. Jacobsen et al. showed that human CFU-D which give rise to granulocyte colonies in fibrin clot DC cultures have different velocity sedimentation and cell cycle properties as compared to CFU-D assayed in vitro. These studies suggested that the CFU-D is a less mature progenitor than the CFU-C and implied a parent-progeny relationship between these two cell types. Niskanen and Cline showed that mouse CFU-D are more sensitive to hypotonic lysis than CFU-C whereas CFU-D and CFU-S are equally sensitive, suggesting a close relationship between the latter two progenitor cells. Observations from this laboratory indicated that both CFU-S and CFU-C are involved in the population of diffusion chamber cultures. CFU-C number varies rapidly with, and this cell appears to be the immediate source of, granulocyte growth in these cultures. The CFU-C in turn appears to originate from the CFU-S in these cultures. In none of these studies, however, has there been the opportunity to observe the sequential expression of granulocytic progenitor cells at different levels of maturity, as has been possible for cells in the erythroid pathway.

The results of the present study demonstrate that at least two classes of granulocytic progenitor cells can be distinguished within a single culture system at different points in time. In the PCDC system progenitors which give rise to granulocytes on day 4 of culture (CFU-D4) are clearly different from those which give rise to colonies on day 7 (CFU-D7). Many of the colonies formed on day 7 are arrayed in aggregates of 3 to 6 smaller colonies, analogous in many respects to erythroid bursts which are derived from primitive BFU-E. Thus, the CFU-D may represent a burst forming unit for the granulocyte line of differentiation, i.e., a BFU-G. By contrast, colonies formed on day 4 are single entities of lower proliferative capacity and hence appear to be derived from more mature progenitor cells.

While the existence of a BFU-G has also been suggested by others, the present study characterizes this early granulocytic precursor cell and defines its relationship to the more mature CFU-D4 as well as to the well characterized progenitor cells, the CFU-S and CFU-C. Differences in the cycling characteristics of CFU-D4 and CFU-D7 as measured by in vitro incubation with Ara-C, in the concentrations of these cells in the bone marrow of mice pretreated with Ara-C or MTX, in their osmotic resistance to water, and in their velocity sedimentation profiles clearly indicate that most day 4 and day 7 colonies arise from different progenitor cells. The similarity of the CFU-D7 to the CFU-S in many of these studies appears to reflect the more primitive nature of the CFU-D7 as compared to the CFU-D4. By the same criteria the latter cell appears to be closely related to the CFU-C as assayed in agar. These relationships, the later expression of the CFU-D7 in culture, and the fact that day 7 colonies generally consist of less mature cells than do day 4 colonies, all suggest a parent-progeny relationship between the CFU-D7 and CFU-D4.

Although the CFU-D7 in many respects behaves like the CFU-S and the CFU-D4 like the CFU-C, these experiments also reveal differences between these pairs of cells. It seems probable that all of these cells are in fact related and lie on a spectrum from primitive pluripotent cells with marked proliferative potential to more differentiated precursors with a limited capacity for growth.

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