Continuous Free-Flow Fractionation of Cellular Constituents in Rat Bone Marrow

By HOWARD C. MEL, LINDA T. MITCHELL AND BO THORELL

THE WELL-KNOWN heterogeneity of bone marrow cells greatly complicates biological, biochemical, and biophysical studies on this tissue. In such a situation two developments would appear highly useful: (1) an unambiguous, reproducible physical classification of the cellular constituents in the heterogeneous mixture, in the tradition established, for proteins, by Svedberg (sedimentation rate) and by Tiselius (electrophoretic mobility); (2) preparative separation of large numbers of cells of the different types, for direct investigation of their characteristic biological (e.g., transplantation, immunological) properties.1

A start has been made on achieving both of these objectives simultaneously. In a previous publication results were presented on apparently anomalously flow-sedimentation of rat bone marrow cells, considering only the two general classes—nucleated and nonnucleated.2 In the present paper we describe the more detailed continuous preparative fractionation of rat bone marrow as judged by morphological criteria. The different fractions will also be characterized by rough uncorrected sedimentation rates (in svedbergs).

No systematic review of the separation literature will be attempted here. Allfrey3 has reviewed literature prior to 1959. Recent remarks by DeDuve,4 and Beaufay and Bertheret5 are relevant to general biophysical problems of cell separations, particularly those based on centrifugation (the most common approach). In an interesting paper dealing with bone marrow, Goodman6 further reviews the literature, and presents her own experimental results. A very recent report is by Stoloff and Weiss.7 The cell separation problem is a difficult one, and Goodman's observation, that none of the past approaches has yielded a satisfactory separation from the standpoint of purity, high yield, and cell viability, appears still to be true today.

METHODS

Preparation of Sample

Male, Long-Evans rats weighing approximately 200 Gm. are decapitated by guillotine, then allowed to exsanguinate. The six long bones are quickly removed, both ends punctured with a No. 18 hypodermic needle and the marrow forced out in chunks or in a single plug, using 3–4 cc. of chilled isotonic saline for each bone. The marrow chunks are dispersed mechanically by forcing the suspension through a No. 60 gauge stainless wire mesh, followed by filtration through a nylon mesh filter bag, then expulsion through a No. 25 hypodermic needle. The cells are centrifuged at 3 C. for 5 minutes at 125 times gravity, then resuspended in saline and made up to the starting sample concentration of $2.6 \times 10^7$.

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cells/cc. At higher cell concentrations with the particular gradient solutions described below, the previously described "clump sedimentation" phenomenon is observed.\textsuperscript{8,9}

In other suspending liquids (e.g., Hanks' solution or isotonic sugar), the cells either became sticky or their condition deteriorated. Since the success of the fractionation procedure depends upon achieving a nonclumpy single-cell suspension, considerable care must be taken at each step. A single rat handled as above yields about $7 \times 10^7$ marrow cells.

**Fractionation Procedure**

The method employed is stable-flow free boundary (STAFL0) sedimentation.\textsuperscript{8,9,10} This is an extension of the earlier STAFL0-electrophoresis\textsuperscript{11} to the use of a gravitational force field. A multilayer, horizontally flowing, free liquid system is stabilized by hydrodynamic feedback principles rather than by use of any solid supporting medium.\textsuperscript{10} This leads to a special kind of plug-laminar horizontal flow. The gravitational force acts transverse to the flow, in the vertical sense, leading to a downward sedimentation movement of cells. Each cell follows a resultant pathway through the main chamber of the flow-cell determined by the horizontal flow rate ($\approx$ the same for all cells) and the vertical sedimentation rate (different for different cells).\textsuperscript{9} If the cell sample is put into the top inlet, an idealized steady-state migration pattern would resemble the wedge-shaped shaded area in figure 1, a schematic drawing of the flow-cell. In practice, patterns closely resembling that of figure 1 are seen. (The RBC have been indicated in the figure as the slowest sedimenting component.)

The inlet solutions feeding the flow-cell are isotonic in saline and have graded amounts of dextran, and are made by diluting 6 per cent Injection Dextran in Normal Saline (Cutter Laboratories, Berkeley, Calif.). The flowing layers are numbered 1--12 from top to bottom and have dextran compositions (in weight/volume per cent) as follows: No. 1, 0 per cent; No. 2, 0 per cent (cell-sample solution); No. 3, 0.50 per cent; No. 4, 0.65 per cent; No. 5, 0.80 per cent; No. 6, 0.85 per cent; No. 7, 0.95 per cent; No. 8, 1.03 per cent; Nos. 9 and 10, 2.1 per cent; Nos. 11 and 12, 3.1 per cent. Under the experimental conditions employed in these experiments, few of the cells sediment below the eighth outlet. The stronger density gradients at the 8--9 and 10--11 boundaries provide a "density shelf" to catch most of those that do. If any large clumps are not disrupted during the sample preparation they may sediment all the way to the bottom. The light horizontal lines inside the main chamber (fig. 1) indicate the initial discontinuities in dextran composition in adjacent layers. At later times (downstream distances) diffusion smooths these boundaries, though less with large molecules like dextran than with small molecules such as sucrose. Dextran is preferred to sucrose as an inert density-gradient-producing solute because it has negligible effect on the osmotic composition of the solution. Presence of even these...
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small amounts of colloid undoubtedly contributes to making a favorable solution environment for the cells.

Solutions are pumped into the main chamber by a ganged rack of 12 syringes. The cell-sample syringe is slowly stirred magnetically. The importance of a small diameter feed-in tube for a cell sample has already been discussed. In order to reduce variations in horizontal linear flow rate near the inlet, caused by this inlet tube having a smaller diameter than that of the others, the cell-sample solution is actually introduced through inlet No. 1. Since this solution has a higher density than the "layer No. 1" saline (actually introduced through inlet No. 2), the two solutions interchange positions immediately upon entering the flow-cell (see fig. 1). Without careful examination of the immediate inlet region, this exchange is not noticed: the top 1/12 layer of the main chamber is clear, the second 1/12 contains the cells. The surprisingly smooth nature of such a doubly bending and crossing inlet flow pattern has been illustrated in a photograph of alternating dye streams, taken in the presteady-state set-up period. To conserve sample during the set-up period, saline is initially substituted for the cell suspension. Upon establishment of the steady-state flow pattern the sample is switched in, in its place.

The flow rate, identical for all solutions, was chosen to give a steady-state residence (sedimentation) time of 32 minutes in the 30 cm. X 1.5 cm. X 0.7 cm. main chamber. This gives a sample flow of $2.2 \times 10^6$ cells/minute. Generally about 10 cc. of each outlet fraction have been collected.

Examination of Fractions

The fractions are often examined directly and photographed using phase contrast microscopy. Because of the greater detail to be seen and the greater general familiarity of most workers with stained smears, the results in this paper are based on counts made from smears. Each fraction is centrifuged and the supernatant removed by suction. A drop of aged rat serum is added to each tube, the pellet gently re-suspended, and several smears made from each fraction. When viability counts are to be made, a portion of the wet pellet is used for that purpose.

After air drying, the slides are first fixed-stained for hemoglobin by Ralph's stain. One set is then panoptically counterstained with Jenner-Giemsa stains, a second set with Giemsa alone. The remaining smears are fixed and retained as spares.

The following morphologic classifications have been used:

1. Erythrocytes (RBC).
2. "Erythroblasts" (EBL): we are using this designation for any nucleated erythroid cell showing a positive Ralph reaction.
3. Mature Granulocytes (WBC): segmented, or "band" (large, thin-ringed nucleus) granulocytes.
4. "Myelocytes" (MYEL): only used for distinctly granulated, nonsegmented (or non-"band") myeloid cells; includes promyelocytes and metamyelocytes; generally larger than WBC.
5. "Immatures" (IM): cells not fitting the other classifications, probably corresponding to "blast" cells; generally large.
6. Lymphocytes (L): used for lymphocytes of all sizes.
8. "Damaged": cells damaged beyond recognition as one of the above classes; many resemble the "amorphous pinkish patches" of Ramsell and Yoffey.

These classifications were chosen to minimize morphologic ambiguities rather than to maximize the detailed descriptive information.

RESULTS

The results of the differential counts for a single experiment (RTM-19) are
Fig. 2.—Relative compositions of collection fractions and sample in rat marrow experiment RTM-19.

presented in figure 2. The data are plotted as the percentage of the total cells in each fraction that are of the given type.

For a comparison of different runs, results for three experiments run under the same conditions are plotted in figure 3. "Damaged" cells (not shown) were usually less than 5 per cent, though in a few instances they were as high as 10 per cent of a fraction. In figure 3, lymphocytes have been shown separately from the immatures. The slides for figure 3 were recounted a number of times in order to have some data involving an irreducible minimum of subjectivity. Between 500 and 750 cells were counted each time for each slide. The results obtained were similar to those found in a large number of repeat experiments in which normal care in counting was exercised. A composite of data from 17 repeat experiments indicates the persisting patterns shown in table 1. The erythrocytes are always most concentrated near the top (fractions Nos. 2, 3). Erythroblasts + S.B. have always shown a peak in fraction No. 4, and quite often a second peak in fraction No. 6. With few exceptions, immatures reach a maximum in fractions Nos. 4 or 5. The myelocytes and mature granulocytes almost always rise to their maximum in the lower fractions Nos. 6–8; we have not seen a systematic separation between these two classes.

The number of cells/cc. in each collection fraction (averaged for three duplicate runs) is shown in table 2. A comparison of total cells/cc. in all collection fractions with the initially measured starting sample concentration reveals a 53 per cent recovery of cells.

In a number of experiments collection fractions were stained with eosin Y.14 "Viability" averages and ranges for five experiments are given in table 3.

DISCUSSION

The results in figure 2 for a single experiment indicate immediately that major changes of composition have been achieved relative to the starting
The actual ranges for the three detailed experiments reported in this paper can be read from figure 3.

Sample, and that great variations in composition have been achieved in the different collection fractions. Figure 3 shows the experimental reproducibility of the migration/fractionation behavior of the different cell classes in the three most carefully scored duplicate runs, carried out with different animals at different times. In spite of biological variability, the numerous steps in handling, and some unavoidable ambiguities in morphology, the cells do fall broadly into categories classifiable (and separable) according to physical (STAFLO-sedimentation) properties.

Since we find few lymphocytes in the rat marrow, curves (IM + L) in figure 2 and (Immature) in figure 3 show no qualitative difference. In grouping the "small blues" with "erythroblasts" instead of with lymphocytes we are being less consistent with the conclusions of Ramsell and Yoffey (who reported many lymphocytes in the marrow of their Lister Hooded rats) than with those of Schooley and Kelly and Schooley, Bryant, and Kelly.16 (The

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Maximum, in Collection Tube No.</th>
<th>Maximum Per Cent*</th>
<th>Starting Samples, Average Per Cent, with S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>2, (3)</td>
<td>94</td>
<td>45.4 ± 4.1</td>
</tr>
<tr>
<td>Erythroblasts + S.B.</td>
<td>4, (2nd pk. in 6?)</td>
<td>33</td>
<td>19.6 ± 8.5</td>
</tr>
<tr>
<td>Immatures</td>
<td>4–5</td>
<td>46</td>
<td>17.5 ± 3.9</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>6–8</td>
<td>53</td>
<td>12.3 ± 3.3</td>
</tr>
<tr>
<td>Mature WBC</td>
<td>6–8</td>
<td>30</td>
<td>8.4 ± 2.8</td>
</tr>
</tbody>
</table>

*The actual ranges for the three detailed experiments reported in this paper can be read from figure 3.
Table 2.—Total Cells/cc. in Collection Fractions
(Averages for Three Runs)

<table>
<thead>
<tr>
<th>Collection Fraction No.</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells</td>
<td>1.5</td>
<td>53</td>
<td>50</td>
<td>24</td>
<td>11</td>
<td>4.2</td>
<td>2.6</td>
</tr>
<tr>
<td>(units: 10^6 cells/cc.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.—Viability Averages and Ranges for Five Experiments

<table>
<thead>
<tr>
<th>Collection Fraction No.</th>
<th>Average Viability, Per Cent</th>
<th>Viability Range, Per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>88.6</td>
<td>81–95</td>
</tr>
<tr>
<td>3</td>
<td>88.4</td>
<td>75–95</td>
</tr>
<tr>
<td>4</td>
<td>89.6</td>
<td>86–94</td>
</tr>
<tr>
<td>5</td>
<td>70.3</td>
<td>55–89</td>
</tr>
<tr>
<td>6</td>
<td>83.0</td>
<td>77–92</td>
</tr>
<tr>
<td>7</td>
<td>77.0</td>
<td>62–86</td>
</tr>
<tr>
<td>8</td>
<td>62.4</td>
<td>48–78</td>
</tr>
</tbody>
</table>

possibility of a meaningful bimodal distribution of erythroblasts merits further investigation.) In any case, relatively little should be concluded about the migration properties of lymphocytes from these experiments. For fractionation-migration studies of lymphocytes, lymph or blood rather than marrow are the logical starting tissues.

The data of experiment RTM-19 (fig. 2) indicate the following enrichments and depletions for the different cell classes: RBC, range of 93.3 per cent to 3.5 per cent, cf. starting 42.1 per cent; erythroblasts + S.B., range of 23.9 per cent to 0.7 per cent, cf. starting 19.6 per cent; immatures (+ L), range of 45.7 per cent to 3.4 per cent, cf. starting 14.8 per cent; myelocytes, range of 41.8 per cent to 0 per cent, cf. starting 14.6 per cent; WBC, range of 16.1 per cent to 0 per cent, cf. starting 7.9 per cent.

For each class of cells relatively high maximum concentrations have been achieved, in some cases over 50 per cent as seen in table 1. These maxima may be compared with the composite average starting sample compositions also included in table 1.

The variability in starting sample composition is indicated by the standard deviations given in table 1. Starting sample for RTM-19 (fig. 2) agrees well with the composite averages (table 1).

The possibility of a systematic variation in viability is suggested by the data of table 3. This deserves investigation but will not be discussed in this paper.

Considering the representative cell concentration data in table 2, fractions Nos. 3 and 4 are seen to contain approximately equal numbers of cells, and more cells than any of the other fractions. The very large change in composition between fractions Nos. 3 and 4 (figs. 2, 3) is therefore particularly noteworthy. This change is most striking and reproducible when considering only the two simple classes: RBC/nucleated, for which reliable data are much more
Table 4.—Examples of Enrichment* by STAFLO-Sedimentation

<table>
<thead>
<tr>
<th>Ratio of Cell Types</th>
<th>Values of Ratio</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Starting Sample</td>
<td>Collected Fraction</td>
</tr>
<tr>
<td>Immature/mature WBC</td>
<td>1.73</td>
<td>∞</td>
</tr>
<tr>
<td>RBC/all nucleated</td>
<td>0.74</td>
<td>21.7</td>
</tr>
<tr>
<td>All nucleated/RBC</td>
<td>1.19 av.</td>
<td>∼ ∞</td>
</tr>
<tr>
<td>EBL+f (+S.B.f)/MYEL+f + WBC</td>
<td>0.97 av.</td>
<td>6.1</td>
</tr>
</tbody>
</table>

*Enrichments and depletion relative to the starting averages can be easily seen in figs. 2 and 3.

†EBL = Erythroblasts; S.B. = "small blue"; MYEL = Myelocytes.

Easily obtained.2 (The physical basis for this "anomalous" differential migration will not be rediscussed here.)

From the preparative standpoint sufficient numbers of cells of any of the fractions Nos. 2-8 (for most common purposes) are relatively easy to obtain without changing the present steady-state parameters. For 10⁷ cells of an immature-rich fraction No. 4, using the figures of table 2, 2 cc. would have to be collected. For the highest RBC-percentage fraction (No. 2), collection of about 67 cc. would provide 10⁷ cells.

To quantitate the relative enrichments achieved in this series of STAFLO-sedimentation experiments, one can calculate (from fig. 2 and all similar curves) ratios of different cell types in all collection fractions. For the ratio (immatures/WBC), a maximum value of approximately ∞ has been obtained, compared with a starting value of 1.73; for the ratio (RBC/nucleated cells), a maximum of 21.7 has been obtained, cf. a starting value of 0.74; for the ratio (nucleated/RBC) a maximum of approximately ∞ has been obtained, cf. the average starting value of 1.19; for the ratio (EBL + S.B./MYEL + WBC), i.e., identifiable nucleated red cells to identifiable nucleated whites, a maximum of 6.1 has been obtained compared with the average starting ratio of 0.97. These figures are tabulated in table 4, together with the experiment and fraction in which each was obtained.

As previously noted, less than 100 per cent of the nominally injected cells are recovered in the collection fractions. In these experiments, recoveries have generally ranged between 50 and 80 per cent. Part or most of the loss undoubtedly is due to the presence of some residual clumps. These will sediment much faster than individual cells, and a few will sometimes reach the bottom of the main migration chamber.

It is beyond the scope of this paper to pursue in detail the physical aspects of the complex sedimentation process giving rise to figures 2 and 3. For the representation, nonnucleated/nucleated, the sedimentation rate differences could be ascribed primarily to differences in cell size.2 For representations of figures 2 and 3, either size or density apparently can be the controlling factor. For example, the erythroblasts + S.B. in fraction No. 4 are smaller than the immatures in fraction No. 5, i.e., this segregation is by size. On the other hand, the mature WBC in fraction No. 6 are smaller than the myelocytes in
Table 5.—Nominal Sedimentation Rates for Different Collection Fractions

<table>
<thead>
<tr>
<th>Collection Fraction No.</th>
<th>Average Extreme Sedimentation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mm./hr.)</td>
</tr>
<tr>
<td>2</td>
<td>1.25</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
</tr>
<tr>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td>6</td>
<td>10.0</td>
</tr>
<tr>
<td>7</td>
<td>12.5</td>
</tr>
<tr>
<td>8</td>
<td>15.0</td>
</tr>
</tbody>
</table>

the higher fraction No. 5, i.e., density appears to be the controlling factor here. Other examples could also be given. In any case, the differential migration and separation is by sedimentation rate, as contrasted with the usual equilibrium (centrifugation) methods for separating cells. Both size and density factors must enter into the rate-type migration. As pointed out elsewhere, however, “size” and “density” are ambiguous concepts when applied to living cells.2,5

In STAFLO apparatus No. 7 used in these experiments each flowing layer is 1.25 mm. thick (high) so that each outlet is on the average 1.25 mm. below the outlet directly above. With the cells entering the main chamber in the position of stream No. 2, cells leaving via outlet No. 2 must have sedimented between 0 and 1.25 mm., or an average of 0.625 mm., during the 32-minute (~ ½ hour) residence time. Thus, the “average extreme sedimentation rate” for cells in collection fraction No. 2 must be approximately 0.625/½ = 1.25 mm./hour. Similar calculations can be made for the other collection fractions; the resulting values are tabulated in Table 5, both in mm./hour and in svedbergs (S). (One svedberg = 10^{-13} seconds.) The values in S-units are seen to fall in the range 3.5 \times 10^2 to 4.2 \times 10^6. Comparison of tables 1 and 5 gives the nominal S-rates corresponding to the different cell-type maxima.

In conclusion, it should be reemphasized that the migration-fractionation experiments reported here are in a relatively early stage. Nevertheless, one objective, to obtain a predictable preparative fractionation of rat bone marrow, appears reasonably well met. Toward the other (analytical) objective—to provide a physical classification for the different cell types—we can offer the above uncorrected, nominal S-rates. Though this characterization represents only a partial achievement of our objective, it may be useful.2,9 As far as we know, other sedimentation data of this kind have not yet been reported. A start on the microelectrophoretic characterization of similar cells has been reported recently by Ruhenstroth-Bauer and collaborators.17 It is hoped eventually to be able to correct data of this kind to some kind of “standard conditions.”

When it becomes clear what kind of improved separation may be required for a given biological purpose, it appears very likely that such an improvement can be achieved. To this end, a systematic study of different gradients, macromolecular content, pH, sample concentrations and flow rates, as well as...
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the combined use of electric and gravitational fields, should prove very fruitful.

SUMMARY

A single-cell suspension of normal rat bone marrow is prepared mechanically. This suspension is continuously fractionated in free solution, under sedimentation rate conditions, using 1 g. only. With a sample flow of $2.2 \times 10^6$ cells/minute and a 32-minute steady-state residence time in the stable-flow free boundary (STAFLO) flow-cell, the cells exit almost entirely into 7 of the 12 collection bottles. Maximum numbers of different cell types are observed, with good repeatability, in approximately descending order from top to bottom as follows: erythrocytes, "erythroblasts," "immatures," "myelocytes," and mature granulocytes. Major changes are effected relative to the starting marrow composition, and large relative enrichments are achieved for certain cell types. In addition to the rapid, mild, preparative aspect of this study, nominal sedimentation rates can be assigned for the different collection fractions, in the range of $3 \times 10^5$ to $4 \times 10^6$ svedbergs, thus making a start on this kind of simple physical classification of the cellular elements in this complex tissue.

SUMMARY IN INTERLINGUA

Es preparate mechanicamente un suspension monocellular de normal medula ossee del ratto. Iste suspension es fractionate continuemente in solution libre, sub conditiones de rapiditate del sedimentation, con le uso de solmente 1 g. Con un fluxo del specimen de $2.2 \times 10^6$ cellulas per minuta e un tempore de residentia in stato stabile de 32 minutas in le cellula de fluxo a confinio libre e fluxo stabile, le cellulas exi quasi totalmente ad in 7 del 12 bottlias de collection. Numeros maximal de differente typos cellular es observate, con bon repetibilitate, in ordine approximativamente descendente ab le capite al pede in le sequente serie: Erythrocytos, "erythroblastos," "immaturos," "myelocytes," e granulocytos matur. Major alterationes es effectuate relative al initial composition medullari, e grande inricchimentos es effectuate pro certe typos cellular. A parte le rapide, leve, preparative aspectos de iste studio, valores nominal de sedimentation pote esser ascribite pro le differente fractiones del collection, in le ordine de $3 \times 10^5$ ad $4 \times 10^6$ svedberg. Assi es compite un comenciamento pro iste typo de simple classification physic del elementos cellular in iste complexe tissu.

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