ISOLATION OF MURINE PLURIPOTENT HEMOPOIETIC STEM CELLS

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Murine hemopoietic tissue provides a model system to study differentiation from a pluripotential stem cell, capable of extensive self-replication, to fully mature, functionally differentiated progeny, including erythrocytes, granulocytes, macrophages, megakaryocytes, as well as B and T lymphocytes (1, 5, 15, 16, 22, 43, 44, 54). The existence of a pluripotent hemopoietic stem cell (PHSC)[1] was first predicted from studies assessing the radioprotective capacity of grafts containing hemopoietic cells (21, 31, 41, 51). The development of the CFU-S assay (colony-forming unit/spleen [44]) provided a means to estimate PHSC numbers in hemopoietic grafts, since at least some of the spleen colonies contain new CFU-S (40, 43) in addition to differentiated progeny of several lineages (16, 22, 54). The CFU-S assay has been invaluable for characterizing the PHSC in terms of their physical properties and cell surface markers (19, 20, 25, 35, 38, 45, 46, 49, 53).

Investigations on hemopoietic cell differentiation are hampered by the fact that PHSC constitute a minor proportion of cells in hemopoietic organs and, therefore, can only be detected in functional assays. Because the functional assays take at least a week, the early differentiation steps occurring in the first few days after activation cannot be studied unless purified PHSC are used. A number of attempts have been made to purify CFU-S on the basis of their physical and surface properties (3, 6, 8, 13, 24, 26, 37–40, 42, 48), but only in the rat system has the purity approached levels required for more direct studies (14, 23).

We have previously described a method to purify CFU-S from adult mouse bone marrow combining two separation procedures (48): first, mouse bone marrow cells are separated by equilibrium density centrifugation; second, the

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CFU-S, colony-forming unit/spleen; CSA, colony-stimulating activity; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FLS, forward light scatter; GM-CFU, granulocyte/monocyte colony-forming unit; HASH, H.HBSS containing sodium azide and serum; H.HBSS, Hepes-buffered Hanks' balanced salt solution; PES, postendotoxin serum; PHSC, pluripotent hemopoietic stem cell; PLS, perpendicular light scatter; PMUE, pregnant mouse uterus extract; WGA, wheat germ agglutinin.

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low density cells are labeled with wheat germ lectin conjugated with fluorescein isothiocyanate (WGA-FITC) and analyzed by a fluorescence-activated cell sorter (FACS) to separate all brightly fluorescent cells with the light scatter characteristics of CFU-S. This method gives 60- to 100-fold enrichment of CFU-S. The highly enriched stem cells have been shown to behave normally with respect to both their homing upon transplantation and their radioprotection capacity (50).

In the present report, we describe the results of experiments to further purify PHSC by combining the previous method with a third procedure that can discriminate between PHSC and other hemopoietic cell types, namely fluorescence-activated cell sorting for H-2K-positive cells (19, 45). We have examined the morphology of cells purified by this method as well as their functional properties in vivo and in vitro.

Materials and Methods

Animals. Male C57BL/Rij(H-2K^b) × C3H(H-2K^k) F_1 hybrid (BC_3) mice were bred and maintained under specific pathogen-free conditions in our institutes.

Preparation of Cell Suspensions. Bone marrow cells were obtained from 7-wk-old mice (BC_3) by flushing the femoral shafts with Hanks' balanced salt solution (Laboratoires Eurobio, Paris) buffered at pH 6.7 with Hepes (10 mM; Merck & Co., Rahway, NJ) and provided with penicillin (10^2 IU/ml) and streptomycin (0.1 mg/ml) (H.HBSS). The cell suspension was filtered through a nylon sieve and kept on ice until use.

Metrizamide Gradient and WGA-FITC Labeling. The first step of the separation procedure consisted of equilibrium density centrifugation on a discontinuous metrizamide gradient. Stock solutions with densities of 1.100 ± 0.005 and 1.055 ± 0.005 g/cm^3 at 4°C were prepared by dissolving metrizamide (Nyegaard, Oslo) in H.HBSS containing 1% wt/vol bovine serum albumin (BSA) (fraction V; Sigma Chemical Co., St. Louis, MO). The pH and osmolarity of the solutions were pH 6.5 ± 0.1 and 300 ± 3 mosmol/kg, respectively. A solution having a density of 1.078 ± 0.001 g/cm^3 was prepared from the two stock solutions. The solutions were filtered through 0.22-μm Millipore filters and stored at −20°C until use. Discontinuous gradients were formed by pipetting 1 ml of the 1.100 g/cm^3 solution onto the bottom of a 15-ml centrifuge tube (round-bottomed, 16 mm internal diam; Falcon Labware, Oxnard, CA), carefully layering 3 ml of the 1.078 g/cm^3 above this cushion, and, subsequently, layering 1 ml of the 1.055 g/cm^3 solution on top. The bone marrow cells were suspended in the latter solution at 5 × 10^7 cells/ml. All solutions and suspensions were kept at 4°C. WGA-FITC (1 mg/ml; Polysciences, Inc., Warrington, PA) was diluted 100-fold in H.HBSS and 200 μl of this dilution was added per milliliter of 1.055 g/cm^3 metrizamide solution containing the cells. The tube was centrifuged (4°C, 1,000 *g*, 10 min) and the cells at the interfaces between the 1.100 and 1.078 g/cm^3 solutions (high density fraction) and between the 1.078 and 1.055 g/cm^3 solutions (low density fraction) were collected. The fractions were diluted with 5 ml of H.HBSS and were centrifuged again (400 *g*, 10 min) to collect the cells, and the cells were resuspended in H.HBSS. The recovery was determined by counting the cells in each fraction using a hemocytometer.

FACS. The low density cells from the top fraction of the discontinuous density gradient were analyzed by a modified FACS II (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) with the laser at 488 nm (0.4 W). FITC fluorescence was measured through a combination of a broad band multicavity interference filter (520–550 nm transmission; Pomfret Research Optics Inc., Stamford, CT) and a 520-nm cut-off filter (Ditric Optics, Inc., Hudson, MA) by an S-20 type photomultiplier. Forward light scatter (FLS) intensity was measured with the laser beam blocking bar in its narrowest orientation. Perpendicular light scatter (PLS) intensity was measured by an S-11 type photomultiplier. The sheath fluid consisted of H.HBSS (without phenol red) of pH 7.0. PLS signals were linearly amplified; a logarithmic amplifier (T. Nozaki, Stanford, CA) was used for the fluorescence signals.

Cells were sorted into 15-ml glass tubes. The wall sides where the sorted cells were deposited were rinsed before and after the sorting with H.HBSS containing 10% fetal calf serum (FCS). The fluorescence measurements were calibrated at pH 7.0 using standard particles that contain known numbers of bound FITC molecules (47). Cells were sorted twice. In the first sorting the electronic windows were set to select all WGA-FITC-positive cells with medium FLS and low PLS intensities as described previously (48).

Removal of WGA-FITC and Labeling with H-2K. WGA-FITC was removed from the cells by incubation in an isotonic solution of 0.2 M *N*-acetyl-D-glucosamine (Polysciences, Inc.) in H.HBSS for 15 min at 37°C. Cells were then washed with H.HBSS supplemented with 0.01% vol/vol sodium azide and 5% vol/vol fetal calf serum ([HASH] Hanks', azide, serum, Hepes) and centrifuged again. The cells were labeled with antibody and fluorochrome using the biotin-avidin system, which was reported to be nontoxic for CFU-S (4). Anti-H-2K^k-biotin (Becton, Dickinson & Co., Sunnyvale, CA) was diluted in HASH and 50 μl was added to the pellet. The final concentration of antibody was 1 μg per 10^6 cells. The cells were incubated for 45 min at 4°C. Subsequently, the cells were washed once with HASH and the pellet was resuspended in 50 μl of an avidin-FITC (Becton, Dickinson & Co.) solution in HASH (1 μg/10^6 cells). After 30 min of incubation at 4°C, the cells were washed again, resuspended in 1 ml H.HBSS and sorted by the FACS. The 30% most brightly fluorescent cells were sorted into tubes and into cylindrical 30-μl wells of Terasaki culture trays (model 726180; Greiner, Nuertingen, Federal Republic of Germany [FRG]) that contained 10 μl of culture medium and hemopoietic regulatory factors.

In Vitro Cloning of Sorted Cells. The one cell per well collection system consisted of an automated microscope stage positioner (SK317; Märzhäuser, Wetzlar, FRG) which was adapted to fit under the FACS II fluid jet handling chamber. The main stream was deflected into a waste tube and the sorted cells were directed straight downward into the wells. The electronics of the automated microscope stage positioner (Märzhäuser) were modified so that the FACS II sorting electronics mastered the timing of the stage movement. The FACS II electronics were modified to allow automated precise sorting of low numbers of cells (1, 2, 4, 10, 40, 100, or 400). This set-up allows accurate limiting dilution analysis to determine the frequency of clonogenic cells in suspensions. Since the numbers of cells deposited per well are accurately known, the statistics are somewhat different from those of the traditional limiting dilution where only the average of the numbers of cells per well is known. The probability for a well without growth (a negative well) equals $(1 - p)^n$, where p is the fraction of clonogenic cells and n the total number of cells per well.

Electron Microscopy. A procedure was developed to prepare samples containing low numbers of cells for examination by electron microscopy. Cells ($1–5 \times 10^3$) were deposited by the FACS into the bottom of a 30-μl well of a 60-well Terasaki culture tray. Subsequently, the tray was centrifuged (5 min, 400 *g*), the supernatant was removed, fixation medium was added, and the tray was centrifuged again (5 min, 400 *g*) and stored for at least 16 h at 4°C. The fixation medium consisted of 2% vol/vol glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4, 300 mosmol/kg). The cells were further processed for electron microscopy as described previously for cell cultures (29).

CFU-S Assay. Recipient mice were irradiated at 20–25 animals per cage in a Gammacell 20 small animal irradiator (Atomic Energy of Canada, Ltd., Ottawa) with a lethal dose of gamma rays (^{137}Cs, 9.5 Gy, 0.86 Gy/min) between 1 and 8 h before transplantation. Less than 0.1 colony per spleen was observed in uninjected irradiated control mice, which all died between 12 and 17 d after irradiation. To determine the CFU-S content of cell suspensions, appropriate dilutions were prepared and 0.5 ml aliquots were injected. The mice were housed five or fewer per cage with food and water ad libitum. Groups of mice were sacrificed 8 and 12 d after transplantation. Their spleens were fixed in Telleyesniczky's solution and macroscopically visible surface colonies were counted and scored as CFU-S. 7–10 mice were used for each determination.

Radioprotection Assay. Unfractionated or sorted bone marrow cells were transplanted at several dilutions into groups of 10 mice each. A dilution of the same cell suspension was used for determining CFU-S content of the graft. The fraction of mice surviving 30

d after irradiation and transplantation was determined. Most of the nonsurviving animals died between 12 and 17 d after irradiation.

GM-CFU Assay. Unfractionated and sorted cells were assessed for the presence of myeloid progenitor cells by in vitro semisolid agar culture techniques as described earlier (9, 10) as well as by limiting dilution procedures in liquid culture using the cell depositing system of the FACS. In brief, the semisolid agar cultures consisted of 0.3% vol/vol agar (Bacto agar; Difco Laboratories, Detroit, MI) in Dulbecco's medium with 13% horse serum (noncommercial batch) and 7% FCS added. 1-ml aliquots of agar medium containing known numbers of cells were plated into 35-mm petri dishes (Corning Glass Works, Corning, NY) that contained 0.1 ml of one or more types of colony-stimulating activity (CSA) preparations. For granulocyte/monocyte (GM)-CFU-1 the CSA consisted of a mixture of pregnant mouse uterus extract (PMUE) and 18-h postendotoxin serum (PES); for GM-CFU-2 only PMUE was added to the plates. A concentration of PMUE that stimulated maximum numbers of colonies was used (17). The PES concentration was such that it induced no colony formation in the absence of PMUE and, therefore, acts as a colony-enhancing activity (18). The cultures were incubated (National Appliance Co., Portland, OR) at 37°C, 100% humidity, and 10% CO_2 in air. The number of colonies per culture dish was determined at day 7 using an inverted microscope. Only colonies containing >100 cells were scored. Triplicate dishes were used for each experimental point. Each experiment was repeated at least three times.

The serum-free liquid culture medium for Terasaki wells consisted of a 1:3 mixture of Ham's F12 (Gibco Laboratories, Grand Island, NY) and Dulbecco's modified Eagle's medium (H-16; Gibco Laboratories) supplemented with alpha-medium constituents as described by Wagemaker and Visser (52) and with nucleosides. The mixture was buffered with 10 mM Hepes and $NaHCO_3$ (2.2 g/l) at pH 6.8–6.9. Penicillin (100 IU/ml) and streptomycin (10 mg/l) were added. The medium was further supplemented with 1% wt/vol bovine serum albumin (BSA) (Sigma Chemical Co.), deionized as described by Worton et al. (53), human transferrin saturated with $FeCl_3$ at 0.3 g/l, 10^{-4} M alpha-thioglycerol, 1.25×10^{-5} M Na_2SeO_3, insulin (Organon, Inc., Oss, The Netherlands) at 10^{-4} g/l, 10^{-5} M lineolic acid, and 3×10^{-5} M cholesterol (Sigma Chemical Co.). PES and/or PMUE were added to this medium at concentrations similar to those for the semisolid agar culture system. Aliquots of 10 μl liquid culture medium with or without CSA were put into each well of a 60-well Terasaki culture tray (Greiner) and the trays were stored at 37°C, 100% humidity, and 10% CO_2 in air for about 1 h before cells were deposited into the wells by the FACS, as described above.

Results

Enrichment for CFU-S. Mouse bone marrow cells were separated by equilibrium density centrifugation on a discontinuous metrizamide gradient and simultaneously labeled with WGA-FITC. The low density cells were analyzed by the FACS and three subpopulations of cells could be distinguished (Fig. 1 *A* and *B*). Calibration of the FACS indicated that the average number of FITC molecules per cell in the upper cluster (Fig. 1 *B*) was equal to 3.4×10^5. Since the WGA-FITC contained about 3.0 FITC molecules per WGA dimer, these cells bound $\sim 10^5$ WGA dimers per cell. When $>3 \times 10^5$ WGA dimers were bound per cell, aggregation of cells and concomitant decreased recovery in CFU-S was observed. The two subpopulations with low fluorescence bound about 1.5×10^4 FITC molecules per cell, whereas unlabeled cells showed a fluorescence intensity equivalent to 5,000 FITC molecules, close to the detection threshold of the FACS (30, 47).

Fluorescent cells with medium FLS and low PLS scatter intensities were sorted (Fig. 1 *A* and *B*) using electronic sorting windows as described previously (48). This fraction comprised 4–6% of all nucleated low density cells. The enrichment

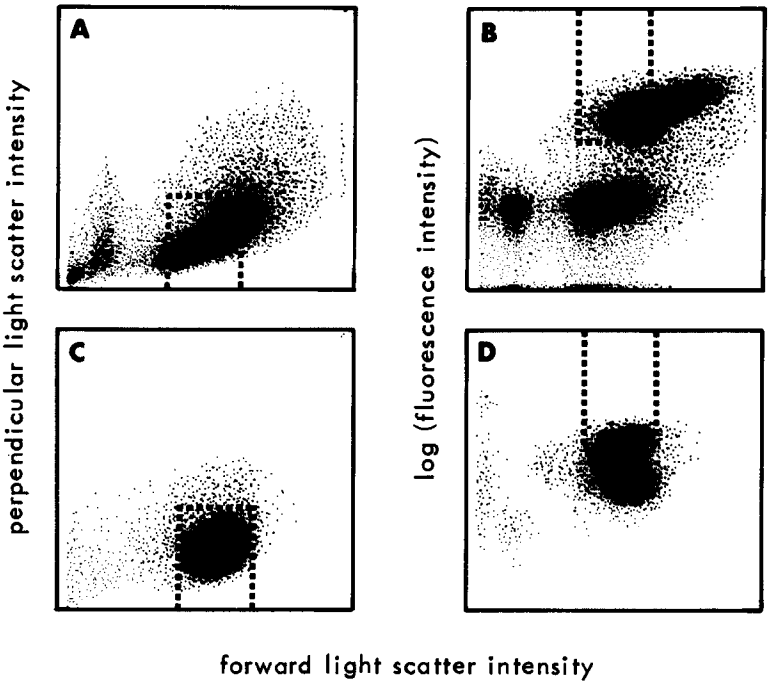

FIGURE 1. FACS sorting windows (dashed-line squares) for isolating CFU-S from mouse bone marrow. (*A*, *C*) PLS vs. FLS; (*B*, *D*) fluorescence vs. FLS. (*A*, *B*) WGA-FITC–labeled low density cells; (*C*, *D*) WGA-FITC-positive low density cells after removal of WGA-FITC and subsequent labeling with anti-H-2K-biotin and avidin-FITC. Details are described in Materials and Methods.

TABLE I

CFU-S Enrichment by Combining Density Separation and Fluorescence-activated Cell Sorting after WGA and H-2K Labeling

Cells	CFU-S per 10^5 cells		Percent recovery		Enrichment factor
	Day 8	Day 12	Nucleated cells	Day 12 CFU-S	Day 12 CFU-S
Unfractionated bone marrow cells	38 (19–77)	49 (21–96)	100	100	1
Low density cells ($\rho < 1.078$ g/cm^3)	190 (90–260)	240 (110–300)	10 (5–15)	60	4.9
Sorted WGA$^+$ low density cells	1,600 (710–2,300)	2,100 (800–3,000)	1.0 (0.7–1.2)	50	43
Sorted WGA$^+$ and H-2K$^+$ low density cells	2,000 (690–4,000)	6,600 (1,900–13,000)	0.3 (0.2–0.4)	40	135

Data represent the mean and range (within parentheses) of between 5 and 15 experiments.

(Table I) obtained by this method was similar to that reported previously (48, 50) using a discontinuous BSA gradient, followed by WGA-FITC labeling and sorting. The WGA-FITC did not noticeably influence the density separation at this concentration.

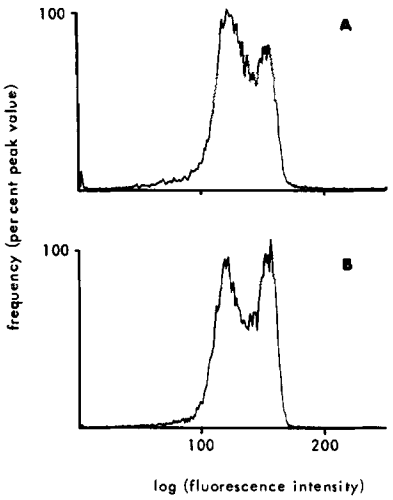

FIGURE 2. Frequency distribution of the fluorescence intensities of anti-H-2K-biotin- and avidin-FITC–labeled cells. (*A*) All cells; (*B*) cells with FLS and PLS within the sorting windows indicated in Fig. 1 *C*. Cells were previously enriched for CFU-S by density centrifugation and sorting on the basis of WGA-FITC fluorescence as described in Materials and Methods.

The sorted cells were incubated with the competitive sugar, *N*-acetyl-D-glucosamine, to remove WGA-FITC. This procedure did not affect the CFU-S assay nor did labeling the cells with anti-H-2K^k-biotin and avidin-FITC. Analysis by the FACS showed that two major subpopulations of cells could be distinguished on the basis of their fluorescence intensities (Fig. 1 *D*). These two subpopulations were better resolved when the fluorescence histogram of cells within the FLS and PLS windows (Fig. 1 *C*) was examined (Fig. 2). The average number of FITC molecules per cell for these subpopulations was 0.7×10^5 and 3.5×10^5, respectively. Since the average number of FITC molecules per bound anti-H-2K molecule was estimated to be 5 ± 1, the cells bound approximately 1.4×10^4 and 7×10^4 anti-H-2K molecules per cell, respectively. Titration experiments indicated that labeling of the cells with anti-H-2K was saturated.

The most brightly fluorescent cells ($30 \pm 5\%$ of the total remaining population) were sorted with windows shown in Fig. 1 *C* and *D* and assayed for CFU-S. Colonies were counted 8 and 12 d after transplantation (Table I). The number of day 12 CFU-S in the sorted fraction was about threefold higher than the number of day 8 CFU-S, suggesting that the sorting procedure selects for delayed-type CFU-S (32). The day 12 CFU-S were enriched three- to fourfold by this step and the overall recovery averaged 40%.

Radioprotection. The ability of the sorted cells to protect lethally irradiated mice was determined by the 30-d survival assay. Fig. 3 shows that 170 ± 30 sorted cells, as compared with $(3.1 \pm 0.7) \times 10^4$ unfractionated cells, were required to protect 50% of the animals against lethal gamma radiation, an enrichment of 180 ± 70–fold. The number of day 12 CFU-S required for 50% radioprotection was 14.9 ± 1.3 for grafts of unfractionated bone marrow cells and 10.6 ± 1.4 for sorted cells (Fig. 4). The average enrichment factor for day

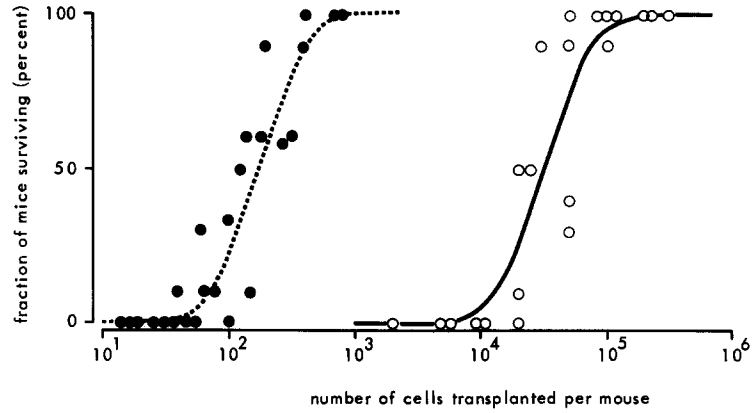

FIGURE 3. 30-d survival of lethally irradiated mice vs. the number of nucleated cells transplanted per mouse. (○, ——) graft of unfractionated cells; (●, – – –) sorted cells that were 130-fold enriched for CFU-S. Lines represent the result of probit analysis.

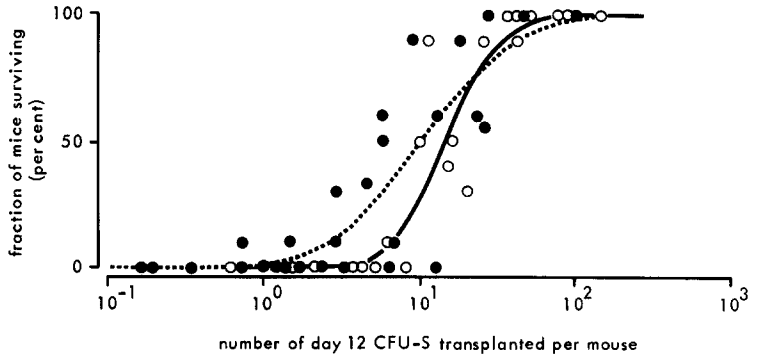

FIGURE 4. 30-d survival of lethally irradiated mice vs. the number of day 12 CFU-S transplanted per mouse. For details, see legend to Fig. 3.

12 CFU-S in these experiments was 130 ± 30, indicating that PHSC were at least coenriched with the CFU-S.

Morphology. The morphology of the sorted cells was examined by light microscopy after May-Grünwald-Giemsa staining and by electron microscopy. All cells could be classified as undifferentiated blasts by light microscopy (Fig. 5). Electron microscopy revealed two predominant cell types in the sorted fraction: between 43 and 59% of the cells resembled the "candidate stem cell" described by van Bekkum et al. (6) (Fig. 6*b* and *d*); 24–43% were similar to that stem cell except for somewhat more cytoplasm and deeper nuclear indentations (Fig. 6*a* and *c*); the remaining 15–17% of the cells were clearly more differentiated (not shown). The two major cell types, which together comprised 85% of the cells, could be partially separated from each other by the FACS on the basis of their PLS intensities. The "candidate stem cell" had relatively high PLS, whereas the cell with more cytoplasm had lower PLS intensity. Preliminary experiments indicated that both of these cell types, after sorting for PLS differences, produced similar numbers of spleen colonies in lethally irradiated recipients, suggesting

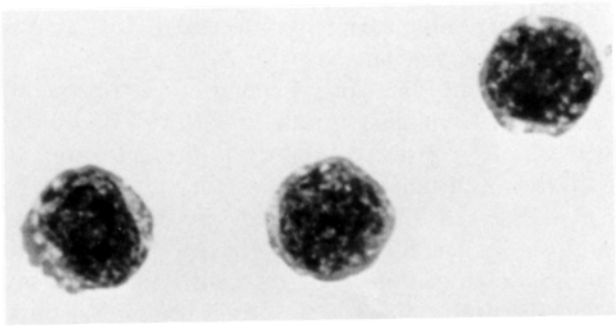

FIGURE 5. Pictures of typical examples of the predominant cell type in sorted suspensions after May-Grünwald-Giemsa staining.

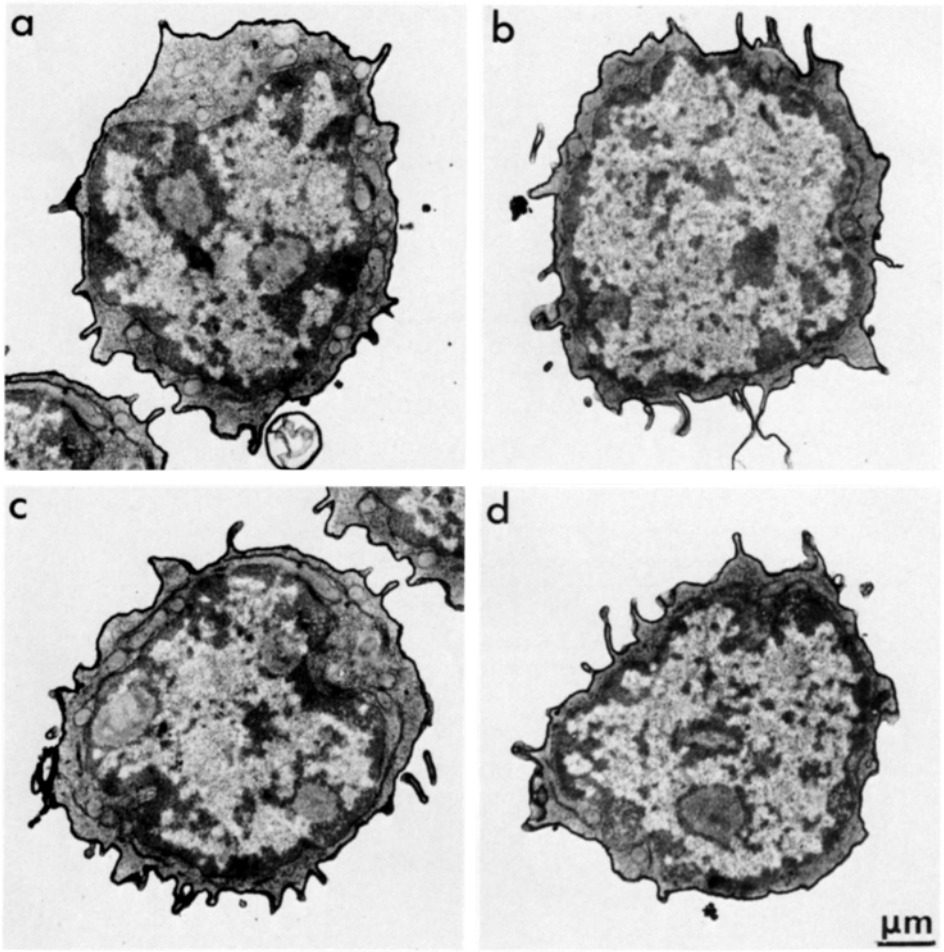

FIGURE 6. Electron microscope pictures of cells from the sorted suspensions. (*b* and *d*) Typical examples of the cells meeting our morphological criteria (CMOMC; ref. 6); (*a* and *c*) typical examples of the cell type that is slightly different from the CMOMC (see Results).

that the criteria for morphological recognition of PHSC as described by van Bekkum et al. (6) may be somewhat too strict.

Viability and DNA Content. The sorted cells were incubated with fluorescein diacetate to determine their viability. Analysis by the FACS indicated that >90% of the cells (94 ± 3%; n = 2) were brightly fluorescent and, therefore, were viable according to dye exclusion criteria.

DNA content was assessed on fixed sorted cells by staining with propidium iodide (33). Fig. 7 shows a comparison of the propidium iodide fluorescence distribution of unfractionated bone marrow cells and of the sorted cells. The histogram (Fig. 7*B*) shows that 98–100% of the sorted cells contained 2nDNA.

In Vitro Culture. Table II gives the incidence of GM-CFU-1 and -2 in unfrac-

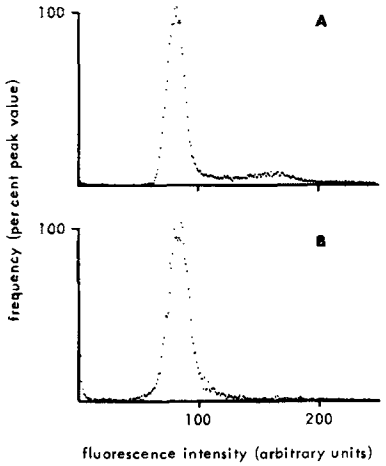

FIGURE 7. Comparison of the DNA histograms of unfractionated (*A*) and sorted (*B*) bone marrow cells.

TABLE II

Enrichment for Myeloid Progenitor Cells by Combining Density Separation and Fluorescence-activated Cell Sorting after WGA and H-2K Labeling

Assay and progenitor cell type	Number of colonies per 10^5 cells		Enrichment factor
	Unfractionated bone marrow cells	Sorted cells	
Spleen colony assay			
day 8 CFU-S	41 ± 6	1,450 ± 210	36
day 12 CFU-S	48 ± 6	6,040 ± 720	127
Semisolid agar assay			
GM-CFU-1	99 ± 11	6,930 ± 820	70
GM-CFU-2	198 ± 18	2,570 ± 290	13
Liquid culture assay*			
GM-CFU-1	420 ± 60	27,000 ± 2000	64
GM-CFU-2	<100	<100	?

Data represent the mean and standard deviation of the results of three experiments.

* Limiting dilution analysis from cells that were counted and deposited by the FACS in Terasaki trays.

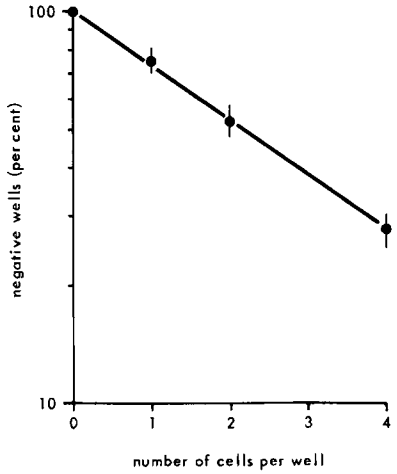

FIGURE 8. Limiting dilution analysis of sorted cells in Terasaki tray culture system with serum-free liquid culture medium containing 18-h PES and PMUE. Wells were scored at day 7.

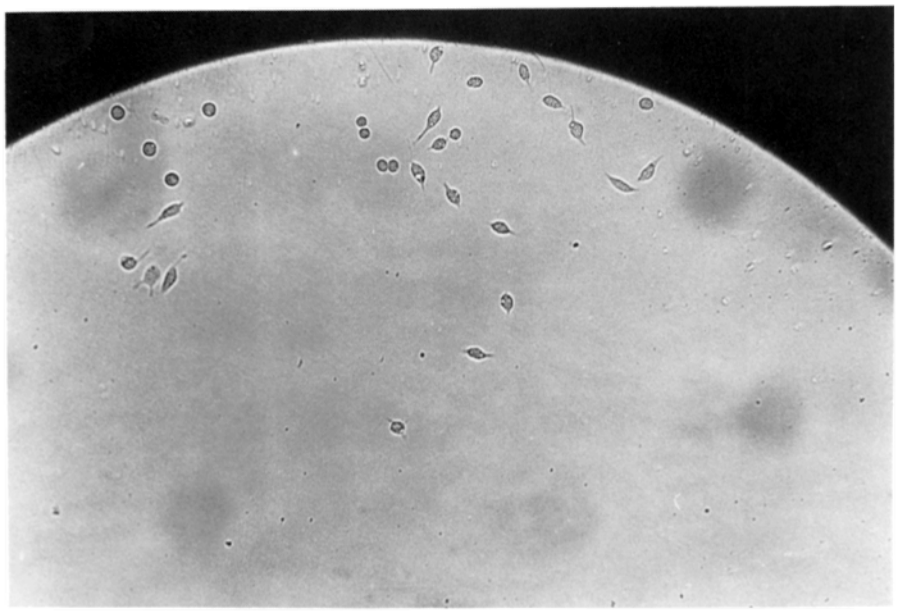

FIGURE 9. Picture of typical growth pattern of sorted cells in microwells in the presence of 18-h PES and PMUE with serum-free liquid culture medium at day 3.

tionated and in sorted cell suspensions as determined in semisolid agar cultures and liquid cultures. The plating efficiency for GM-CFU-1 was higher in liquid cultures than in agar cultures, both for unfractionated and for sorted cells (Table II). The enrichment factor for GM-CFU-1 in both culture systems was about half that of day 12 CFU-S. Some enrichment for GM-CFU-2 was observed in the agar cultures, although most are removed by the density separation (not shown; cf. 9, 19). No colonies were detected in the liquid cultures in the presence of

PMUE alone, whereas, in the presence of both 18-h PES and PMUE, between 25 and 30% of the sorted cells produced myeloid progeny. Limiting dilution analysis showed that this was a single-cell event (Fig. 8), indicating that no accessory cell activity was required.

In the single-cell cultures, no cells were observed after 24 h of incubation in the absence of CSA. When only PMUE was added, 20–30% of the wells contained the cell for up to 3 d, after which it disappeared. With both 18-h PES and PMUE, 30 ± 3% of the wells contained 2–32 dividing cells at day 3, and between 10^2 and 10^4 monocytes and granulocytes at day 14. No obvious morphological changes were observed during the first 2 d of culture, but by day 3 about 25% of the remaining cells stretched and attached to the bottoms of the wells (Fig. 9). When these attached cells were stained with May-Grünwald-Giemsa, they appeared to be undifferentiated blast cells (not shown). Only after 2 wk of culture were fully differentiated monocytes and granulocytes observed, suggesting that this culture technique detects a very early hemopoietic progenitor (11).

Discussion

The results of both the CFU-S and the 30-d survival assays indicated that 120–200-fold enrichment for PHSC can be achieved by our new combination of separation methods. In three experiments with the highest enrichment, an average of 10 ± 1 day-12 spleen colonies was observed per 100 injected sorted cells. This gives a minimum value for the seeding efficiency (f factor) of sorted CFU-S in spleen of 0.1, or twice the value measured in classic retransplantation experiments (7, 28, 34). The latter method, in fact, measures the f factor for the second transplantation. It has been demonstrated, however, that quiescent CFU-S enter the cell cycle within hours after transplantation (27) and that the f factor for proliferating CFU-S may be only half that for quiescent CFU-S (36). Therefore, an f factor for quiescent CFU-S in the first transplantation twice that for proliferating CFU-S in the second transplantation would be reasonable. The incidence of 10 spleen colonies per 100 injected cells would then indicate a purity for spleen colony-forming cells of 100%. The average purity of the PHSC in the overall series of enrichment experiments would be 65%, with a range of 35–110%.

The morphology of the sorted cells, as judged by light microscopy, indicated that they were homogeneous. Examination at the level of resolution of the electron microscope, on the other hand, revealed that two distinct but morphologically similar cell types were predominantly present, both of which can give rise to spleen colonies. There are two alternative hypotheses to explain these data: (*a*) one of the cell types is the PHSC, the other one a committed hemopoietic progenitor cell which may give rise to spleen colonies in irradiated recipients but with more limited proliferative capacity (7); or (*b*) both cell types are PHSC, one type being quiescent PHSC in the G_0 phase of the cell cycle, the other type PHSC in the G_1 phase of the cell cycle. The observation that the sorting procedure yielded a similar enrichment factor for CFU-S and for the cell type that provides radioprotection makes the first hypothesis unlikely. The second hypothesis, on the other hand, might explain the fact that the sorted cells form three times as many spleen colonies at 12 d as at 8 d after infusion, if it is assumed that the day

12 spleen colonies arise from quiescent PHSC and that day 8 colonies originate from proliferating PHSC, as suggested previously (2). The morphologically predominant cell type, which resembled the "candidate stem cell" described earlier (6), should represent quiescent PHSC that give rise to day 12 colonies in this model.

30% of the sorted cells were induced to proliferate in the liquid culture system. This percentage can probably be increased by improving the culture conditions and by using other sources of stimulating factors, since CSA and the enhancing factor used are specific for inducing myeloid differentiation. It is interesting to note that no proliferation was seen in the serum-free single-cell cultures when PMUE alone was added, whereas colonies were present in the serum-containing agar cultures in the same experiment. This suggests that PMUE-stimulated colony formation by primitive progenitor cells is dependent on factor(s) present in serum. Such a mechanism would explain the apparently contradictory findings that CSF-1 (the major colony-stimulating factor present in PMUE) binds only to mononuclear phagocytic cells and their precursors (12) but that PMUE can induce proliferation of purified multipotential progenitors from fetal liver in liquid cultures containing serum (39). The increased survival of sorted cells in PMUE-containing cultures, compared with unstimulated cultures, would argue that PMUE can affect the survival of primitive progenitors. However, whether this is due to CSF-1 or to the other substances in the rather crude preparation used in these studies remains to be determined.

Summary

A method is described to purify pluripotent hemopoietic stem cells (PHSC) from adult mouse bone marrow. The method consists of three separation steps. First, bone marrow cells are centrifuged in a discontinuous metrizamide gradient and simultaneously labeled with wheat germ agglutinin-fluorescein isothiocyanate (WGA-FITC). Second, the low density cells are analyzed by a fluorescence-activated cell sorter (FACS) and the WGA-positive cells with medium forward and low perpendicular light scatter intensities are sorted. The WGA-FITC is removed from the cells by incubation with *N*-acetyl-D-glucosamine. Finally, the sorted cells are incubated with anti-H-2K-biotin and avidin-FITC and sorted a second time to enrich cells with high H-2K density.

The sorted cells gave rise to 2 spleen colonies per 100 injected cells at 8 d and 6.6 colonies per 100 cells at 12 d after transplantation into lethally irradiated syngeneic recipients. The average enrichment factor for day 12 CFU-S (colony-forming unit/spleen) was 135 (range, 90–230; $n = 15$) and was similar to that for the cell type that provides radioprotection (180 ± 70), indicating that these functional properties were copurified. Indirect evidence suggests that the spleen-seeding efficiency (f factor) of these cells is 0.10 and, therefore, the average purity of the sorted PHSC was 65% (range in 15 experiments, 35–110%). The sorted cells were all in the G_1 or G_0 phase of the cell cycle. They appeared to be undifferentiated blasts by morphological criteria. Electron microscopy revealed that the sorted cells consisted primarily of two cell types, possibly representing G_0 and G_1 cells.

The FACS was used to deposit single selected cells into individual microwells

of Terasaki trays. 32% of the sorted cells could be induced to form myeloid progeny in vitro. This procedure should be useful for direct studies on the regulation of hemopoietic cell differentiation.

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