ANALYSIS OF RAT HEMOPOIETIC CELLS ON THE
FLUORESCENCE-ACTIVATED CELL SORTER

I. Isolation of Pluripotent Hemopoietic Stem Cells
and Granulocyte-Macrophage Progenitor Cells*

BY IRVING GOLDSCHNEIDER, DONALD METCALF, FRANK BATTYE, AND
TOM MANDEL

From the Department of Pathology, University of Connecticut Health Center, Farmington, Connecticut 06032; and the Cancer Research Unit, Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria 3050, Australia

Pluripotent hemopoietic stem cells (PHSC) are operationally defined by their ability to generate all of the cellular components of the lymphohemopoietic system while retaining the capacity for extensive self-replication. The existence of PHSC has been documented by the in vivo spleen colony-forming unit (CFU-S) assay (1) and by cell tracer studies (2, 3). The major obstacles to the isolation of PHSC have been their paucity in hemopoietic tissues (4), their close physical similarities to myeloid and erythroid progenitor cells (5, 6), and the absence of a stem cell-specific isotypic marker (7).

Myeloid progenitor cells are committed to differentiation along one or at most two pathways of leukocyte development. They can be identified and enumerated operationally by their ability to produce colonies of differentiated leukocytes in semisolid medium in the presence of specific colony-stimulating factors (CSF) (8). Consequently they are referred to as in vitro colony-forming cells (CFC). To date, progenitor cells have been identified for neutrophilic granulocytes (G) and macrophages (M) (GM-CFC), eosinophils (EO) (EO-CFC) and megakaryocytes (MEG) (MEG-CFC). Cluster-forming cells, which produce small aggregates of G and/or M in vitro, are thought to be progeny of GM-CFC.

Single-cell transfer experiments have demonstrated the clonal origin of G and M (9). Results of subcloning studies have suggested that progenitors of G are ancestral to progenitors of M (10). This view has been reinforced by experiments in which developmentally discrete subsets of GM-CFC have been partially resolved by velocity sedimentation (11).

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**Abbreviations used in this paper** CFC, in vitro colony-forming cell(s), CFU-S, in vivo spleen colony-forming unit, CSF, colony-stimulating factor(s), EBSS, Eagle's balanced salt solution; EO, eosinophil(s), FACS, fluorescence-activated cell sorter, FCS, fetal calf serum; FITC, fluorescein isothiocyanate, G, neutrophilic granulocyte(s); M, macrophage(s) or monocyte(s), MEG, megakaryocyte(s), PHSC, pluripotent hemopoietic stem cells, PMN, polymorphonuclear leukocytes; RBC, erythrocyte(s), SCM, spleen-conditioned medium, TdT*, terminal deoxynucleotidyl transferase positive

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In the present study we use the fluorescence-activated cell sorter (FACS) (12) to isolate PHSC from rat bone marrow and to identify three subsets of GM-CFC. We also describe two populations of presumptive modulator cells that influence the seeding efficiency and differentiation of PHSC in the CFU-S assay.

The separation protocols derive from earlier observations that Thy-1 antigen is present on many hemopoietic cells in the rat (13), which include PHSC (14, 15), presumptive thymocyte progenitors (16), early members of the B lymphocyte series (17–19), and, possibly, progenitors of erythrocytes and myeloid cells (20). These observations are confirmed and extended in the present study by detailed analysis on the FACS of the distribution of Thy-1 antigen among rat bone marrow cells. The results indicate that, in this species, Thy-1 antigen is present on all lineages of lymphohemopoietic cells, and that, within each lineage, the concentration of Thy-1 antigen is inversely related to the state of differentiation of the member cells.

Materials and Methods

Animals. 7- to 9-wk-old male and female DA strain rats were used as donors of bone marrow cells. Comparable results have been obtained with Lewis strain rats (data not shown). In some experiments, donor rats were given cortisone acetate 50 mg i.m./100 g of body wt 3 h before harvest of the bone marrow cells. 5- to 7-wk-old syngeneic rats given 750 rad of whole body x-irradiation 6 h before cell transfer were used as recipients in the CFU-S assays. Irradiated rats were maintained on oxytetracycline, 1 mg/ml drinking water.

Preparation of Bone Marrow Cell Suspensions. Bone marrow cells were collected from individual femoral shafts by flushing the marrow cavity with Elsen's balanced salt solution (EBSS). The dispersed cells were filtered through a single thickness of porous lens paper to remove debris, and washed in two changes of cold EBSS. Cells from two or three different animals were pooled for each experiment. Viability counts were performed with 0.1% trypan blue. In some experiments, erythrocytes (RBC) were lysed by suspending 2 × 10⁶ nucleated bone marrow cells in 10 ml of 0.168 M NH₄Cl for 7 min at room temperature. The cells were then centrifuged at 2,000 g for 7 min at 4°C and washed in two changes of cold EBSS. The RBC ghosts were removed by layering the cells on 2 ml of fetal calf serum (FCS) and centrifuging at 2,000 g for 7 min.

Cell Sorting. Bone marrow cells were separated according to relative low-angle (1.5–15°) light scatter and relative fluorescence intensity for Thy-1 antigen on a FACS (FACS II, Becton, Dickinson & Co., Rutherford, N.J.) as described previously (14).

Immunofluorescence. 2 × 10⁶ nucleated bone marrow cells were incubated for 20 min at 4°C with a 1:160 dilution (1.9 µg/ml) of fluorescein isothiocyanate (FITC)-F(ab')₂ rabbit anti-Thy-1 antibodies in 0.25 ml EBSS. The cells were washed three times and diluted to 5 × 10⁸ cells/ml in EBSS for sorting on the FACS. Control experiments with an equivalent concentration of FITC-F(ab')₂ rabbit anti-Thy-1 serum absorbed with rat thymocytes stained <2% of the nucleated bone marrow cells, none of which was PHSC or CFC. The FITC-F(ab')₂ rabbit IgG antibody to purified rat brain Thy-1 antigen was the generous gift of Dr. R. J. Morris (Imperial Cancer Research Fund Laboratories, London, England). The antiserum had an immunofluorescence titer of 1:640 against 8 × 10⁶ rat bone marrow cells/ml. Proof of specificity for Thy-1 antigen has been presented elsewhere (14, 17).

Cytology. Cell smears were prepared with the aid of a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa) and stained with May-Grünewald-Giemsa stain. Differential cell counts were routinely performed on 1,000 cells/smear. Cell diameters were determined with a calibrated ocular micrometer. Results represent the means of three to six separate experiments.

Electron Microscopy. Cells were collected from the FACS directly into fixative (2% glutaraldehyde and 2% paraformaldehyde in 0.08 M cacodylate buffer). The sample was centrifuged to form a pellet, the fixative was removed, and the fixed cells were resuspended in horse serum diluted 1:1 with water. This mixture was then placed into a cellulose nitrate centrifuge tube (9% × 1 % in.) and gently centrifuged to sediment the cells. Excess horse serum was removed.
and 2.5% glutaraldehyde was carefully layered on top to cross-link the horse serum surrounding the cells. The bottom of the tube was then cut off, and, after postfixation in 2% OsO₄ followed by 2 h in 2% aqueous uranyl acetate, the sample was dehydrated in a graded series of acetone. The acetone dissolved the cap of cellulose nitrate around the cell pellet, and, after embedding in Spurr’s resin, the sample was cut, stained, and examined in a Philips EM 300 (Philips Electronic Instruments, Inc., Mahwah, N. J.). This collection procedure enabled 10^4-10^5 cells to be processed without loss for electron microscopy.

CFU-S Assay for PHSC (1). 1.0 X 10^4 to 1.0 X 10^6 nucleated bone marrow cells in 1.0 ml of EBSS plus 10% FCS were injected intravenously into irradiated (750 rad) syngeneic recipient rats. Spleens from recipients were harvested 12 d later, fixed in 5% acetic acid-10% formalin-85% ethanol solution, and examined for macroscopically visible colonies. Paraffin-embedded spleen sections were stained with hematoxylin and eosin and examined for colony morphology. The number of cells injected was chosen to yield between 10 and 60 discrete colonies/spleen. Preliminary experiments confirmed the linearity of the dose-response relationship noted in other studies (14, 20).

CFC Assay for GM-CFC. Rat bone marrow cells were cultured in semisolid agar (final concentration of 0.3%) with a modification of the technique published by Bradley and Siemienowicz (21). 8 X 10^4 to 1 X 10^5 nucleated cells were cultured for 7 d (37°C, 10% CO₂ in air) in 35-mm plastic Petri dishes (T. C. plates; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif) in a final vol of 1 ml of agar medium. The agar medium was an equal volume mixture of 0.6% bacto-agar and double-strength Dulbecco’s modified Eagle’s medium, prepared as described previously (8). Human or rat plasma, heated to 56°C for 30 min and centrifuged at 3,000 rpm for 10 min to remove precipitate, were added at a final concentration of 20% vol/vol. Colony formation was stimulated by the addition of 0.1 ml GM-CSF diluted in 5% FCS in normal saline. Rat spleen-conditioned medium (SCM) induced with phytohemagglutinin was used as the standard source of CSF for GM-CFC. The culture conditions for producing rat SCM were identical to those described for mouse SCM (22). Active batches of CSF were pooled, aliquoted, and stored at -20°C. Results were comparable to those obtained with highly purified mouse GM-CSF (kindly provided by Dr. A. W. Burgess, Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia).

Enumeration of colonies (≥40 cells) and clusters (<40 cells) in semisolid agar cultures and cytological examination of cells in individually harvested colonies was conducted according to standard procedures of this laboratory (8).

Complement-mediated Cytotoxicity. 2.5 X 10^6 nucleated rat bone marrow cells were incubated (30 min at 4°C) in 50 μl of serially twofold-diluted anti-Thy-1 serum. The cells were washed three times in EBSS and incubated (30 min at 37°C) in 50 μl of normal guinea pig serum (agarose absorbed, final dilution, 1:6) as a complement source. The cells were washed twice and assayed for CFC activity. Three anti-Thy-1 sera were tested: (a) rabbit IgG anti-rat brain Thy-1 antigen (14, 17), (b) mouse anti-Thy-1.1 (C3H anti-AKR thymocytes), kindly provided by Dr. I. McKenzie (Austin Hospital, Heidelberg, Victoria, Australia), and (c) mouse anti-Thy-1.2 (AKR anti-C3H thymocytes), kindly provided by Dr. K. Shortman (Walter and Eliza Hall Institute of Medical Research). Complement controls and thymocyte-absorbed antiserum controls were included in each experiment.

Results

Distribution Profiles of FACS-separated Bone Marrow Cells

Rat bone marrow cells form three distinct peaks according to relative low-angle light scatter, a function of cell size (Fig. 1). Peak I contains mature RBC; peak II contains mainly lymphocytes and late erythroblasts; and peak III contains a mixture of undifferentiated cells, early erythroblasts, and members of the myeloid cell series (Table I). Of the latter, myeloblasts and myelocytes are concentrated in the right side of peak III.
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Fig. 1. Relative size distribution of rat bone marrow cells as determined by low-angle light scatter. Vertical threshold markers delineate cell fractions that were separated for functional and morphological study. These fractions are designed by roman numerals to indicate the peaks from which the cells were obtained: L, left side of peak; R, right side of peak. The mean diameter (M) of cells (± SD) in each peak is indicated. The mean percentage of nucleated cells in each fraction is given in Table III.

Table I

Classification of Rat Bone Marrow Cells Separated According to Relative Low-Angle Light Scatter and Fluorescence for Thy-1 Antigen

| Classification of cells | Unfractionated bone marrow | Peak II* | Peak III* |
|-------------------------|-----------------------------|----------|-----------|
|                         | Total | 11 | 5 | 0 | 10 | 15 | 15 | 0 |
| Undifferentiated cells   | 9 (3) | 5 | 2 | 6 | 7 | 0 | 15 | 10 | 15 | 15 | 0 |
| Lymphocytes             | 22 (10) | 48 | 51 | 47 | 58 | 34 | 3 | 5 | 3 | 6 | 1 |
| Myeloblasts/promyelocytes | 6 (8) | 0 | 0 | 0 | 0 | 0 | 3 | 1 | 6 | 5 | 0 |
| Myelocytes              | 24 (40) | 3 | 2 | 2 | 1 | 14 | 14 | 3 | 4 | 4 | 2 | 6 | 5 |
| Myelocytes (all stages) | 3 (8) | 0 | 0 | 0 | 1 | 6 | 9 | 7 | 11 | 1 | 13 |
| Monocytes (all stages)  | 5 (5) | 1 | 0 | 0 | 0 | 0 | 7 | 7 | 4 | 3 | 5 |
| Erythroblasts (all stages) | 26 (22) | 43 | 46 | 46 | 32 | 44 | 14 | 16 | 15 | 45 | 5 |
| Mitotic figures         | 1 (1) | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 2 | 2 | 0 |

* See Fig. 1 for description of fractions. Means of three experiments. Peak I (not shown) contained mature RBC.
‡ Not processed on the FACs. Numbers in parentheses refer to percentage of cell types in bone marrow from cortisone acetate-treated rats (10 mg/m; 100 g of body wt; 30 h previously).

Fig. 2 shows the fluorescence-distribution profile of DA rat bone marrow cells incubated with excess FITC-F(ab')2 antibodies to Thy-1 antigen. A mean of 47.1% of nucleated bone marrow cells were Thy-1+. Results in Table I show that the great majority of undifferentiated cells, myeloblasts, early erythroblasts, and mitotic cells are Thy-1+; as are ~75% of the bone marrow lymphocytes. Most of the latter cells have previously been shown to be surface Ig+ (17, 18). The great majority of myelocytes, mature granulocytes, monocytes, and late erythroblasts are Thy-1-.

The relative size distribution of the Thy-1+ bone marrow cells is shown in Fig. 3 and Table II. Approximately 64% of peak II cells and 26% of peak III cells were Thy-1+, accounting for 76 and 24% of total Thy-1+ bone marrow cells, respectively.

Treatment of donor rats with cortisone acetate caused a selective depletion of lymphocytes and undifferentiated cells, and a 4.5-fold decrease in the percentage of Thy-1+ bone marrow cells (Tables I and II).
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Fig. 2. Relative fluorescence distribution of rat bone marrow cells incubated with FITC-F(ab')₂ anti-Thy-1 serum. The marker defines the threshold above and below which cells were sorted into Thy-1⁺ (fluorescing) and Thy-1⁻ (nonfluorescing) populations, respectively. In this figure, 46.6% of the nucleated cells was Thy-1⁺.

Fig. 3. Relative size distribution of Thy-1⁺ (fluorescing) rat bone marrow cells (broken line) superimposed on relative size distribution of total nucleated bone marrow cells (solid line) as determined by low angle-light scatter. RBC (formerly peak I) were lysed with 0.168 M NH₄Cl. The small peak immediately to the left of peak II represents dead cells and cell debris. In this figure, 69% of peak II cells and 30% of peak III cells were Thy-1⁺.

PHSC (CFU-S)

Separation on the FACS. Approximately 89% of PHSC were distributed between peaks II and III of the light-scatter profile as determined by the CFU-S assay (Table III). Of these, 80% were in the left side of peak III. Approximately 12% of the total CFU-S was present in the right side of peak III, as was 97% of the GM-CFC (see below).

Results in Table IV show that CFU-S are strongly Thy-1⁺. Virtually all CFU-S were recovered in the upper 25th percentile of cells that were separated according to relative fluorescence intensity for Thy-1 antigen (Fig. 4); and >98% was recovered in the upper 10th percentile of Thy-1⁺ cells.

Analysis of the relative size distribution of cells in the upper 25th percentile for Thy-1 fluorescence revealed two distinct but overlapping populations (Fig. 5A). The distribution of the larger cell type roughly corresponded to that of peak III cells; the distribution of the smaller cell type corresponded to that of cells in the valley between peaks II and III, which include cells that contain the enzyme terminal deoxynucleotidyl transferase (23). Pretreatment of donor rats with cortisone acetate caused a 96% reduction in the population of smaller cells (Fig. 5B), and resulted in a twofold increase in CFU-S activity (Tables III and IV).
### TABLE II

**Distribution of Thy-1+ Cells in Rat Bone Marrow According to Relative Low-Angle Light Scatter**

| Fraction* | Untreated bone marrow cells† | Cortisone-treated bone marrow cells† |
|-----------|------------------------------|-------------------------------------|
|           | Percentage of Thy-1+ cells/ | Percentage of Thy-1+ cells/          |
|           | fraction of total Thy-1+     | fraction of total Thy-1+             |
|           | %                           | %                                   |
| I         | <1                          | <1                                  |<1          |
| II        | 64 ± 4.2                    | 75.9                                | 7.3†       |
| III       | 26 ± 4.9                    | 24.1                                | 32.3†      |
| IIIa      | 25 ± 3.6                    | 11.6                                | ND         |
| IIIb      | 25 ± 3.7                    | 12.5                                | ND         |
| UnfractionatedII | 47 ± 6.6                   | —                                   | 10.4       |

* See Fig. 1 for description of fractions
† Means ± SD of four to six experiments (untreated bone marrow cells), mean of three experiments (cortisone-treated bone marrow cells). See Figs. 2 and 3 for distribution profiles of Thy-1+ cells. ND, not determined
§ Expressed as a percentage of Thy-1+ cells in unfractionated bone marrow
¶ Bone marrow cells processed on FACS but not separated into fractions
†† Fractions IIa and IIIb combined

### TABLE III

**Distribution of CFU-S in Rat Bone Marrow According to Relative Low-Angle Light Scatter**

| Fraction* | Untreated bone marrow cells† | Cortisone-treated bone marrow cells† |
|-----------|------------------------------|-------------------------------------|
|           | Percentage of total nucleated cells | Number of CFU-S/10^6 cells | Percentage of total nucleated cells | Number of CFU-S/10^6 cells |
| I         | <1                           | 0                                  |<1          | 0          |
| IIa       | 30 ± 6.5                     | 19 ± 3.0                            | 23         | 93†       |
| IIIb      | 24 ± 3.6                     | 83 ± 16.9                           | 25         | 87†       |
| IIIb      | 24 ± 3.9                     | 12 ± 3.4                            | 31         | 52        |
| UnfractionatedII | 27 ± 6.5                   | —                                   | —          | —         |

* See Fig. 1 for description of fractions
† Means ± SD of four to six experiments (untreated bone marrow cells), mean of three experiments (cortisone-treated bone marrow cells). Values for CFU-S are not adjusted for seeding efficiency
§ 95% of CFU-S originally present in unfractionated bone marrow were recovered in fractions IIa, IIIb, and IIIb
¶ Not processed on the FACS
†† Fractions IIIb and IIIb combined

Hence, the final protocol for isolating PHSC consisted of selecting the upper 10th percentile of Thy-1+ cells in fractions IIa and IIIb (Fig. 1) of bone marrow from cortisone-treated rats. This resulted in a mean 320-fold enrichment of CFU-S, which was 78% of the predicted enrichment (Table IV). In contrast, when bone marrow
Table IV

Distribution of CFU-S in Rat Bone Marrow According to Relative Fluorescence Intensity for Thy-1 Antigen

| Thy-1+ cells percentile* | Untreated bone marrow cells | Cortisone-treated bone marrow cells |
|--------------------------|-----------------------------|-------------------------------------|
|                          | Number of CFU-S/10^6 cells | Observed enrichment | Predicted enrichment | Number of CFU-S/10^6 cells | Observed enrichment | Predicted enrichment |
| 91-100                   | 3,693 ± 713                | 151                    | 45                   | 7,802 ± 450                | 320                    | 410                   |
| 76-100                   | 1,542 ± 260                | 63                     | 18                   | ND                        | ND                     | 164                   |
| 76-90                    | 25 ± 5.9                   | 1                      | —                    | 29 ± 11.7                 | 1                      | —                     |
| 1-76                     | 0.9 ± 0.5                  | <1                     | —                    | 0.0                       | <1                     | <1                    |
| 1-100                    | 119 ± 28                   | 4.9                    | 4.5                  | 1,117 ± 186               | 46                     | 41                    |
| 0%                       | 0.0                        | <1                     | —                    | 0.0                       | <1                     | —                     |
| Unfractionated           | 24.4 ± 3.9                 | —                      | —                    | 45.3 ± 11.4               | 19                     | 16                    |

* Thy-1+ cells in fraction IIb + IIIb (Figs 1 and 3, Table II) were separated into percentiles according to relative fluorescence intensity as illustrated in Fig 4.
† Means ± SD of four to six experiments. CFU-S values not adjusted for seeding efficiency.
§ Number of times greater than value for unfractionated bone marrow cells. ND, not determined.
¶ Thy-1- cells; ~52% of cells in fraction IIb + IIIb from untreated bone marrow and 92% of cells from cortisone-treated bone marrow.

Fig. 4. Relative fluorescence distribution of rat bone marrow cells incubated with FITC-F(ab')2 anti-Thy-1 serum. FACS analysis of cells in fractions IIb and IIIb (Fig. 1). The numbers in parentheses indicate the percentiles of Thy-1+ cells in the fractions between the vertical threshold markers according to relative fluorescence intensity. In this figure, 53% of the total nucleated cells was Thy-1+

cells from untreated rats were used, the observed enrichment of CFU-S (151-fold) was 3.3 times greater than the predicted enrichment.

Morphology of candidate PHSC. (a) Light microscopy. Approximately 76% of the cells in the stem cell-rich fraction of bone marrow from cortisone-treated rats was undifferentiated mononuclear cells. These cells had a broad rim of basophilic agranular cytoplasm surrounding a centrally placed round to oval leptochromatic nucleus that contained one or more nucleoli (Fig. 6). The mean cell diameter in cytocrifuge-processed smears was 11.2 ± 1.3 μm. Although there was some heterogeneity with respect to cell and nuclear shape and degree of cytoplasmic basophilia, no discrete subsets were distinguished at the light microscopic level. The population of undifferentiated mononuclear cells was restricted to the upper 10th percentile of Thy-1+ cells.

Most of the remaining cells in the stem cell-rich fraction were polychromatophilic...
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Fig. 5 Relative size distribution of strongly Thy-1* (upper 25th percentile) rat bone marrow cells (broken line) superimposed on relative size distribution of total nucleated bone marrow cells (solid line) as determined by low-angle light scatter. (A) Bone marrow from untreated rats (B) Bone marrow from rats injected with cortisone acetate, 50 mg/100 g body wt, 36 h before harvesting.

erthroblasts (mean diameter 9.5 ± 1.2 μm). Many showed evidence of early hemo-
globinization in the perinuclear region as determined by a positive reaction with dimethylbenzydine (24).

(b) Electron Microscopy. Two subsets of undifferentiated mononuclear cells were distinguished ultrastructurally in the stem cell-rich fraction. These were present in roughly equal proportions. Type 1 cells were generally round cells with smooth margins and contained a round leptochromatic nucleus that frequently had a prominent central nucleolus (Fig. 7a). The cytoplasm of these cells contained many monoribosomes. Type 2 cells were similar in size to type 1 cells, but differed in that their nuclei were more irregular in outline and had more condensed chromatin and smaller, less obvious nucleoli (Fig. 7b). The cell outline of type 2 cells was also more irregular than that of type 1 cells; and the cytoplasm was more electron dense and contained a greater concentration of organelles.

Fig. 6 PHSC candidates May-Grunwald-Giemsa stain (a and b) Undifferentiated mononuclear cells from 310-fold and 328-fold enriched suspensions of CFU-S. Selected to illustrate typical features of candidate PHSC. Note the moderate amount of basophilic cytoplasm, scattered intracytoplasmic vacuoles, and lucent perinuclear zone, and the large round to oval leptochromatic nucleus with one or more nucleoli (5b, insert). × 775.

Fig. 7 Electron micrographs of undifferentiated cell types from a fraction of rat bone marrow cells enriched 310-fold for PHSC. (a) Type 1 cells have a generally electron-lucent cytoplasm that contains many monoribosomes, a few mitochondria, and occasional vacuoles. The cell outline is relatively smooth and the nucleus is leptochromatic and contains a well-developed nucleolus. (b) Type 2 cells have a somewhat more electron-dense cytoplasm and an irregular nucleus with a more condensed chromatin pattern. × 5,800.

Fig. 8. Cell types present in fractions of rat bone marrow cells enriched for GM-CFC (Thy-1*) and GM-cluster-forming cells (Thy-1') May-Grunwald-Giemsa stain. See Table I for differential cell counts (a) Thy-1* cells, fraction IIIr. Undifferentiated mononuclear cells, myeloblasts, basophilic erythroblasts, large lymphocytes, and a mitotic cell are represented (b) Thy-1' cells, fraction IIIr. Myelocytes, PMN, monocytes, and a plasma cell are represented × 775.
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**Table V**
Classification of Hemopoietic Spleen Colonies Formed by Rat Bone Marrow CFU-S Separated on the FACS*

| Colony type          | Unfractionated bone marrow cells§ | Percentage of colonies formed by Thy-1* cells§ |
|----------------------|----------------------------------|---------------------------------------------|
|                      | 1-100th percentile     | 76-100th percentile | 91-100th percentile |
| Untreated bone marrow|                                 |                                    |                     |
| Erythrocytic         | 62                  | 60                  | 69                  | 77                  |
| Granulocytic         | 15                  | 14                  | 14                  | 7                   |
| Megakaryocytic       | 8                   | 9                   | 9                   | 4                   |
| Mixed                | 7                   | 10                  | 8                   | 3                   |
| Undifferentiated     | 8                   | 7                   | 1                   | 9                   |
| Cortisone-treated marrow |                 |                                    |                     |
| Erythrocytic         | 58                  | 60                  | ND                  | 92                  |
| Granulocytic         | 18                  | 20                  | ND                  | 6                   |
| Megakaryocytic       | 5                   | 0                   | ND                  | 0                   |
| Mixed                | 10                  | 10                  | ND                  | 3                   |
| Undifferentiated     | 8                   | 10                  | ND                  | 0                   |

* Means of three experiments ND, not determined
§ Not processed on the FACS
§§ Separated from fraction IIr + IIIl according to relative fluorescence intensity for Thy-1 antigen (Table IV)

1+ cells on the FACS did not alter these ratios, except for the apparent absence of megakaryocytic colonies in recipients of cortisone-treated bone marrow. However, separation of the upper 25th percentile of Thy-1+ cells, and especially of the upper 10th percentile, increased the erythroid:granulocytic ratio to 10-15:1. Moreover, there appeared subjectively to be a marked increase in the average size (cellularity) of all colony types and in the proportion of mature cell types present within each colony.

**GM-CFC**

**Effect of Anti-Thy-1 Serum and Complement.** Treatment of adult rat bone marrow cells with anti-Thy-1 serum and complement reduced the number of GM-CFC between 60 and 75% as assayed by the CFC assay (Table VI). This was true whether rabbit heteroantiserum to purified rat brain Thy-1.1 antigen or mouse alloantiserum to Thy-1.1 antigen was used. Neither complement alone nor antiserum alone had any effect; neither did anti-Thy-1.2 alloantiserum plus complement Absorption of the rabbit or mouse anti-Thy-1.1 serum with rat thymocytes or Thy-1.1+ (but not Thy-1.2+) mouse thymocytes removed the cytotoxic antibodies to GM-CFC, further attesting to the specificity of the reaction.

GM-cluster-forming cells were not affected by treatment with rabbit anti-Thy-1 serum plus complement, even when exposed to an antibody concentration 20-fold greater than that used against GM-CFC.

**Separation of GM-CFC Subsets on the FACS.** GM-CFC and cluster-forming cells are among the largest cells in rat bone marrow as judged by relative light scatter. Results in Table VII show that >99% of GM-CFC and >98% of GM-cluster-forming cells are present in the right side of peak III. In addition, all EO-CFC and MEG-CFC
TABLE VI

| Antiserum (Dilution) | Complement | Number of colonies/10^3 cells* | Number of clusters/10^3 cells* |
|----------------------|------------|-------------------------------|-------------------------------|
| -                    | -          | 101 ± 11.3                    | 668 ± 144                     |
| -                    | +          | 108 ± 13.4                    | 619 ± 117                     |
| Rabbit anti-Thy-1.1 (1:10)‡ | -         | 99 ± 9.6                      | 655 ± 76                      |
| Rabbit anti-Thy-1.1 (1:10)‡ | +         | 26 ± 9.0                      | 661 ± 120                     |
| Mouse anti-Thy-1.1 (1:5)§  | -         | 98 ± 8.7                      | ND                            |
| Mouse anti-Thy-1.1 (1:5)§  | +         | 42 ± 6.2                      | ND                            |
| Mouse anti-Thy-1.2 (1:5)¶  | -         | 95 ± 11.4                     | ND                            |
| Mouse anti-Thy-1.2 (1:5)¶  | +         | 102 ± 10.8                    | ND                            |

* Determined on day 7 of in vitro culture. Means ± SD of four to six experiments.
‡ Rabbit IgG against rat brain Thy-1 1 antigen Cytotoxicity titer against rat GM-CFC, 1:10
§ Cytotoxicity titer against rat GM-CFC, 1:10
¶ Cytotoxicity titer against Thy-1 2 mouse thymocytes, 1:20.

TABLE VII

| Fraction* | Number of colonies/10^3 cells§ | Enrichment∥ | Number of clusters/10^3 cells∥ | Enrichment∥ |
|-----------|-------------------------------|-------------|-------------------------------|-------------|
| I         | 00                            | <1          | 00                            | <1          |
| II L      | 0.0                           | <1          | 0.0                           | <1          |
| II R      | 0.2 ± 0.4                     | <1          | 0.2 ± 0.5                     | <1          |
| III L     | 39 ± 5.4                      | <1          | 15.0 ± 23.3                   | <1          |
| III R     | 587.5 ± 150.0                 | 4.7         | 813.2 ± 166.5                 | 1.3         |
| Unfractionated¶ | 125.4 ± 23.3 | —           | 612.2 ± 124.8                | —           |

* See Fig 1
‡ Determined on day 7 of in vitro culture. Means ± SD of four to eight experiments
§ Number of times greater than value in unfractionated bone marrow cells. All GM-CFC and 32% of GM-cluster-forming cells were recovered after separation on the FACs.
¶ Not processed on the FACs.

that were identified were restricted to this fraction, although a systematic study of these cell types using optimized culture conditions was not undertaken.

Results in Table VIII show that >95% of GM-CFC are Thy-1+ and that >85% of GM-cluster-forming cells are Thy-1-. Both figures are probably conservative inasmuch as there was a moderate degree of overlap between Thy-1+ and Thy-1- cells in the fluorescence profile.

Isolation of the Thy-1+ cells in fraction III R resulted in a 12.7-fold enrichment of GM-CFC, close to the predicted 16-fold enrichment. No significant further enrichment was obtained by separating the Thy-1+ cells according to relative fluorescence intensity. However, there was a marked shift in the proportions of colony types that formed in vitro.

Results in Table IX show that 67% of GM-CFC in the upper 25th percentile for Thy-1 fluorescence formed G colonies, whereas 71% of GM-CFC in the lower 75th percentile formed M colonies. Progenitor cells that produce mixed colonies, which
TABLE VIII

Distribution of GM-CFC and Cluster-forming Cells in Rat Bone Marrow According to Relative Fluorescence Intensity for Thy-1 Antigen

| Thy-1⁺ cells in fraction IIIₐ percentile* | Number of colonies/10⁵ cells‡ | Enrichment§ | Number of clusters/10⁵ cells‡ | Enrichment§ |
|-----------------------------------------|-------------------------------|-------------|-------------------------------|-------------|
| 91-100                                  | 1,224 ± 100                   | 9.8         | 0.0                           | <1          |
| 76-90                                   | 1,632 ± 183                   | 13.0        | 0.0                           | <1          |
| 51-76                                   | 1,509 ± 248                   | 12.0        | 0.0                           | <1          |
| 1-50                                    | 1,104 ± 194                   | 8.8         | 361 ± 60                      | <1          |
| 76-100                                  | 1,788 ± 349                   | 14.3        | 0.0                           | <1          |
| 1-75                                    | 1,240 ± 140                   | 9.9         | 179 ± 25                      | <1          |
| 1-100                                   | 1,588 ± 366                   | 12.7        | 221 ± 21                      | <1          |
| 0 I                                     | 50 ± 5.6                      | <1          | 1,289 ± 327                   | 2.1         |

* Thy-1⁺ cells in fraction IIIₐ (Table VII) were separated into percentiles according to relative fluorescence intensity. Approximately 25% of cells in fraction IIIₐ were Thy-1⁺.
‡ Day 7 in vitro culture. Means ± SD of four experiments.
§ Number of times greater than value in unfractionated bone marrow cells.
¶ Thy-1⁻ cells.

TABLE IX

Distribution of GM-CFC Subsets According to Relative Fluorescence Intensity for Thy-1 Antigen

| Thy-1⁺ cells in fraction IIIₐ percentile* | Percent in vitro colonies‡ |
|-----------------------------------------|-----------------------------|
|                                         | G   | M   | Mixed | Others |
| %                                       |     |     |       |        |
| 91-100                                  | 73.6| 10.4| 8.5   | 7.5    |
| 76-90                                   | 67.8| 12.4| 10.7  | 9.1    |
| 51-75                                   | 5.1 | 50.2| 28.1  | 16.6   |
| 1-50                                    | 2.9 | 77.9| 12.4  | 6.8    |
| 76-100                                  | 66.7 (90)† | 13.1 (8) | 12.0 (23) | 8.2 (38) |
| 1-75                                    | 3.7 (10)‡ | 71.2 (92) | 18.8 (77) | 6.3 (62) |
| 1-100                                   | 23.2| 48.2| 18.9  | 9.7    |
| Unfractionated§                         | 20.5| 62.0| 11.4  | 6.1    |

* Table VIII, footnote.
† From day 7 cultures. Mixed colonies contain both G and M, the minority population representing >10% of total cells in the colony.
‡ Not processed on the FACS.
¶ Numbers in parentheses indicate the percentage of total colonies of each type that is formed by CFC in the indicated fraction.

contain both G and M, were found in all fractions, but the highest percentage (28%) was present in the 51-75th percentile of Thy-1⁺ cells.

When the distribution of these various subsets of GM-CFC is expressed as a percentage of total progenitor cells of each type, the following results are obtained: 90% of the G-CFC were present in the upper 25th percentile of Thy-1⁺ cells; 92% of the M-CFC were present in the lower 75th percentile; and 45% of the mixed GM-CFC were in the 51-75th percentile.

Mixing experiments in which cells from the upper 25th and lower 75th percentile...
of Thy-1+ cells were combined at ratios of 1:3, 1:1, and 3:1 showed strictly additive results in terms of the per centages of G, M, and mixed GM colonies that developed.

**Morphology of Thy-1+ and Thy-1- Cells in Fraction IIIr.** More than 90% of cells in the upper 25th percentile of Thy-1+ cells in fraction IIIr were undifferentiated mononuclear cells and basophilic erythroblasts; most of the cells in the lower 75th percentile were immature myeloid cells, predominantly myeloblasts and promyelo-cytes, and basophilic erythroblasts. The undifferentiated mononuclear cells in fraction IIIr were larger (mean diameter 12.6 ± 2.0 µm) than the candidate PHSC in fraction IIIl and had eccentrically placed, indented nuclei, and more abundant basophilic cytoplasm (Fig. 8a).

The great majority of Thy-1- cells in fraction IIIr were myelocytes, metamyelocytes, and mature G and M (Fig. 8b).

**Discussion**

The results of this study permit the following conclusions regarding normal hemopoiesis in the rat: (a) PHSC, GM-CFC, and GM-cluster-forming cells exist as discrete cell populations in bone marrow; (b) the heterogeneity of in vitro GM colonies reflects a corresponding heterogeneity of GM-CFC; and (c) the concentration of Thy-1 antigen on hemopoietic precursor cells is inversely related to their relative state of maturity.

These conclusions seem justified in view of the virtually complete separation of these cell populations on the FACS according to relative light-scatter and relative fluorescence intensity for Thy-1 antigen. Fractions that contain 89% of total CFU-S can be separated from fractions that contain 97% of total GM-CFC solely on the basis of relative light scatter. Given that CFU-S and GM-CFC as routinely assayed exist in approximately equal proportions in bone marrow (~0.2% of nucleated cells), it is apparent that this degree of functional dissociation must be accounted for by a comparable degree of cellular dissociation. GM-cluster-forming cells, being Thy-1-, can be separated from CFU-S and GM-CFC, all of which are Thy-1+. These relationships are summarized in Fig. 9.

Other attempts to separate these heretofore operationally defined cell populations have been only partially successful. In mouse bone marrow, CFU-S were found to be slightly smaller, more dense, and less adherent on average than GM-CFC (5, 25); and GM-cluster-forming cells were slightly larger, less dense, and more adherent than GM-CFC (11). This stickiness of GM-cluster-forming cells may explain their selective loss on the FACS in the present experiments (Table VII). Pretreatment of donor mice with Freund's adjuvant, irradiation, or vinblastine permitted a greater degree of separation of CFU-S and GM-CFC, but even the best experiments showed significant overlapping (9, 26). Marked enrichment of GM-CFC has been achieved with monkey and human bone marrow (27, 28), but assays for CFU-S do not exist in these species. Similarly marked enrichment of a subset of CFU-S from the endosteal surface of rat bone marrow has been achieved (29), but GM-CFC activity was not determined.

In the present study, the observed enrichment of CFU-S approximated the predicted enrichment when bone marrow cells from cortisone-treated rats were separated on the FACS according to relative intensity of Thy-1 fluorescence. This did not occur when untreated bone marrow cells were used. Rather, the observed enrichment exceeded the predicted enrichment by 3.3-fold; and the percentage of morphologically identi-
Fig. 9 Gated analysis of rat bone marrow cell subsets according to relative low-angle light scatter (size) and relative fluorescence intensity for Thy-1 antigen on the FACS. Composite diagram based on data from elsewhere (23) (TdT+ cells) and this paper. "Fractions" (abscissa) refers to gated fractions separated according to relative light scatter (Fig. 1). "Percentile" (ordinate) refers only to gated fractions of Thy-1+ (fluorescing) cells (Fig. 4). All Thy-1- cells are given a value of 0, even though they may exhibit small amounts of nonspecific (background) fluorescence by FACS analysis. See text for description of the various designated cell subsets. Lymphs, lymphocytes.

Fiable PHSC candidates (14%) correlated with the predicted enrichment of CFU-S (45-fold) rather than with the observed enrichment (151-fold). The cause of this discrepancy is not known, but several intriguing possibilities exist. Table IV shows that the deviation from the expected enrichment occurred when the cells in the 76–100th percentile for Thy-1 fluorescence were separated from the cells in the 1–75th percentile. This suggests that a population of cells in the lower 75th percentile of Thy-1+ cells normally acts to decrease the seeding efficiency (or proliferative capacity) of CFU-S to spleen.

Assuming that this hypothesis is correct, why did the same phenomenon not occur with cortisone-treated bone marrow cells? One highly speculative explanation is that there are two sets of accessory cells that normally regulate the seeding efficiency of CFU-S. The first of these is the weakly Thy-1+ suppressor cell population mentioned above; the second is a population of strongly Thy-1+, cortisone-sensitive, amplifier cells.

Another phenomenon that requires explanation is the increased erythroid:myeloid spleen colony ratio caused by CFU-S from rat bone marrow depleted of weakly Thy-1+ cells. This phenomenon occurred with bone marrow from cortisone-treated as well as untreated rats, which suggests that a population of cortisone-resistant modulator cells was involved. It is possible that these modulator cells, being weakly Thy-1+, belong to the aforementioned population of suppressor cells.

The above predictions need to be tested experimentally. However, there is evidence for the presence of a Thy-1+ amplifier cell in mouse bone marrow (30, 31). This cell type is required to maximize the development of hemopoietic colonies in the spleens of recipients. It can be replaced by viable thymocytes or by thymosin (32). Moreover, preliminary evidence suggests that this amplifier cell may, itself, be regulated by a
Thy-1+ suppressor cell in normal mouse bone marrow. As in the present study, removal of the putative suppressor cell increased the number of spleen colonies ~2.5-fold; removal of both the suppressor and amplifier cells did not significantly alter the number of spleen colonies that developed. Also, as in the present study, perturbations of presumptive modulator cells altered the ratio of erythroid:myeloid spleen colonies.

Numerous morphological candidates have been proposed for the PHSC (33–35). Most have fallen within the "lymphocyte-transitional" cell category defined by Yoffey (36). Unfortunately, the degree of stem cell enrichment in these earlier studies was insufficient to permit a direct correlation between PHSC activity and a particular morphological cell type. Moreover, techniques that were commonly used to enrich for PHSC also enrich for myeloid, erythroid, and lymphoid progenitor cells (5, 6, 37–40). This may account for the fact that the number of lymphocyte-transitional cells in bone marrow greatly exceeds that of PHSC.

Recently, van Bekkum et al. (6) have proposed that a population of undifferentiated mononuclear cells present in density gradient-enriched fractions of mouse and rat bone marrow is the PHSC candidate. This cell type is similar ultrastructurally to the type 1 PHSC candidate that we have isolated on the FACS. The ratio of candidate PHSC (identified morphologically):CFU-S (identified functionally) was consistently 1:2 in van Bekkum's studies (6), which suggests that many CFU-S do not resemble type 1 cells. Several authors have demonstrated that cycling and noncycling subsets of mouse CFU-S can be distinguished by differences in sedimentation rates and buoyant densities (Visser et al. [41]). Presumably, cycling and noncycling CFU-S would differ morphologically. Ross et al. (42) have described two candidate PHSC from the blood of dogs. These two PHSC candidates strongly resemble the type 1 and type 2 PHSC candidates from rat bone marrow. It is of interest, therefore, that the combined percentage of type 1 and type 2 cells (mean 76%) in the stem cell-rich fraction from rat bone marrow closely approximates the predicted percentage of CFU-S (mean 78%) in this fraction. The latter figure is based on an assumed seeding efficiency of 0.01 for rat bone marrow CFU-S (4), and is derived according to the formula: number of spleen colonies/10^n bone marrow cells × enrichment of CFU-S × 100 + seeding efficiency × 10^6.

The identity of GM-CFC in rodents is uncertain. In the mouse, cells that generate M colonies in vitro tend to be larger than cells that form mixed colonies, and these, in turn, tend to be larger than cells that form granulocytic colonies (11). Moreover, in single cell transfer studies in vitro (3, 10), cells from early granulocytic colonies can form colonies of M; and cells from more mature colonies can form GM-clusters. We have separated these progenitor cell subsets from rat bone marrow. As anticipated, G-CFC were associated with undifferentiated mononuclear cells, M-CFC with myeloblasts, and GM-cluster-forming cells with myelocytes.

Our findings, therefore, are consistent with the view that progenitors of G are the least-mature members of the GM-cell lineage, and that these bear a precursor-product relationship to progenitors of M and to cluster-forming cells. As in other studies (11), the results of mixing experiments were strictly additive, which indicates that the patterns of colony formation observed after cell separation is a result of the existence of subpopulations of GM-CFC rather than to the activities of modulator cells or to a stochastic process. The asynchrony of colony development, as illustrated by the presence of three colony types on day 7 of culture, is further evidence of the
heterogeneity of GM-CFC; less-mature GM-CFC taking longer than more-mature GM-CFC to generate macrophages. Data presented elsewhere (43) suggest that the rate of this transition is, in part, determined by the concentration of CSF in the cultures; high concentrations favoring the prolonged production of G, low concentrations favoring the early production of M.

It is not possible at this time to explain the physiological significance of the inverse relationship that we have observed between relative fluorescence intensity for Thy-1 antigen and relative maturity of members of the GM-cell series in rat bone marrow. There is no known function for Thy-1 antigen, and its distribution among lymphohemopoietic cell populations varies widely between species (13-19, 44, 45). However, it is clear that both the absolute amount of Thy-1 antigen (as determined by relative fluorescence intensity in the presence of excess FITC-anti-Thy-1 antibodies) and the concentration of Thy-1 antigen are greater on immature than on mature hemopoietic cells. Thus, in the rat as in the mouse, the progressive maturation of myeloid cells from the level of the PHSC is characterized by a progressive increase in cell size; yet the relative amount of Thy-1 antigen decreases as maturation progresses. In morphological terms, the relative concentration of Thy-1 antigen can be depicted as follows: undifferentiated cells (11.2-μm mean diameter) > undifferentiated cells (12.6-μm mean diameter) > myeloblasts > myelocytes > polymorphonuclear leukocytes/monocytes. In developmental terms, the relative concentration of Thy-1 is: CFU-S > G-CFC > mixed GM-CFC > M-CFC > GM-cluster-forming cells > mature polymorphonuclear leukocytes (PMN)/monocytes. These relationships are schematically illustrated in Fig. 9.

Results of ongoing studies indicate that an inverse relationship also exists between Thy-1 concentration and maturation of other myeloid cells, RBC, and lymphocytes in the rat. In the EO series, the relative concentration of Thy-1 antigen is: CFU-S > EO-CFC > myeloblasts/myelocytes > EO. Similarly, early erythroblasts are Thy-1⁺, and late erythroblasts and mature RBC are Thy-1⁻. In the T lymphocyte series, putative thymocyte progenitors (terminal deoxynucleotidyl transferase-positive [TdT⁺] bone marrow cells) (23) and cortical thymocytes are strongly Thy-1⁺, whereas medullary thymocytes are weakly Thy-1⁺, and a large majority of peripheral T cells are Thy-1⁻ (46). Thy-1 antigen is also restricted to the least-mature members of the B lymphocyte series, being present on complement receptor-negative B cells in fetal liver, bone marrow, and peripheral lymphoid tissues, but not on memory B cells or antibody-forming cells (19). It is likely, therefore, that Thy-1 antigen will prove to be a useful marker with which to identify and isolate precursor cell subsets belonging to most of the lymphohemopoietic cell lineages in the rat. Moreover, our recent observations suggest that antisera to nonallelic determinants on the Thy-1 molecule may prove useful for the isolation of lymphohemopoietic precursor cells in the mouse (47).

Summary

A scheme is presented whereby pluripotent hemopoietic stem cells (PHSC) from rat bone marrow can be enriched 320-fold with the aid of the fluorescence-activated cell sorter. This scheme is based on the observations that PHSC are strongly positive for Thy-1 antigen (upper 10th percentile); have light-scattering properties (size distribution) between those of bone marrow lymphocytes and myeloid progenitor cells; and are relatively resistant to cortisone. It is estimated that PHSC may constitute
80% of the cells isolated according to these parameters. Candidate PHSC are described at the light and electron microscopic levels.

At least two populations of accessory cells appear to influence the number and/or the nature of the hemopoietic colonies that form in the in vivo spleen colony-forming unit assay. Putative amplifier cells are strongly Thy-1\(^+\) and cortisone sensitive; putative suppressor cells are weakly Thy-1\(^+\) and cortisone resistant.

Three subsets of granulocyte (G) -macrophage (M) progenitor cells (in vitro colony-forming cells [CFC]) are identified on the basis of relative fluorescence intensity for Thy-1 antigen: G-CFC are strongly Thy-1\(^+\); M-CFC are weakly Thy-1\(^+\); and cells that produce mixed G and M CFC have intermediate levels of Thy-1. GM-cluster-forming cells and mature G and M are Thy-1\(^-\). The results suggest that G-CFC are bipotential cells that give rise to G and M-CFC; and that the latter produce mature M through a cluster-forming cell intermediate.

Thy-1 antigen is also demonstrated on members of the eosinophil, megakaryocyte, erythrocyte, and lymphocyte cell series in rat bone marrow. In each instance, the relative concentration of Thy-1 antigen is inversely related to the state of cellular differentiation.

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