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The Kinetics of Granulopoiesis in Long-Term Mouse Bone Marrow Culture. Part I.

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Abstract. The spontaneous stratification in long-term bone marrow cultures was illustrated and quantified. The cultures were separated into three hematopoietic layers: nonadherent cells in the supernatant medium, lightly adherent cells on top of the stromal layer, and remaining cells buried within the stromal layer. The cells of each layer were subcultured for 10 days in plastic tubes that inhibit the formation of a stromal layer. Daily samplings with absolute and differential cell counts were obtained. We identified three families of cell disappearance curves and cell types: CFU-s, hemocytoblasts, myeloblasts, and promyelocytes (G1, 2); myelocytes (G3); and postmitotic granulocytes (G4). Also, the numbers of mitotic and necrotic cells were determined. The longest half-time of CFU-s was 2.5 days. Lacking stromal support, CFU-s disappeared faster than other differentiated cells. Generally, these cells maintained their numbers for the first week of subcultures, which was attributable to a temporarily maintained balance of cell death and fresh cell production. After more than 7 days, there was a rapid decline of all differentiated cell types.

Introduction

The following study is a product of collaborative planning. Previous mathematical models predicted the outcome of published experiments [1]. In contrast, using a specially designed experiment, we have developed a model of granulopoiesis. Long-term primary cultures of mouse bone marrow [2] were chosen because they are easily harvested and provide favorable conditions for growth,

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differentiation, and maintenance of hematopoietic stem cells [colony-forming units-spleen (CFU-s)], demonstrated by high weekly yields of granulocytes and CFU-s [3, 4]. In stratified cultures, a nonadherent layer of mostly mature granulocytes, suspended in the growth medium, overlies an adherent layer containing a three-dimensional stromal network rich with granulocytic precursors [5]. The adherent cells are further subdivided into those that can be washed or shaken free and those that even harsher mechanical procedures cannot dislodge.

This study shows that granulopoiesis continues independently for about a week if each layer is subcultured separately under conditions preventing stroma formation. According to the hypothesis that precursor cells in each layer determine the course of residual granulopoiesis during subculture, a single set of assumptions should describe the kinetics of granulopoiesis in each subcultured population. Accordingly, this experiment was conducted to obtain sufficient data to construct families of curves depicting survival and growth of the recognizable elements in the granulopoietic cell series. Using the available knowledge about granulopoiesis in vivo, a mathematical model was constructed and then applied to predict the course of various experimental curves, given only their initial values. The experiments are described in Part I, and in Part II the model is presented and its independent predictions compared with the experimental results from Part I.

Materials and Methods

Bone Marrow Cultures

Fisher's medium was supplemented with 20% horse serum, 1×10^{-5} *M* hydrocortisone, penicillin (80 μg/ml), and streptomycin (80 μg/ml); all from GIBCO Laboratories (Grand Island, NY). The marrow contents of one femur and tibia of one young adult C3H male mouse (Jackson Laboratories, Bar Harbor, ME) were flushed with 11 ml of conditioned medium onto a 25 ml plastic tissue culture flask (Corning). The number of flasks varied from 10 to 90 depending on the experimental design. Marrow was incubated at 33°C in an atmosphere of 7% CO_2 in air. At the weekly "feeding," the supernatant medium and suspended cells were replaced with 7 ml of fresh and 4 ml of conditioned, cell-free medium.

Plateau-Phase Subcultures

Figure 1 shows the sequential harvesting of three cell-containing layers from plateau-phase cultures. Nonadherent (NA) cells were pipetted off the supernatant medium. Next, the lightly adherent (LA) cells were obtained by two 14 ml washes with medium, with vigorous shaking (15 sec, by hand) during the first wash. This procedure removed all cells that could be loosened. Other methods, such as "scrubbing" (with microbeads, cytodex 1, Pharmacia) or trypsinization (0.25% for 10 to 15 sec; trypsin from GIBCO), yielded

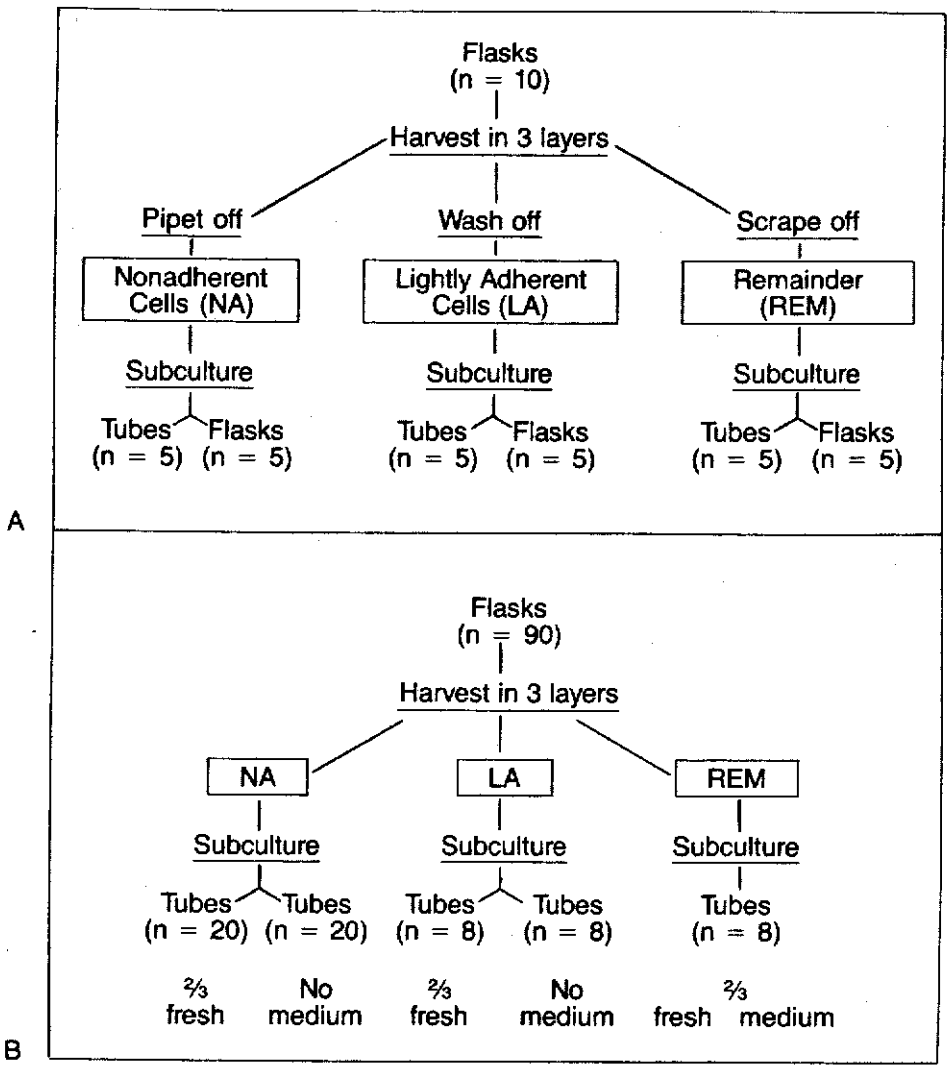

Fig. 1. (A) Experiment A: primary cultures in plateau phase (3 days after last feeding). Endpoints were total cell counts and differential counts from cytospin smears, obtained daily (except day 5 and 6) through 7-10 days. The samples were 0.5 ml/culture, pooled for each group. Only the tube culture differentials were evaluated in this study. Tubes and flasks were subcultured in ⅔ fresh medium. (B) Experiment B in which NA, LA, and REM cells were not obtained simultaneously (see Materials and Methods). The design was repeated 3 and 7 days after the last feeding of the 90 primary cultures. The endpoints were total cell counts and CFU-s on days 0, 1, 2, 3, 4, and 7. Cytospin smears were obtained on day 0 only.

additional granulopoietic cells only if applied so harshly that the supporting stroma was disrupted.

Cells not removed by pipetting or washing constituted the remainder (REM) layer; these cells were obtained by scraping the bottom of the flask with a sterile rubber blade. The sequential harvests from 10 flasks were pooled, centrifuged at 200 *g* for 4 min, and resuspended in fresh medium. Hemocytometer counts and cytospin smears were obtained. Appropriate dilutions were prepared in medium (2 parts fresh:1 part conditioned) to subculture 5×10^6 or 6×10^6 cells in either 50 ml plastic tubes or 25 ml tissue culture flasks. Three days after the last feeding, the subcultures were initiated as shown in Figure 1A and B. Daily samples (0.5 ml) were obtained from each of five tubes or flasks, and pooled to determine the cell count and prepare cytospin smears for differential counts (obtained daily, except on days 5 and 6). On day 7 of the subculture, fresh, serum-free medium was added to bring the total volume to 11 ml. Subcultures were discontinued after 7 to 10 days when the cell counts were $< 2 \times 10^5$.

Feeding Interval and CFU-s Disappearance Time

To establish the disappearance function of subcultured cells and CFU-s in tubes, another experiment was performed. Understanding the disappearance function was important, for if it were independent of the time since feeding, a delay in feeding would affect the cell survival. Three days after feeding, supernatant (NA) cells from 90 plateau-phase cultures were subcultured in 40 tubes (5×10^6 cells per tube). Half of the tubes contained cells in 2 parts fresh: 1 part conditioned medium; the other half contained cells in pure conditioned medium. Two weeks later, the experiment was repeated with nonadherent cells from the same donor cultures. However, this time the subcultures were initiated seven days (not three) after feeding.

The four treatment groups consisted of nonadherent cells, subcultured with or without feeding and beginning three or seven days after the last feeding. The feeding schedule of the original (donor) cultures was then shifted, so that two weeks later half of the cultures would be three days, and half seven days, past their last feeding when their adherent layers were harvested and subcultured. For each assay, one to seven subcultures were pooled depending on cell density. Cellularity and CFU-s were then determined.

Assay of CFU-s

Appropriate dilutions of single-cell suspensions were injected intravenously into lethally irradiated (1250 rad in split dose) C3H mice. Eight days later their spleens were removed and the colonies counted.

Microscopy

Within 24 h, the air-dried smears were stained with May-Grünwald-Giemsa. At least 1000 cells per sample were scored using traditional criteria [6]: mitotic cells were scored as either stromal or hematopoietic. Occasional mitosis in metamyelocytes was observed, even though they are generally considered postmitotic. As suggested by *Bessis* [6], necrosis was recorded separately for stromal and hematopoietic cells, including only those cells which were neither disintegrated nor being phagocytized. Kodak film (technical pan 2415) was used for the phase photomicroscopy, and an AMR scanning electron microscope (Burlington, MA) was used in an independent morphological study.

Results

Stroma-Bound Versus Stroma-Adherent Hematopoiesis

In culture, all hematopoiesis occurred in and on the stromal layer. Immature hematopoietic cells were packed so tightly into nest-like clusters that they shaped each other's surfaces, forming a "cobblestone" pattern (Fig. 2A, B). As the cells matured, they became rounder and apt to float in suspension. During preliminary studies, we found that the removal of hematopoietic cells from the stromal layer was always incomplete. Numerous (from 2 to 20) immature cell clusters remained on the stroma after the lightly adherent cells had been washed off. These residual cells could only be freed by procedures so vigorous that the stroma was damaged and disrupted as well.

Our inability to separate all hematopoietic cells from the stroma was eventually explained by a morphological study. The three-dimensional stromal architecture was clearly apparent under phase-contrast optics (Fig. 2C), but the tissue's optical transparency prohibited the observation that thinly spread reticulum cells actually covered nests of hematopoietic cells. Opaque to scanning electron microscopy, the covering reticulum cells reveal the hematopoietic cells buried beneath them through accidental tears (Fig. 2D). Thus three hematopoietic layers exist in long-term cultures: nonadherent, lightly adherent, and stroma-bound.

Table I reviews the overall cell composition in each of the three layers and shows the significant fraction of the hematopoiesis that was stroma-bound. This REM layer contained 72% immature cells (BL + G1 + G2 + G3) and showed high mitotic activity (1.3%) and no evidence of cell death.

Fig. 2. Immature granulopoietic cells in bone marrow culture. (A) Phase contrast view of a cluster of myelocytes, one in mitosis. (B) Scanning electron microscope (EM) view of a similar cluster. The cells are in the characteristic, densely packed "cobblestone" pattern. Parts of reticulum cells are visible in the lower right corner and in the upper third of the picture. (C) A phase optics view of the culture stroma. The elongated histiocytes cross the field at numerous angles and create a delicate meshwork in which a solitary myelocyte undergoes mitosis. The seemingly unstructured lower left field is part of a fully spread reticulum cell. (D) The sheet-like nature of reticulum cells becomes apparent in scanning EM when overlying blood cells are washed away. Clusters of blood cells beneath the sheet can be recognized through accidental tears in the sheet. Since the sheets are transparent to light, this area would have appeared similar to (A) under phase optics. Specimen at a tilt angle of 50 degrees. Magnifications are ×2100, ×2000, ×1300, ×2000.

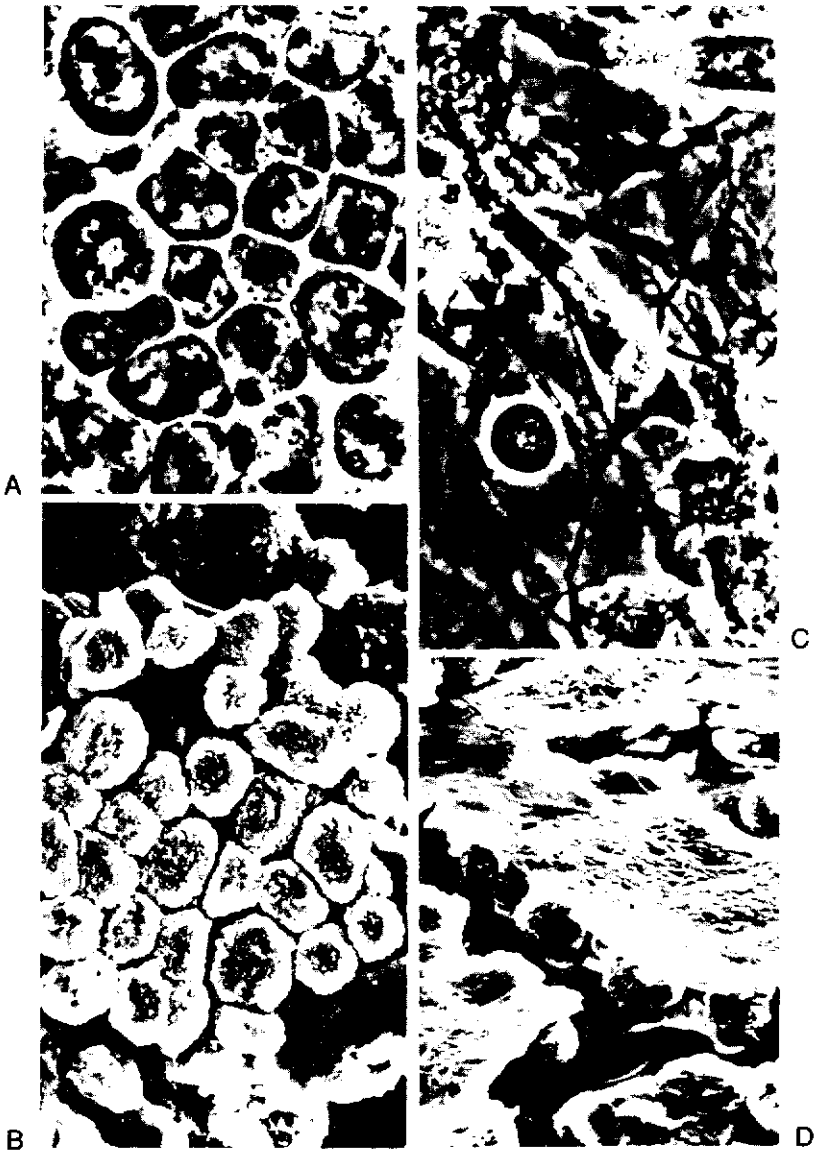
A
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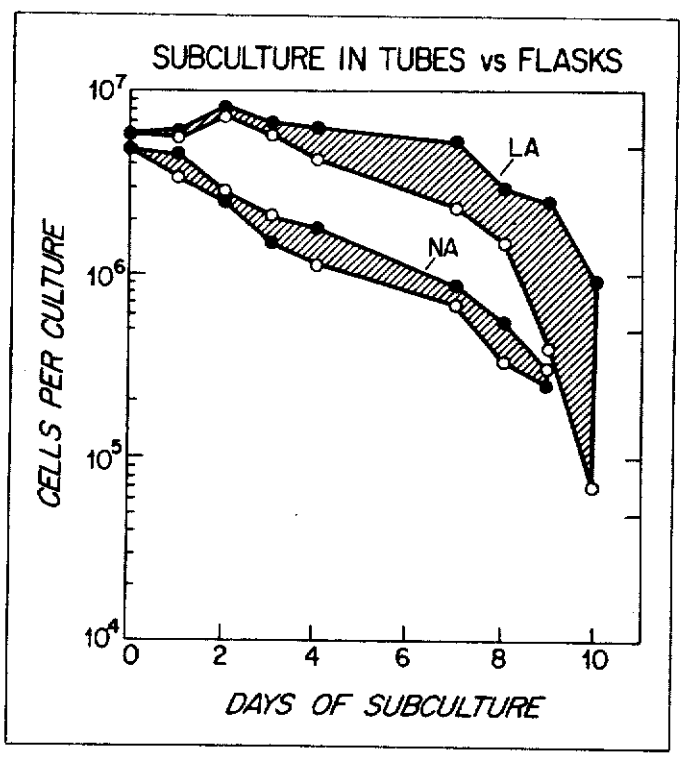
Table I. Composition of cell layers in long-term cultures

Source[a] of cells	Stromal cells (10^3)	mitosis	Hematopoietic cells ($\times 10^3$)[b] (%) G4	G3	G1,G2	BL	Meg	Mit	Nec	Total ($\times 10^6$)
NA	598	0	4730 (84)	512 (9)	136 (2)	222 (4)	31 (0.5)	0 (0.0)	19 (0.3)	6.23 (100)
LA	784	8	4580 (58)	2610 (33)	430 (5)	118 (2)	17 (0.2)	101 (1.3)	8 (0.1)	8.66 (100)
REM	2030	7	470 (28)	998 (60)	123 (7)	30 (2)	18 (1.1)	21 (1.3)	0 (0.0)	3.70 (100)
Total	3412	15	9780 (64)	4120 (27)	689 (5)	370 (2)	56 (0.4)	122 (0.8)	27 (0.2)	18.59 (100)

[a] NA = nonadherent; LA = lightly adherent; REM = remains stroma-bound.
[b] G4 = late granulocytes; G3 = myelocytes; G1, 2 = myeloblasts, promyelocytes; BL = undifferentiated blast cells; Meg = megakaryocytes; Mit = mitotic; Nec = necrosis.
Initial composition of the three culture layers as harvested before subculturing.



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Comparison of Tube and Flask Subculture

To observe granulopoietic cells during completion of their terminal maturation program, we needed a system in which no stem cells were maintained; so we used tubes in which no stromal layer could form. However, to determine if other important differences were introduced by using tubes, we subcultured aliquots of NA and LA suspensions in both vessel types and compared the cell disappearance curves (Fig. 3). Cells disappeared at equal rates from both culture vessels, unless the suspensions contained cells capable of stroma formation, such as when LA cells were placed in flasks. They established a stroma in five days and grew into fully productive cultures in four weeks. (A second transfer into fresh flasks was unsuccessful because no stroma developed.) Nonetheless, NA cells disappeared at the same rate in flasks and in tubes, indicating that the survival conditions were similar for at least seven days.

Time Between "Feedings" and CFU-s Survival

The fractional survival of cells (Fig. 4A) and CFU-s (Fig. 4B) was calculated for different subculture feeding conditions. Table II presents the half-life values, calculated from the regression lines. A comparison of the six regression lines shows that the CFU-s disappeared much faster than did the cell counts. This difference could be due to differentiation of CFU-s into precursors, or to some CFU-s dying under the stroma-free condition to which they were subjected, as Part II discusses [7]. Both CFU-s and differentiated cells disappeared more slowly when they came from the LA and REM layers than when they came from the nonadherent culture phase. Presuming that the disappearance curves represent a net effect of new cell production versus cell death, this indicates that the balance between cell birth and death processes varied depending on the cell composition of the subcultured layer. Differences among CFU-s, depending on whether they settled on or within the stroma, may exist as well. However, the data are not sufficient to allow this conclusion without further experiments.

Failing to supply fresh medium on the day of subculture did not result in early cell losses, indicating the nutrients were not exhausted during 10 to 14 days. When subculturing was begun on different days, cells from the NA and the LA layers disappeared more slowly when subcultured three days after feeding, rather than seven days. This effect could be caused by the high proliferative activity typically

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Fig. 3. Disappearance curves of cells subcultured in flasks (●) or tubes (○). Cells from the nonadherent culture layer (NA) disappeared at equal rates from flasks and tubes. Cells from the lightly adherent (LA) layer disappeared more slowly from the flasks because they were able to form a supporting stroma.

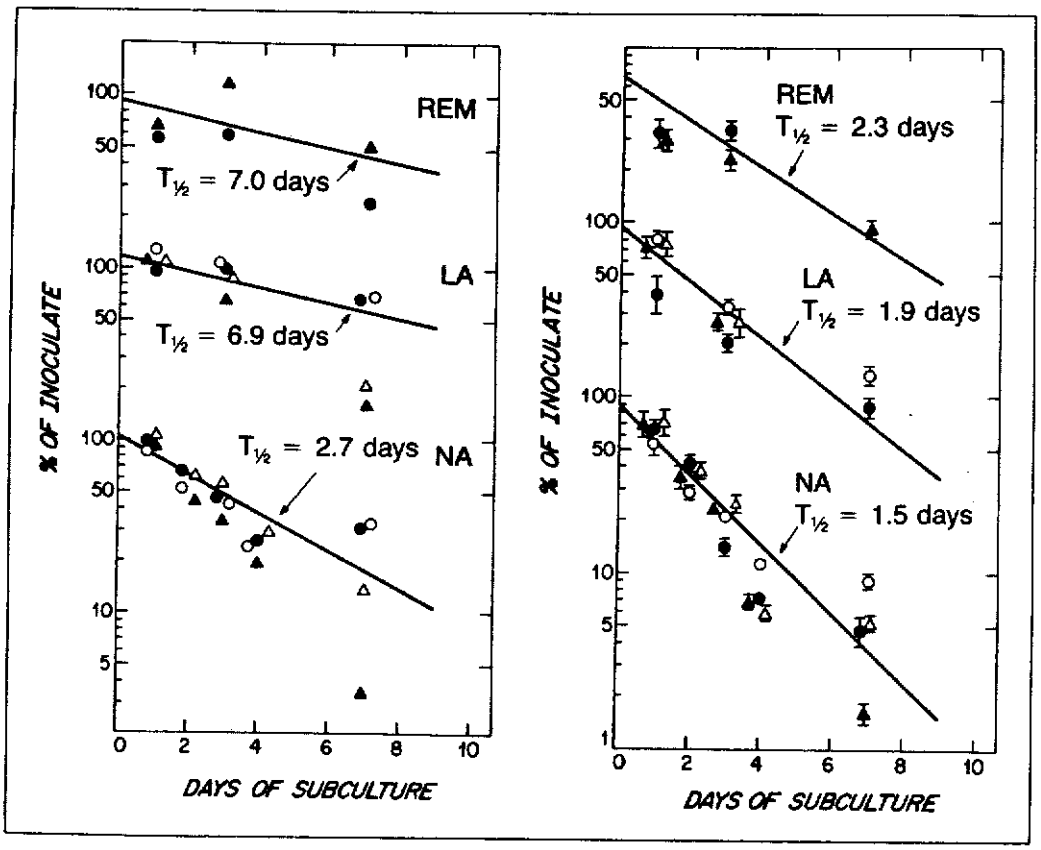

Fig. 4. Cell (A) and CFU-s (B) disappearance during seven days of subculture in plastic tubes. Nonadherent (NA), lightly adherent (LA), and remaining (REM) cells from Dexter-type cultures were separately subcultured in four variations: 3 days after feeding, with (•) and without (○) fresh medium; or 7 days after feeding, with (▲) and without (△) fresh medium. The time survival fractions are shown. Regression lines were drawn, using the average slopes and intercepts from the four treatment groups.

seen two to three days after feeding [4]. But since REM cells showed an opposite effect, the significance of the difference between day 3 and day 7 half-lives is not certain. The average disappearance times (Table II) are valid estimates of overall cell disappearance for longer culture periods.

Subculture of the Three Layers

The differential cell disappearance curves were prepared for 10-day subcultures of three culture layers in tubes. The overall cell counts were fitted to a simple exponential function so that overall cell disappearance rates could be

Table II. Disappearance half-life of cells and CFU-s in subculture

	Day 3		Day 7		Mean ± SE
	Fed	Not fed	Fed	Not fed	
Cell subculture					
NA	3.5[a]	3.8	1.4	2.2	2.7 ± 0.6
LA	12.3	9.8	2.5	2.9	6.9 ± 2.5
REM	3.9	ND	10.1	ND	7.0
CFU-s subculture					
NA	1.5	2.0	1.1	1.5	1.5 ± 0.2
LA	2.2	2.4	1.6	1.6	1.9 ± 0.2
REM	2.3	ND	2.4	ND	2.35

[a] $T_{1/2}$ values were calculated (in days) from linear regression analysis of the log-transformed survival fractions in Figures 4 and 5.

ND = not done.

obtained. The cellular components of each cultured layer, as determined by the absolute differential cell counts, were examined. Their time curves, not fit to a mathematical function, are shown in Figure 5. Presumably, these curves reflect at least some of the underlying cell birth, death, and transformation processes that gave rise to the overall disappearance rates. The cell types shown in Figure 5 are: undifferentiated blast cells or hemocytoblasts (BL); early granulocytic precursors, i.e., myeloblasts and promyelocytes (G1, G2); myelocytes (G3); and late granulocytes, i.e., metamyelocytes, band forms, and segmented forms (G4). The total cellularity (solid line) reflected the most numerous cell population, G4, and also included stroma cells (ranging from 1×10^5 to 33×10^5) and megakaryocytes (Meg; ranging from 2×10^3 to 30×10^3).

During the first week of subculture, there was significant proliferative activity, demonstrated by an increase in mitotic figures (Fig. 5A, C), a wave of immature neutrophils (Fig. 5A, B), and a rise in postmitotic cells (Fig. 5B, C). A proliferative burst was in progress and generated peak values in G4 after two or three days. The decline of immediate precursors of G4 throughout the experiment (Fig. 5B, C) is explained by the diminishing influx of stem cells and progenitors. The transient low before the proliferative burst in the nonadherent layer (Fig. 5A) is not clear to us. Although we cannot rule out an undetected counting error, or an increased death rate, a possible explanation for the loss is an initial failure to divide as suggested by the lack of mitoses. The early decrease of G4 in the

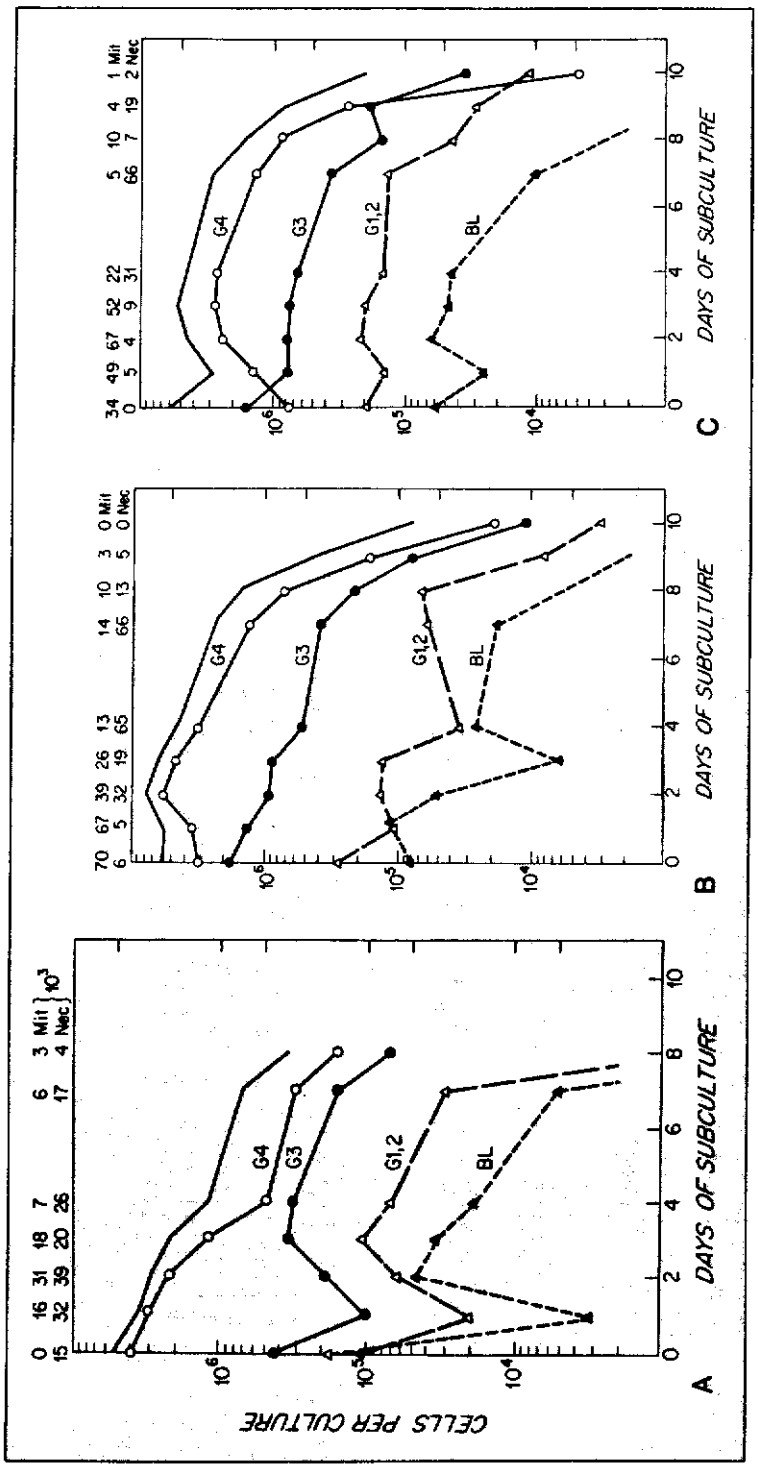

Fig. 5. Maintenance of granulopoiesis in tube subcultures. The number of undifferentiated blast cells (BL), myeloblasts and promyelocytes (G1, G2), myelocytes (G3), and postmitotic neutrophils (G4) is shown. The total cellularity, including stromal cells, is given as a solid line. The number of mitotic and necrotic cells (Mit, Nec) is indicated at the top of the graph. (A) For NA cells, with mitotic and necrotic cell numbers $\times 10^3$. (B) For LA cells. (C) For the REM cells.

nonadherent layer can be explained as follows. As long as all three layers were in direct physical communication in the flask, the nonadherent cells came largely from the LA layer. When the NA layer was disconnected from its main cell source, its G4 population declined because the end cells disintegrated faster than they were acquired. The small amount of cell birth within the NA layers became noticeable 2 days after subculturing and led to a slowing of cell decline from day 4 to 7.

The proliferative wave was exhausted after four to five days, and after seven days cells were rapidly lost from all cultures. If the lack of renewal on the stem cell and progenitor levels was the only reason for the terminal loss, only senescent deaths of the postmitotic cells would result. However, one would then expect a gradual depletion of precursors first and G4 cells last, which was not observed. The physiological culture conditions in tubes lasted apparently no longer than one week.

Discussion

Part of the granulocytic production in long-term bone marrow culture takes place within the stromal layer where hematopoietic cells are actually buried under stromal cells [8, 9]. We found proliferative cell nests (Fig. 2A), some of which could not be removed by mechanical or chemical means without disrupting the stroma. The thin, transparent reticulum cell sheets that covered such cells were visible by scanning electron microscopy (Fig. 2D). This report presents an account of the cell composition and kinetics characteristic of three hematopoietic layers.

Although the same cell types were found in each layer, their distributions constituted a gradient where the lowest, stroma-bound layer contained 70% immature cells, while the uppermost, nonadherent layer contained more than 80% postmitotic cells (Table I). Identical subculture of the individual layers led to expected population changes, assuming that the mitotic programs inherent in the cultured cells were executed. Part II of this study validates the predictability of those changes [7].

Stem cells appeared to follow slightly different programs, depending on whether they were stroma-bound, lightly adherent, or nonadherent before the subculturing. The contribution of cell death, self-renewal, and differentiation to the stem cell disappearance rates in subculture is not clear. But in view of the identical experimental conditions, it is unlikely that there were different death rates of CFU-s. Differences in disappearance rates of CFU-s for different layers would suggest that different self-renewal (or differentiation) probabilities are

correlated with the tendencies of stem cells to settle within or on top of the stroma, or to float above it.

Fresh medium elicits an immediate burst of stem cell proliferation [8] that leads to elevated cell counts in two days [4]. Three days after feeding, the proliferative wave was in progress at the start and ebbed after four to five days. We expected that adding fresh medium at the time of subculturing would reinforce and prolong the proliferative burst. Whether it did is not entirely certain, since failing to feed the subcultures at their initiation did not result in earlier cell disappearance. The eventual cell loss during the second culture week may have been hastened because we did not add serum to the fresh medium on day 7; we wanted to avoid imposing the stimulus of another proliferative wave on the then-terminal cell population.

The intimate enclosure of hematopoietic precursors and stem cells between stromal folds illustrates the importance of the microenvironment [10-12]. Yet, stem cell heterogeneity seemed to persist in the identical microenvironments of the subcultures, suggesting that a predetermined program was being followed. This study supports the view that an adequate microenvironment is required for the predetermined cell development programs to be induced and executed. In a flask culture, for instance, an adequate microenvironment exists for eliciting the proliferative program of individual CFU-s, as shown by clonal cultures [3, 13]. This cannot be accomplished in tubes, although the microenvironment of the fiber appears to be adequate to complete the terminal division and maturation program.

Acknowledgments

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