Heterogeneity in Human Neutrophil, Macrophage, and Eosinophil Progenitor Cells Demonstrated by Velocity Sedimentation Separation

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Progenitor cells of neutrophils, monocyte-macrophages, and eosinophils in human marrow were enumerated in agar cultures stimulated by placental conditioned medium or white cell underlayers. Fractionation of marrow populations by velocity sedimentation showed that the profiles of neutrophil and macrophage colony-forming cells shifted from a peak of 8–9 mm/hr in 7-day cultures to a peak of 6–7 mm/hr in 14-day cultures. This shift was due to degeneration of some early colonies formed by rapidly sedimenting cells and the delayed formation of colonies by slowly sedimenting cells. Eosinophil colony formation was delayed until the second week of incubation. Further evidence of heterogeneity was the observation that rapidly sedimenting colony-forming cells were more responsive to stimulation than more slowly sedimenting cells. In the macrophage and eosinophil populations, cluster-forming cells were partially segregable from colony-forming cells. The observed heterogeneity was similar to that described previously in the mouse and suggests that separate subpopulations of progenitor cells may exist within each hemopoietic family that could possibly give rise to functionally different progeny.

The development of semisolid culture techniques for growing colonies of differentiating neutrophils, monocyte-macrophages, and eosinophils has permitted analysis of the properties and behavior of individual progenitor cells in the marrow or peripheral blood of humans. A striking feature of hemopoietic colonies grown in vitro has been the variability in their size and shape, indicating the probable existence of a significant degree of heterogeneity among progenitor cells within even one hemopoietic family. In previous studies in mice, evidence of this heterogeneity was obtained from studies on marrow populations fractionated by velocity sedimentation separation. It was shown that the more rapidly sedimenting progenitor cells tended to form macrophage colonies, while more slowly sedimenting cells tended to form neutrophil and eosinophil colonies. Furthermore, more rapidly sedimenting colony-forming cells were notably more responsive to stimulation by the specific regulator, granulocyte-macrophage colony-stimulating factor (GM-CSF), than more slowly sedimenting cells. Parallel studies using cell fractionation either by velocity sedimentation or buoyant density separation showed that certain subpopulations of colony-forming cells responded to one form of GM-CSF but not another.
These observations raise the possibility, of potential importance in various diseases, that heterogeneity in progenitor cells may lead to the formation of subpopulations of functionally different end cells. This paper presents data indicating that the situation in humans appears to be essentially the same as in the mouse and that heterogeneity exists within neutrophil, monocyte, and eosinophil progenitor populations.

MATERIALS AND METHODS

Bone Marrow Cultures

Cultures of 100,000 unfractionated bone marrow cells (1 ml) were prepared in 35-mm Petri dishes containing Dulbecco's modified Eagle medium in 0.3% agar. Final serum concentration was 20%. The medium was prepared by mixing equal volumes of double-strength Dulbecco's modified Eagle medium and 0.6% Bacto agar in water (the latter boiled for 2 min to dissolve and held at 37°C). The formula for the double-strength Dulbecco's modified Eagle medium was as follows: Dulbecco's modified Eagle medium H16 instant tissue culture powder, 10 g (Gibco, Grand Island, N.Y.); 390 ml double glass-distilled water; 3 ml L-asparagine (20 g/ml); 1.5 ml DEAE dextran (75 g/ml); 0.375 ml penicillin (200,000 U/ml); 0.375 ml streptomycin (200 mg/ml); 4.9 g sodium bicarbonate; 63 ml horse serum; 187 ml fetal calf serum.

Marrow cells used were portions of diagnostic specimens from various patients who were found subsequently to have had no detectable hemopoietic disease. These human investigations were performed after review and approval of the Ethics Committee of the Walter and Eliza Hall Institute in accord with an assurance filed with, and approved by, the Department of Health, Education and Welfare.

Colonies in these cultures were stimulated by the addition of 0.1 ml of human placental conditioned medium or the use of rhesus monkey peripheral blood underlayers containing $1 \times 10^6$ nucleated cells. Cultures were allowed to gel at room temperature and were incubated for 7-14 days at 37°C in a fully humidified atmosphere of 10% CO₂ in air. Routine colony counts were performed using a dissection microscope, scoring aggregates of 40 or more cells as colonies and 3-40 cells as clusters.

Staining for Eosinophil Colonies

Intact culture dishes were stained differentially for eosinophil colonies with eosin yellow, orange G, and phloxine using the technique described previously. After fixing, mounting on microscope slides, and drying, the cultures were covered with microscope oil and scored at ×100 and ×1000 magnifications. For convenience, a yellow Wratten K1 filter was used to accentuate the red granules in the eosinophils. Aggregates of more than 40 cells were classified as colonies and aggregates of 3-40 cells as clusters.

Separation of Cells by Velocity Sedimentation

In these experiments up to $100 \times 10^6$ marrow cells were suspended in 24 ml of phosphate-buffered saline (PBS of human tonicity pH 7.5) supplemented with 3.3% (v/v) fetal calf serum. This suspension was applied to a buffered step gradient, consisting of 7%-25% fetal calf serum in PBS in an autoclavable glass chamber 11 cm in diameter (Johns Scientific, Toronto). After sedimentation at 4°C for 2-3 hr, the cone volume was discarded and 10-ml fractions were collected and concentrated to 1-2 ml by centrifugation. Cell counts were performed on each fraction and a constant number of cells from each fraction were cultured in agar as described above.

RESULTS

Figure 1 shows a typical result of fractionating a marrow population from a patient with no demonstrable hemopoietic disease using velocity sedimentation separation. Red cells in the suspensions served as a useful internal marker and sedimented with a peak velocity of 2.5-3.5 mm/hr. The sedimentation profile of the nucleated cells was usually more complex, with most cells sedimenting at
5-9 mm/hr. As shown in the example, in cultures scored on day 7 of incubation, the colony-forming cells appeared to segregate as a single homogeneous peak with a peak velocity of 7.5-8.5 mm/hr, and a shoulder in the more rapidly sedimenting fractions that might have represented cells in the S, G2, or M phase of the cell cycle.

In cultures of fractionated mouse marrow cells, cluster-forming cells were shown to sediment significantly more rapidly than colony-forming cells. Figure 2 shows the mean profiles of colony- and cluster-forming cells from marrow...
samples from four patients with no demonstrable hemopoietic disease. From these data it was clear that, unlike the situation with the mouse, the sedimentation profiles for total cluster-forming cells did not differ significantly from that of total colony-forming cells. Similar data were obtained from an analysis of total colonies and clusters in day 14 cultures.

To score such human marrow cultures simply by counting total colonies and clusters is no longer adequate, since they may be composed of neutrophils, monocytes, or eosinophils. Figure 3 shows the mean data from velocity sedimentation experiments using four human marrows from patients with no demonstrable hemopoietic disease. All of the colonies and clusters grown at day 7 from each fraction were classified according to cellular composition. Each type of colony-forming and cluster-forming cell appeared to segregate as a relatively uniform population with a peak sedimentation velocity of approximately 8 mm/hr. Note that, as shown previously, eosinophil colony development exhibited an initial lag. By day 7 the only eosinophil aggregates present contained less than 40 cells in size and were classifiable merely as clusters. It should be noted that the profile for eosinophil cluster-forming cells differed from the other profiles in exhibiting a distinct secondary peak in the more rapidly sedimenting cell region (9–12 mm/hr).

![Fig. 3. Profiles of neutrophil, macrophage, and eosinophil colony-forming (•) and cluster-forming (○) cells in day 7 cultures stimulated by placental conditioned medium. Each profile represents mean data from cultures of marrow cells from four patients with no detectable hemopoietic disease. Data normalized to percentages of levels in fraction with maximum frequency of colony- or cluster-forming cells. Note absence of eosinophil colonies in day 7 cultures.](attachment://image.png)
It was shown previously that between 7 and 14 days of incubation of human marrow cultures there is a progressive fall in the absolute number of neutrophil colonies and a marked rise in the number of eosinophil colonies to comprise 20%–30% of all colonies. An analysis at day 14 of the velocity sedimentation profiles of cells forming colonies and clusters is shown in Fig. 4. These profiles showed a number of differences from the day 7 profiles obtained from the same four marrows (Fig. 3). The profiles for neutrophil, macrophage, and eosinophil colony-forming cells were significantly displaced to the more slowly sedimenting regions compared with the day 7 profiles. Furthermore, although single peaks of colony-forming cells were present in each group, macrophage colony-forming cells were distinctly more rapidly sedimenting than either neutrophil or eosinophil colony-forming cells. It will also be noted that although the distribution profiles of all cells forming clusters (combined, regardless of type) did not differ from the profile of all cells forming colonies (top portion of Fig. 4), when each hemopoietic population was analyzed separately, clear differences were seen. Many of the cells forming macrophage and eosinophil clusters were more rapidly sedimenting than the majority of corresponding colony-forming cells. This difference was most evident for eosinophils, for which a distinct second peak of cluster-forming cells was present with a sedimentation velocity of 11–12 mm/hr.

Fig. 4. Profiles of neutrophil, macrophage, and eosinophil colony-forming (e) and cluster-forming (c) cells in day 14 cultures stimulated by placental conditioned medium. Each profile shows mean data from same four patients as in Fig. 3. Data normalized to percentages of levels in fraction with maximum frequency of colony- or cluster-forming cells. Note shift in profiles of colony-forming cells to slowly sedimenting regions compared with distribution in Fig. 3.
When the absolute number of colonies developing in these cultures was considered, the basis for these changes in profiles was found to differ for each hemopoietic class. For neutrophils, there was a reduction between days 7 and 14 in the number of clusters and colonies formed by every fraction. The reduction, however, was least for slowly sedimenting cells, causing a shift in the velocity sedimentation profile, with a new peak between 5–7 mm/hr. This peak did not represent new colony-forming cells, merely cells forming early colonies that were able to survive longer in culture.

For macrophages, between days 7 and 14 there was an absolute increase in the number of colonies and clusters. Thus the new day 14 peak of 6–8 mm/hr represented a large number of new colonies and clusters formed from slowly sedimenting cells during the second week of incubation. Alternatively, since some granulocyte colonies can transform to macrophage colonies, some of the additional colonies might have arisen from colonies scorable on day 7 as neutrophil colonies.

There was also an increase between days 7 and 14 in total numbers of eosinophil colonies and clusters, although there was actually a slight decrease in the number of eosinophil clusters formed by rapidly sedimenting cells (8–12 mm/hr). This decrease was more than compensated for by a large increase in clusters formed by slowly sedimenting cells (4–8 mm/hr) and the enlargement of many of these clusters to form aggregates scored as colonies.

The conclusion is that for each of the three hemopoietic progenitor populations, heterogeneity exists in that some progenitors form early colonies which may or may not persist during the second week of culture, while others form colonies that are slow to develop but are prominent by the end of the second week. These different types of colony-forming cells are able to be partially segregated from one another using velocity sedimentation separation.

To exclude the possibility that this was a special phenomenon due to the use of placental conditioned medium, parallel analyses were performed in cultures stimulated by peripheral blood underlayers. The poor visibility associated with the presence of cells and debris in the underlayers prevented a complete analysis by colony type. As shown in Fig. 5, however, the profiles for total colony-
forming cells at days 7 and 14 showed very clearly the same phenomenon described above in cultures stimulated by placental conditioned medium. The peak of colony-forming cells at day 7 was 8–9 mm/hr, but by day 14 the peak was significantly shifted to the more slowly sedimenting regions with a peak of 5.5–6.5 mm/hr. In this experiment, the absolute frequency of day 14 colonies was $165/10^9$ cells and of day 7 colonies $50/10^9$ cells. Thus the new peak of slowly sedimenting cells was not due simply to the selective survival of a subset of slowly sedimenting cells but to the formation of a new set of colonies by these slowly sedimenting cells.

Responsiveness to CSF

With mouse marrow cells, it has been shown that the most rapidly sedimenting neutrophil and macrophage colony-forming cells are also the most readily stimulated by CSF and that there is a progressive fall in responsiveness with decreasing sedimentation velocity. Replicate cultures were performed of fractionated human marrow populations in which the proliferation of colony-forming cells in each fraction was stimulated by optimal or suboptimal concentrations of placental conditioned medium. For each fraction, colony counts in suboptimally stimulated cultures were expressed as a percentage of colony counts in optimally stimulated cultures. In a typical example shown in Fig. 6, the ratio of suboptimally to optimally stimulated colony numbers at day 14 fell progressively from the more rapidly sedimenting fractions to the more slowly sedimenting fractions, indicating that, as in the mouse, the rapidly sedimenting colony-forming cells were also more responsive to stimulation than the slowly sedimenting cells.

A similar type of result was obtained if colonies were scored on day 7 or if peripheral blood underlayers with different colony-stimulating activities were compared.

DISCUSSION

The present observations have documented the existence of heterogeneity in human neutrophil, macrophage, and eosinophil progenitor populations. Although the differences were not as clear as those for the mouse, at least a proportion of macrophage and eosinophil cluster-forming cells were more rapidly sedimenting and partially segregatable from the corresponding colony-forming cells. Macrophage colony- and cluster-forming cells tended to have a slightly faster sedimentation velocity than neutrophil colony- and cluster-forming cells.
(seen better in day 14 cultures). Rapidly sedimenting cells were more responsive to stimulation than slowly sedimenting cells. All three of these characteristics have previously been demonstrated in corresponding hemopoietic populations in the mouse marrow.

The present observations have also documented a remarkable shift in the profiles of the colony-forming cells according to whether the cultures were scored at day 7 or 14 of incubation. In view of the variation between different laboratories in the day on which cultures are scored, an important conclusion from this finding is that observations on days 7 and 14 are not monitoring the same colony-forming cell populations and that the data obtained may not necessarily be comparable. A corresponding study has not yet been published for mouse marrow cells, but in this laboratory a small shift has been observed in velocity sedimentation profiles in the direction of slowly sedimenting cells if day 14 cultures are compared with day 7 cultures (Metcalf D: Unpublished data).

The present data indicate that the distribution patterns of neutrophil, macrophage, and eosinophil progenitor cells differ and emphasize that human marrow cultures can no longer be scored simply by counting total colony and cluster numbers, regarding all colonies and clusters as being composed of the same cells.

The function served by the heterogeneity in hemopoietic progenitor populations is obscure at the moment. It is conceivable, however, that the heterogeneity indicates the existence within specific hemopoietic families of distinct subpopulations. If sufficiently sensitive functional tests could be developed it might be possible to show that the functions of the end cell progeny of these various subpopulations differ significantly from one another. Thus the segregation of distinct subpopulations may serve to create a flexible system in which demand for a particular subtype of end cell can be met most efficiently.

The heterogeneity documented in progenitor (colony-forming) cell populations in the present experiments implies that a significant degree of commitment or specialization has already occurred in these populations in reaching the progenitor cell stage. It is likely that placental conditioned medium contains separate factors stimulating neutrophil–macrophage and eosinophil proliferation, and if the operation of these factors is restricted to regulation of cell proliferation subsequent to the progenitor cell stage, they will have little direct influence in determining the number of cells entering each progenitor compartment.

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REFERENCES

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