Rhodamine123 Reveals Heterogeneity Within Murine Lin⁻, Sca-1⁺ Hemopoietic Stem Cells

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Summary

Murine bone marrow Lin⁻, Ly6A/E⁺ cells have been fractionated on the basis of rhodamine123 retention into Rh123med/hi and Rh123lo subpopulations. These populations have different responses to hemopoietic growth factors with respect to in vitro colony formation. Cells from either fraction were not stimulated by only granulocyte-colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), interleukins 1 and 6 (IL-1 and -6), or leukemia inhibitory factor (LIF) alone. The Rh123med/hi, but not the Rh123lo fraction, contained cells that could be stimulated by either stem cell factor (SCF) or IL-3 alone. When combinations of growth factors were added, the Rh123med/hi fraction produced more colonies, and responded to a wider range of factor combinations than the Rh123lo population. When tested in vivo, both populations contained no detectable day 8 colony-forming unit-spleen (CFU-S), and similar frequencies of day 13 CFU-S. When transplanted into lethally irradiated recipients (100 cells/recipient), significant numbers of donor cells (67–73%) were found in the peripheral blood of Rh123lo recipients. Both myeloid and lymphoid cells were of donor origin. By comparison, the Rh123med/hi population produced recipients with 1–2% donor cells in peripheral blood, the majority of which were lymphoid.

The publication by Spangrude et al. (1) of a method for enriching murine bone marrow cells on the basis of low levels of Thy-1 expression (Thy-1⁻), absence of expression of a panel of antigens defining mature hemopoietic lineages (Lin⁻), and expression of Sca-1 (the Sca-1 antigen, also known as Ly6A/E), has generated considerable discussion concerning the degree of purity and identity of pluripotential hemopoietic stem cells (PHSC) (1–8). Cells with the Thy-1⁻, Lin⁻, Ly6A/E⁺ phenotype were significantly enriched for CFU-S(d12) (cells capable of forming spleen colonies 12 d after transplantation of lethally irradiated recipients), the implication being that some of the CFU-spleen (S) were in fact PHSC and could therefore reconstitute the hemopoietic system of lethally irradiated recipients (1). Support for this concept was difficult to assess as the results from only one animal grafted with Thy-1⁻, Lin⁻, Ly6A/E⁺ cells, 6 wk previously, were shown (1). The Thy-1⁻, Lin⁻, Ly6A/E⁺ population has subsequently been tested at limiting cell numbers (down to one cell) for long-term repopulating ability, but only 1 of 40 to 1 of 13 cells generated detectable blood cells in vivo for periods of up to 3–7 wk, with very few cells producing progeny for much longer (9). Only 1 of 280 mice transplanted was able to supply sufficient cells, of donor origin, for secondary transplantation (9). The Thy-1⁻, Lin⁻, Ly6A/E⁺ fraction has been further subdivided on the basis of rhodamine123 retention (10). When compared with the Rh123hi subset, it was shown that the Rh123lo cells had a lower frequency of CFU-S(d13), a greater ability to reconstitute spleen and bone marrow 13 d after transplantation, and that these Rh123lo subset-derived marrow cells were able to produce donor cells in the peripheral blood of secondary recipients for periods of at least 8 wk (10). Finally, when the thymus of secondary recipients was examined, donor cells were detected in both myeloid and lymphoid populations, the conclusion being that Rh123lo, Thy-1⁻, Lin⁻, Ly6A/E⁺ cells were multipotential (10), albeit the data obtained was from examination of only the thymus, a tissue not normally assayed for multipotential cells. When assayed in vitro, with limited combinations of growth factors, a similar frequency of cells with colony-forming ability was observed with both Rh123 subsets (10).

We have divided Lin⁻, Ly6A/E⁺ cells into Rh123lo and Rh123med/hi fractions, and show that these have different responses to hemopoietic growth factors with respect to in vitro colony formation. In addition, a minimum of 1 in 40 cells of the Rh123lo population was able to engraft lethally irradiated recipients, producing both myeloid and lymphoid cells for at least 19 wk. In contrast, cells of the Rh123med/hi frac-

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1 Abbreviations used in this paper: CFC, colony-forming cell; CFU-S, CFU-spleen; HPP, high proliferative potential; LIF, leukemia inhibitory factor; PHSC, pluripotential hemopoietic stem cell; SCF, stem cell factor.
tion, after engraftment, predominantly repopulated the lymphoid lineages.

Materials and Methods

Animals. Mice were used at 6–12 wk of age, and were of the following strains: C57BL/Ka-Thy-1.1 (Thy-1.1, Ly5.2), C57BL/Ka-Thy-1.2/Ty-1.2, Ly5.2), and C57BL/6-Ly-5.1-Pep (Thy-1.2, Ly5.1). They were bred at The Walter and Eliza Hall Institute of Medical Research animal facility under specific pathogen-free conditions and were maintained on acidified water.

FACS®. To prepare cells for FACS® sorting, C57BL/Ka-Thy-1.1 mice (Ly5.2) (10–12 animals) were killed by cervical dislocation, and the cells from both femurs and tibiae were flushed out using a 2.5-ml syringe with a 21-gauge needle. After washing, the bone marrow cells were resuspended in 10 ml balanced salt solution containing 5% BSS.FCS and 0.1 μg/ml rhodamine123 (Eastman Kodak Co., Rochester, NY), and incubated at 37°C for 30 min. After washing once, the cells were resuspended in 10 ml fresh BSS.FCS, and incubated at 37°C for another 30 min. The cell suspension was then diluted further by the addition of 10 ml of BSS.FCS, and layered on top of Ficoll (Pharmacia Inc., Piscataway, NJ), and centrifuged at 1,600 g for 10 min at room temperature. Light density viable bone marrow cells were recovered at the interface and washed with 10 ml of BSS.FCS. After washing and resuspension in 3 ml of BSS.FCS, the cells were incubated on ice for 15 min with a mixture of rat mAbs, GK1.5, 53.6.72, 53.7.3.13, RA3.6B2, M1/70.15.11.5, and RB6-8C5, reactive with specific antigens on mature T, B, and myeloid cells (neutrophils and monocytes/macrophages). After washing with 10 ml BSS.FCS and centrifugation through an FCS underlayer, the cells were resuspended in 1 ml of BSS.FCS and mixed together with 1 ml of pre-washed magnetic Dynabeads (Dynabeads M450; Dynal Inc., Oslo, Norway), and incubated for 20 min at 4°C with constant mixing. After diluting the mixture by adding 5 ml of fresh BSS.FCS, the beads, together with the cells bound to them, were removed magnetically. The procedure was repeated twice to both bead and bead-free cell populations. The bead-free cell fractions were pooled, centrifuged, and were then stained sequentially for 15 min each in 0.5 ml vol on ice with PE-conjugated goat anti-rat IgG (mouse absorbed), biotinylated E13 161.7 mAb and Texas red-avidin. Each staining step was followed by a washing step with 10 ml of BSS.FCS, and the bone marrow cells were pre-incubated with 50 μg normal rat Ig for 2–3 min before the addition of biotinylated E13 161.7 Ab. After the final wash, the cells were resuspended in 3 ml of BSS.FCS containing 10 μg/ml propidium iodide (Calbiochem Corp., La Jolla, CA), and filtered through a 63-μm pore size nylon screen. FACS® was performed using an unmodified FACStar-Plus® instrument (Becton Dickinson Immunocytometry Systems, San Jose, CA). All cells were kept at 4°C throughout the whole procedure. After completion of cell sorting, the deflected cells were centrifuged at 1,500 rpm for 5 min, resuspended, and counted using a hemocytometer.

In Vitro Cultures. Marrow Lin−, Ly6A/E+ cells fractionated into Rh123lo or Rh123med/hi subpopulations as indicated in Fig. 1 (bottom), were cultured at 100–200 cells/culture in duplicate 35-mm petri dishes. The cells were immobilized in 1 ml of agar medium and stimulated by various combinations of the following stimuli at 1,000 U/ml unless specified otherwise: murine IL-3, GM-CSF, M-CSF, or human G-CSF, prepared in this laboratory and kindly provided by Dr. N. A. Nicola. Human IL-6 (kindly provided by Dr. A. Gearing, Oxford University, Oxford, UK) was used at 100 U/ml. Colonies of >50 cells were scored at day 12 of incubation. The cell types within colonies were determined in situ after fixation of cultures with 2.5% glutaraldehyde, air-drying, and staining for acetylcholinesterase and with hematoxylin and Luxol Fast blue.

Transplantation. Recipient animals, C57BL/6-Ly-5.1-Pep (Ly5.1) were exposed to 11.0 Gy of γ radiation from a 137Cs source (Atomic Energy, Ottawa, Canada) at a dose rate of 31 Gy/min. The 11.0-Gy dose was delivered in two equal exposures, given 3 h apart. Irradiated recipients were transplanted intravenously with indicated numbers of Rh123lo, Lin−, Ly6A/E+, or Rh123med/hi, Lin−, Ly6A/E+ cells in the absence of any filler cells. Animals were maintained on aqueous antibiotics (neomycin sulphate, 1.1 grams/liter for at least 3 wk after transplantation. Hemopoietic reconstitution was determined by the percentage of donor-derived (Ly5.2+) nucleated cells in the peripheral blood of recipient (Ly5.1) animals using Ly5.2 allelic-specific mAb, S450-15.2 (kindly provided by Dr. I. Mackenzie, University of Melbourne, Victoria, Australia), together with rat mAbs, RA3–6B2 (for B cells) and GK1.5 and S3–6.72 (for T lymphocytes). Forward and perpendicular light scatter were used to define granulocytes and monocytes/macrophages, and this was confirmed by using the myelomonocytic-specific rat mAbs, RB6–8C5 and M1/70.

Results

Isolation of Rh123 Subsets of Ly6A/E+, Lin− Cells. We have isolated murine Lin−, Ly6A/E+ cells as these have been shown to be phenotypically and functionally similar to Thy-1lo, Lin−, Ly6A/E+ cells (11). Although the Lin−, Ly6A/E+ population contained only 30–50% Thy-1lo cells and 200 ng/ml, respectively. Recombinant human IL-1 (kindly provided by Dr. A. Gearing, Oxford University, Oxford, UK) was used at 100 U/ml. Colonies of >50 cells were scored at day 12 of incubation. The cell types within colonies were determined in situ after fixation of cultures with 2.5% glutaraldehyde, air-drying, and staining for acetylcholinesterase and with hematoxylin and Luxol Fast blue.

Figure 1. Fractionation of Lin−, Ly6A/E+ cells by Rh123 retention. (Top) Relative cell number vs. relative Rh123 fluorescence of Lin−, Ly6A/E+ marrow cells (solid line) compared with normal bone marrow cells (dashed line). (Bottom) Relative Rh123 fluorescence vs. forward light scatter of Lin−, Ly6A/E+ cells. The two windows show regions from which the two Rh123 subpopulations were collected.
and a twofold-lower frequency of day 13 CFU-S, their ability to confer long-term in vivo hematopoietic engraftment was not significantly impaired compared with the homogenous Thy-1\(^+\), Lin\(^-\), Ly-6A/E\(^+\) cells (11). We have further subfractionated the Lin\(^-\), Ly-6A/E\(^+\) marrow population using the fluorescent dye, rhodamine123. Fig. 1 (top) shows the distribution of Ly6A/E\(^+\), Lin\(^-\) cells with reference to their Rh123 fluorescence intensity. The Ly6A/E\(^+\), Lin\(^-\) population can be divided into two recognizable populations, namely Rh123\(^-\) and Rh123\(^{med/hi}\), based on relative forward light scatter characteristics (Fig. 1, bottom). When compared with a standard reference cell population, lymph node cells, which had a mean forward light scatter at channel number 109, the Rh123\(^{-}\) population had a mean forward light scatter at channel number 118, and the Rh123\(^{med/hi}\) population had one at 130. Morphologically, the majority of the Rh123\(^{-}\) cells were small mononuclear cells with a chromatin pattern resembling that of small lymphocytes, whereas those of the Rh123\(^{med/hi}\) population were medium-sized blasts.

**In Vitro Colony Formation.** To evaluate the in vitro colony-forming cell (CFC) content of these populations, cells (150 cells/culture) from each population were cultured in semi-solid agar medium stimulated with recombinant cytokines, rhodamine123. Results represent mean + SD of quadruplicate cultures scored on day 12 of incubation. (A) is data from cultures of Rh123\(^{lo}\). (B) data from Rh123\(^{med/hi}\) cells.

![Figure 2. Colony formation in vitro by rhodaminel23 fractions of Lin\(^-\), Ly6A/E\(^+\) bone marrow cells. Results represent mean + SD of quadruplicate cultures scored on day 12 of incubation. (A) is data from cultures of Rh123\(^{lo}\). (B) data from Rh123\(^{med/hi}\) cells.](image)

**Table 1. CFU-S Content of Rh123\(^{lo}\) and Rh123\(^{med/hi}\) Fractions of Lin\(^-\), Ly6A/E\(^+\) Cells**

| No. of cells transplanted | Percent survival\(^*\) (30 d) | No. of spleen colonies\(^\dagger\) |
|---------------------------|--------------------------------|--------------------------------|
|                           | Day 8                          | Day 13                          |
| 100 Rh123\(^{lo}\)        | 90                             | 0 ± 0                           | 4.5 ± 1.7                      |
| 100 Rh123\(^{med/hi}\)    | 90                             | 0 ± 0                           | 4.5 ± 0.6                      |

\(^*\) Ten animals transplanted in each group.  
\(^\dagger\) Mean number of spleen colonies ± SD per 100 transplanted cells.

TABLE 1. CFU-S Content of Rh123\(^{lo}\) and Rh123\(^{med/hi}\) Fractions of Lin\(^-\), Ly6A/E\(^+\) Cells
cells were absent in recipients transplanted with Rh123<sup>med</sup>/hi, Lin<sup>-</sup>, Ly6A/E<sup>+</sup> cells (Fig. 3). Even in recipients transplanted with 1,500 Rh123<sup>med</sup>/hi cells, although displaying an increased level (a median of 12.0 ± 1.9% measured 17 wk posttransplantation) of donor-derived nucleated peripheral blood cells, the majority of these were lymphoid (B and T) cells (Fig. 3). Only one of four recipients examined, showed the presence of about 8% donor cells in the circulating myeloid population (Fig. 3).

Discussion

The Thy-1<sup>-</sup>, Lin<sup>-</sup>, Ly6A/E<sup>+</sup> cell population has been fractionated on the basis of rhodamine123 retention (10). It was concluded, albeit on a limited number of assays, that there was no difference between the Rh123<sup>lo</sup> and Rh123<sup>hi</sup> subsets with regard to in vitro CFC numbers (10). A similar result has also been reported recently, although once again using only a limited number of growth factor combinations (13). However, it was also shown that the Rh123<sup>lo</sup> population was capable of a 13-d marrow repopulation, and that these marrow cells contained sufficient donor cell reserves to repopulate secondary lethally irradiated recipients for up to 8 wk, although multilinage development was only tested for and demonstrated in the thymus (10). These results suggested that the Rh123<sup>lo</sup> population was less mature than the Rh123<sup>hi</sup> fraction, but because the cells had been passaged through primary recipients for 13 d before secondary transplantation, it was not possible to determine the direct repopulating ability of the Rh123<sup>lo</sup> cells, or the frequency of such cells. Although the experiments were incomplete, it was suggested that the differences observed in vivo may have been due to localization in differing microenvironments, rather than to inherent differences between the Rh123 subsets (8).

The results presented here, demonstrate that the two Rh123 fractions are functionally different. The in vitro results demonstrate that in the same environment, there is a differential responsiveness to individual and combinations of growth factors. The in vivo results also demonstrate that there are functional differences between the two Rh123 subpopulations. The Rh123<sup>lo</sup> population repopulated both myeloid and lymphoid lineages, whereas the Rh123<sup>med</sup>/hi population predominantly engrafted the lymphoid lineages. Taken together, these in vitro and in vivo results suggest that there is genuine intrinsic heterogeneity within the cells of the Lin<sup>-</sup>, Ly6A/E<sup>+</sup> phenotype that accounts for the differing behavior in vitro and after engraftment in vivo.

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