Cell-Cell Interaction in Vitro: Studied by Density Separation of Colony-forming, Stimulating, and Inhibiting Cells From Human Bone Marrow

By J. S. Haskill, R. D. McKnight, and P. R. Galbraith

Cell-cell interaction in vitro was studied by separating normal human bone marrow cells into fractions on the basis of their buoyant density in a linear gradient of bovine serum albumin and by assaying the colony-forming ability of marrow fractions and combinations of marrow fractions in methylcellulose medium without an added extrinsic source of colony-stimulating factor. In this culture system unfractionated marrow cells seldom formed colonies, but colonies grew from the low-density fractions, suggesting that an inhibitor had been removed by cell fractionation. When cells from the low-density fraction were mixed with the cells from the middensity and highdensity fractions, stimulation and inhibition of colony size respectively were observed. These observations suggest that cell-cell interaction may play a role in regulation of granulopoiesis in vitro and suggests an approach to the investigation of granulopoiesis in disease.

Human Bone Marrow Cells clone in vitro when an appropriate culture medium and source of colony-stimulating factor (CSF) are provided. Each colony arises from a single colony-forming cell (CFC).

Using a cell separation technique in which normal human bone marrow cells were subjected to fractionation on the basis of their density in a linear gradient of bovine serum albumin (BSA), we found that normal human CFC, unlike murine CFC, gave a fairly homogeneous density profile and were restricted to the low-density region of the gradient. In addition, CFC-enriched fractions grew colonies in the absence of an added extrinsic source of CSF. Unfractionated bone marrow seldom grew colonies under identical conditions, suggesting an inhibitor had been removed by the cell separation procedure. By recombining bone marrow fractions we have obtained evidence suggesting that normal bone marrow contains both colony-inhibiting cells (CIC) and colony-stimulating cells (CSC). Preliminary studies of interacting cell populations suggest an approach to study the control of normal and possibly leukemic granulopoiesis.

MATERIALS AND METHODS

Bone Marrow

Bone marrow aspirated from the sternum or iliac crest of human donors was collected in sterile heparin tubes and sedimented at 500 g for 10 min. The supernatant nucleated cell-rich plasma was removed, washed twice in tissue culture medium, and the concentr-
tion of nucleated cells was determined to permit appropriate dilution of the cell suspension to be made before the cells were plated in culture or subjected to cell fractionation.

Cell Fractionation

Equilibrium density gradient centrifugation was carried out in continuous linear gradients of bovine serum albumin (BSA). Less than $3 \times 10^8$ bone marrow cells were contained in each density gradient to avoid aggregation. Cell fractions were collected and washed twice in culture medium. Cell suspensions obtained from each fraction were appropriately diluted with culture medium after aliquots had been taken for total and differential nucleated cell counts. The cell suspensions (or mixtures of cell suspensions) were then plated in culture.

Cell Culture

Methylcellulose medium was used throughout (Grand Island Biological Company Medium 1066 supplemented with single strength additions of glutamine, pyruvate, nonessential amino acids, and 12% fetal calf serum). Double-strength culture medium diluted with 3% methylcellulose (Dow Chemical Company, Viscosity 4000 USP Grade) in water, containing the bone marrow cell suspension, was plated on bacteriologic grade Petri dishes (Falcon Plastics 1008, 35 x 10 mm). When specified, the bone marrow cell suspensions in methylcellulose medium were placed over feeder layers prepared by the method of Robinson and Pike. Cultures were gassed with 10% CO$_2$ in air (with a bicarbonate concentration of 2.1 g liter). This resulted in a pH of 7.1.

Feeder Layers

Normal human peripheral blood collected in sterile heparin tubes was allowed to stand for 1 hr at room temperature. Leukocyte-rich plasma was then harvested, and the leukocyte concentration was adjusted so that the final concentration was $10^6$ nucleated cells per milliliter of 0.5% agar.

Colony Counts

Colony counts were made (duplicate or triplicate plates) on day 14. Colonies containing less than 35-40 cells were not scored. The average number of cells per colony was determined (duplicate or triplicate plates) by sucking up individual colonies with a finely drawn pasteur pipette and streaking them out on a microscope slide upon which they were stained (Wright’s or peroxidase). Sampling was attempted by selecting the first ten colonies counted on each plate.

RESULTS

Cell Separation Studies in Normal Subjects

Density Profile of Marrow Granulocytes: A typical density profile obtained when normal bone marrow cells were subjected to cell separation is shown in Fig. 1. The most immature granulocytic cells (blasts and promyelocytes) occupied the light-density region, the most mature cells (bands and segmented forms) the heavy-density region, and cells of intermediate maturity (myelocytes and metamyelocytes) occupied and middensity region of the gradient. Lymphoid cells, although scattered throughout the gradient, were present in highest proportion in the light-density fractions. Nucleated erythroid cells were also scattered throughout the gradient.

Density Profile of Normal Colony Forming Cells: The ability to form leukocyte colonies in vitro (40 or more cells) was restricted to cells isolated from the light-density region of the gradient A (density, 1.055-1.064), although there was slight spillover into the intermediate fractions A$^1$ (density, 1.064-1.066) (Fig. 2). Colony size varied in the fractions. The largest colonies with an average size of 200 cells were found in the peak colony-forming fraction.

Under identical culture conditions, colony formation seldom occurred from unfractionated
Fig. 1. Normal bone marrow: Equilibrium density distribution profile of total nucleated cells (expressed as a percentage of the largest fraction). The percentage composition of each fraction with respect to granulocytic cells (above) and lymphoid, monocyto-, and nucleated erythroid cells (below) are also shown.

Bone marrow, suggesting cell fractionation had removed an inhibitor. This prompted studies in which cell fractions were recombined.

Recombination of Bone Marrow Fractions: Bone marrow cells obtained from the light-density (1.059–1.064) region of the BSA gradient (A cells) were mixed in increasing dilution: first, with cells obtained from the mid-density (1.064–1.066) region (A1 cells), and second, with cells obtained from the high-density (1.075–1.082) region (B cells) (Fig. 2). In each cell suspension the total number of nucleated cells plated was constant at 2 × 10^6. The results of a typical experiment are shown in Fig. 3.

Since the CFC-enriched fraction (A) was progressively diluted by fractions poor in CFC (A1 and B), the mean number of colonies per plate decreased. Therefore, changes in colony size was taken as an index of inhibition. Increasing dilution by A1 cells did not inhibit, but rather enhanced colony size, suggesting colony-stimulating activity in this fraction. In contrast, dilution by B cells decreased colony size, suggesting inhibitory activity by high-density cells.

In order to exclude the possibility that inhibition of colony size was simply a result of
Marrow fraction recombination: A mixture plot at constant cell number \((2 \times 10^5)\). The CFC-enriched fraction (A) was plated with increasing proportions of cells from the intermediate-density fraction \(A^I\) and from the high-density fraction (B). The numbers given beside the experimental points indicate the per cent PMN (bands and segmented forms) in each cell mixture. With increasing dilution of the CFC-enriched fraction (A) the number of colonies per plate decreased, but did not reach zero when \(A^I\) cells constituted 100\% of the population plated, since the \(A^I\) fraction contained a small number of CFC. In spite of this, colony size increased when \(A^I\) cells were added, suggesting stimulation of growth of a smaller number of CFC.

Fig. 4. Marrow fraction recombination: Effect of increasing numbers of normal B cells plated with \(2 \times 10^5\) A cells. The B-cell fraction contained 95\% PMN. When identical cell mixtures were plated over a feeder layer, the inhibition of colony formation was overcome and colonies of 40 to 1000 cells grew. Maximum colony size was 400 cells in the absence of feeder layers. (In order to demonstrate an inhibitory effect, the B-cell fraction must be highly enriched with respect to PMN. If a poor separation is achieved the B-cell fraction may provide variable degrees of 'stimulation'.)

DISCUSSION

These preliminary observations suggest that normal human marrow contains at least three cell types, which, because of differences in density, may be separated; and, because of their interactions in vitro, may be assayed. Marrow fractions enriched with low-density CFC appear to form colonies without an added extrinsic source of colony-stimulating factor (CSF). The colony-stimulating cells (CSC) and colony-inhibiting cells (CIC) appear to be of intermediate and high density, respectively. Although the identity of these cells...
has not been firmly established, circumstantial evidence based upon cell fraction morphology accuses PMN (bands and segmented forms) of inhibition. However, the fraction showing stimulatory activity, although highly enriched with myelocytes and metamyelocytes, also contained a small proportion of lymphoid cells, making it difficult to identify the stimulating cells. In fact, Moore and Williams have presented evidence that the cells that produce CSF in feeder layers are either lymphocyte-like or monocytic, and more recent observations support their contention.

The concept that PMN possess inhibitory properties is not new. Paran et al. and Shadduck have shown that intact granulocytes inhibit the activity of a standard conditioned medium as source of colony stimulating factor. Disrupted granulocytes were ineffective, and with prolonged incubation in vitro the conditioned medium regained stimulatory activity, a phenomenon that is open to several interpretations. Granulocytes aging in vitro might have lost their inhibitory activity, gaining stimulatory properties. Alternatively, age-related granulocyte disruption in vitro might be associated with simple loss of inhibitory properties, and/or predominance of CSF-producing cells, with a shift in the balance of forces toward stimulation. It is perhaps noteworthy that Paran et al. and Shadduck harvested rat granulocytes that had migrated into the peritoneal cavity, and cell populations collected in this way may have had a reduced proportion of CSF-producing cells.

It is not possible from our studies to state whether stimulating and inhibiting activities result only from production and diffusion of cell products or if these activities may also be mediated by direct cell-cell interaction. While it is clear that CSF generated in agar feeder layers is diffusible and can stimulate target cells, no similar diffusability of colony-inhibiting factor has been detected. The relationship of CSC and CIC to colony-stimulating and -inhibiting activity present in sera of some patients remains to be clarified.

It is suggested that serum CSF may be a granulopoietin analogous to erythropoietin, because it increases in the serum in response to neutropenia. CSF activity has been found in extracts of several tissues and as yet its primary site of production (i.e., in response to neutropenia) has not been ascertained. Perhaps it is generated locally in response to local needs. It has also been suggested that a neutrophil-releasing factor, present in the blood of neutropenic patients, provides a mechanism for rapidly mobilizing marrow PMN stores. However, it is not yet clear if cell-cell interaction in vitro fits into the overall scheme of control of granulopoiesis in vivo. If, as appears likely, that generation of CSF and neutrophil-releasing activity provide long-range control mechanisms to ensure an adequate supply of granulocytes in an emergency, it is possible that cell-cell interactions might provide a short range means of maintaining homeostasis under normal conditions. Studies on cell-cell interaction in vitro may provide an approach to the study of normal and leukemic granulopoiesis.

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