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Further Enrichment and Analysis of Rat CFU-s

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Abstract. Using the monoclonal antibody W3/13, which recognizes a determinate expressed on a sialoglycoprotein, rat marrow cells with the phenotype Thy-1 antigen upper 20% positive ($Ox7^{20}$) and high molecular weight leukocyte common antigen negative ($Ox22^-$) were separated into W3/13 dim ($W3/13^d$) and W3/13 bright ($W3/13^b$) subpopulations by single-laser cell sorting. The spleen colony-forming unit (CFU-s) was found in the $W3/13^d$ fraction. A 468-fold enrichment of CFU-s was achieved. Only 20% of the $Ox7^{20}$, $Ox22^-$, and $W3/13^d$ cells were in the S phase of the cell cycle as compared to 56% of $Ox7^{20}$, $Ox22^-$, and $W3/13^b$ cells. Using Indo-1, it was not possible to demonstrate increases in cytosolic Ca^{++} levels within the enriched CFU-s population by colony-stimulating factors (CSFs) or interleukins 1, 2, and 3. However, challenge with the Ca^{++} ionophore, ionomycin, demonstrated apparent heterogeneity of intracellular Ca^{++} management within the enriched CFU-s population. The source of this heterogeneity is not known. Only a 12-day CFU-s was detected in the rat, and it was predominantly, but not exclusively, a Rhodamine 123 (Rh123) dull cell.

Introduction

Previously, sorting rat marrow cells for the Ox7 upper 20% positive ($Ox7^{20}$) and cells other than those expressing high levels of high molecular weight leukocyte common antigen ($Ox22^-$), resulted in a 100-fold enrichment of the rat spleen colony-forming unit (CFU-s) [1], a 350-fold enrichment of cells capable of protecting rats from the lethal effects of ionizing radiation [2] and a 282-fold enrichment of the marrow prothymocyte [3]. The $Ox7^{20}$ and $Ox22^-$ population was found to be phenotypically heterogeneous. It could be separated into two subpopulations by dual laser flow cytometry using the monoclonal antibody W3/13 [2]. The purpose of this study was to characterize the rat CFU-s with respect to the W3/13 dim ($W3/13^d$) and bright ($W3/13^b$) subpopulations of the $Ox7^{20}$ and $Ox22^-$ cell populations.

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Materials and Methods

Animals

Lewis male rats were obtained from Charles River Laboratory (Kingston, RI) at 4 weeks of age and used at 8 weeks of age in an AAALAC-accredited facility. Rats were euthanized by CO_2 gas inhalation.

Biologicals

Rhodamine 123 (Rh123) and Indo-1/AM were purchased from Molecular Probes (Junction City, OR); pokeweed mitogen (PWM) and 4'6'-diamidino-2-phenylindole dihydrochloride (DAPI) from Sigma Chemical Company (St. Louis, MO); human granulocyte colony-stimulating factor (G-CSF), mouse recombinant interleukin (IL) 1 and 3 from Amgen Biologicals (Thousand Oaks, CA); rat IL-2 from Accurate Chemical and Scientific Corporation (Westburg, NY); ionomycin and trifluoperazine dimaleate (TFP) from CALBIOCHEM Corporation (San Diego, CA). CMRL-1066, RPMI-1640, Opti-1 medium, fetal calf serum (FCS) and Dulbecco's phosphate-buffered saline (PBS) from GIBCO Laboratories (Grand Island, NY).

Flow Cytometry and Sorting

The fluorescence-activated cell sorter (FACS-II; Becton-Dickinson Immunocytometry Systems, Mountain View, CA) was upgraded to a dual laser, six-parameter instrument. A forward light scatter measurement, and 3 immunofluorescence measurements were initiated with the first argon laser (488 nm); fifth and sixth fluorescence measurements were made following excitation with a second ultraviolet argon laser. Filters were purchased from Omega Optical Inc. (Brattleboro, VT). The FACS-II electronics were used to delete the crossover between fluorescence channels. Either the DAPI-DNA fluorescence or Indo-1 violet/blue fluorescence ratio was recorded on the spatially separated delayed channel. The forward light scatter (FWLS) signal was used to trigger the electronics of the FACS-II, but was not recorded on the Consort 40 computer system because of space limitations.

Cell Staining

Single cell preparations and immunofluorescence cell staining with allophycocyanin (APC)-Ox7 Fab', phycoerythrin B (PhyB)-Ox22 Fab', PhyB-W3/13 Fab', and fluorescein isothiocyanate (FITC)-W3/13 IgG were performed as described previously [1, 2]. For DNA staining, the immunofluorescent-tagged cells were suspended in 1 ml PBS containing 200 μg TFP [4] and 3 μg of DAPI for 45 min at 4°C. For either Rh123 or Indo-1/AM staining, cells were incubated at 37°C at a concentration of 1×10^6 cells/ml in 10 ml of 10% FCS-PBS at an Rh123 concentration of 0.1 μg/ml or Indo-1/AM concentration of 0.3 μg/ml for 45 min. The cell suspension was washed twice and tagged with the appropriate immunofluorescent reagents as described above.

CFU-s Assay

A total of 1×10^2 to 3×10^6 cells in 1 ml were injected i.v. into irradiated recipient rats (9 Gy total body radiation at 0.04 Gy per min ^{60}Co). The spleens were removed 12 days later and fixed in Bouin's solution [1].

CFC Assay

Cells were cultured in vitro by a modified version of the double agar technique described by *Bradley et al.* [5]. Pokeweed conditioned medium (PWCM) was prepared by incubating rat spleen cells in RPMI-1640 at 5% CO_2 at 37°C for 7 days. PWCM was mixed in

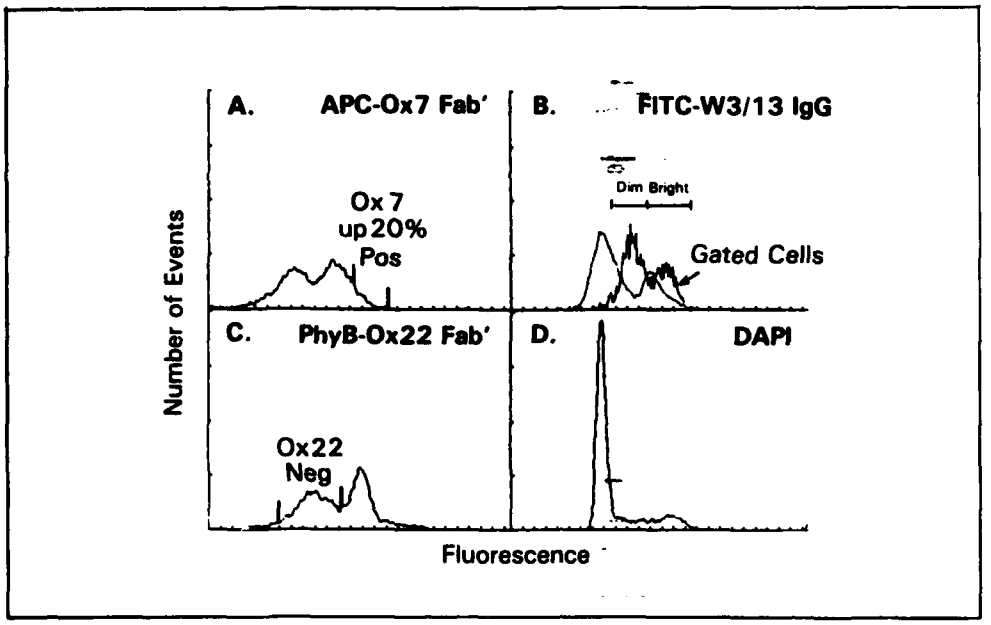

Fig. 1. Five-parameter analysis of rat marrow cells. The primary argon laser was tuned to the 488 nm spectral line and was used to generate the forward light scatter signal and excite the 3 fluorochrome-conjugated monoclonal antibodies. The second argon laser was tuned to the UV and was used to excite the DAPI-DNA complex. By gating on the APC-$Ox7^{20}$ and PhyB-$Ox22^{-}$ cells, a new FITC-W3/13 immunofluorescence histogram was generated and is shown by the line labeled "Gated Cells" in quadrant B.

the bottom layer (equal volumes of 1% BactoAgar and modified 2× CMRL-1066 medium) and layered into a Petri dish. Cells were sorted directly onto the bottom layer. The top layer (equal volumes of 1.5% BactoAgar, 2× CMRL-1066 medium and Opti-1 medium) was immediately added following the sort. Cultures were incubated at 5% CO_2 at 37°C for 5 days at which time the colonies were counted.

Results

Purification

Sorts were performed on either FITC-W3/13 IgG- or PhyB-W3/13 Fab'-labeled rat marrow cells. Sort windows were set to include W3/13 negative, dim, and bright cells. Greater than 96% of CFU-s were recovered in the $W3/13^{d}$ fraction using either immunofluorescent reagent (data not shown). A second series of sorts for CFU-s was performed, gating on all 3 immunofluorescence parameters: APC-$Ox7^{20}$ Fab', FITC-$W3/13^{-}$ IgG, and PhyB-$Ox22^{-}$ Fab' (Fig. 1A-C). Cells were sorted into 1 ml of 2% FCS-PBS and immediately injected i.v. into a single irradiated recipient rat. There was a linear relationship between cells injected and spleen colonies detected (Fig. 2). A 468-fold enrichment of CFU-s was achieved. The sizes of the $Ox7^{20}O$, $Ox22^{-}$, and $W3/13^{d}$ or $W3/13^{b}$ subpopulations were 0.213% ± .019% and 0.115% ± .017% (mean ± SE) of the total marrow cellularity, respectively.

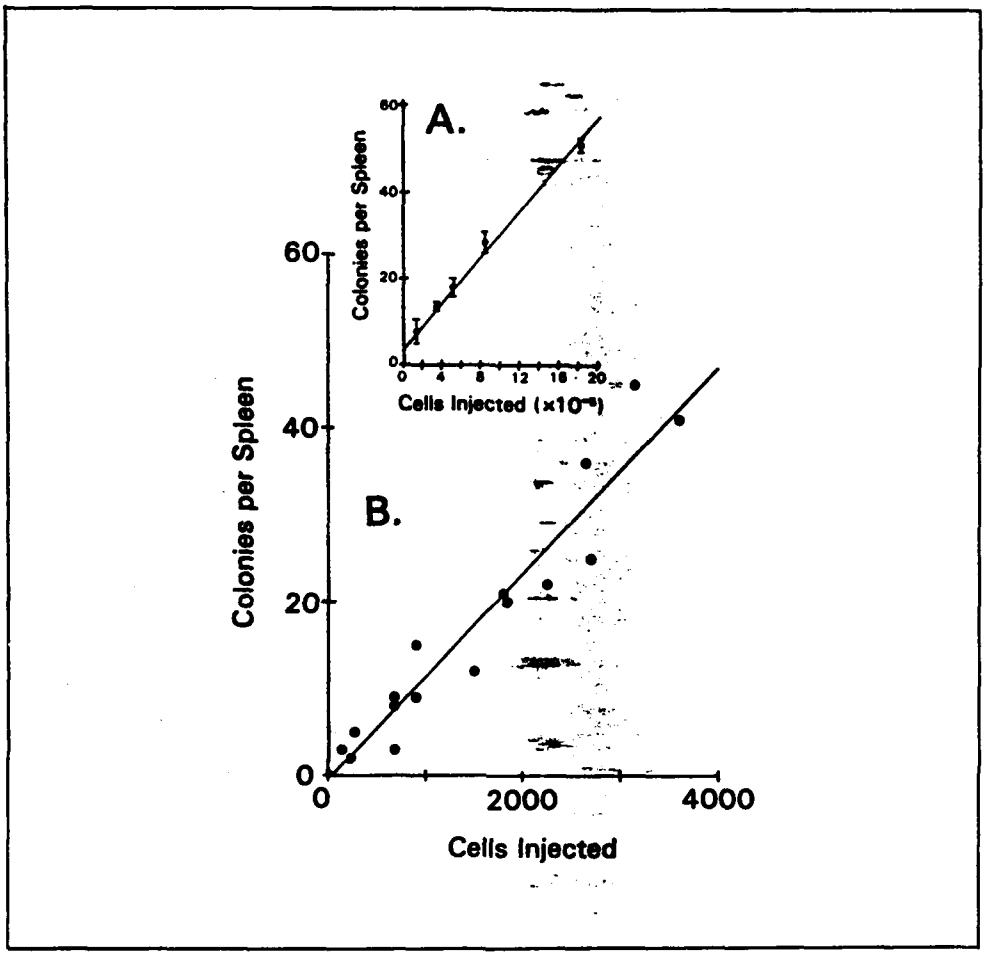

Fig 2. CFU-s dose-response curve for A) normal marrow cells and B) for enriched APC-Ox7^{20}, PhyB-Ox22^{-}, and FITC-W3/13^{d} sorted cells. Based on 25.6 CFU-s per 10^{6} cells for triple-labeled, unsorted marrow cells, a 468-fold purification of CFU-s was realized.

Cells were sorted directly into 35 mm Petri dishes containing the bottom layer, and immediately following the sort, the top layer was added. The number of colonies developing was linear to the number of Ox7^{20}, Ox22^{-} and W3/13^{d} cells sorted and plated. Approximately 1 of 7 cells generated a colony (Fig. 3). Very few of the Ox7^{20}, Ox22^{-} and W3/13^{b} sorted cells developed into colonies (Fig. 3). Theoretically, those colonies that developed were small (less than 50 cells in size) and should have been classified as clusters.

Characterization

The DAPI-DNA histogram for total marrow cells is shown in Figure 1D. The DAPI-DNA histograms for the Ox7^{20}, Ox22^{-}, and W3/13^{d} or W3/13^{b} subpopulations (as shown in Fig. 1B) are presented in Figures 4A and B, respectively. Twenty per cent of the W3/13^{d} population were in the S phase of cell cycle, while the W3/13^{b} population had 56% of its cells in the S phase of cell cycle.

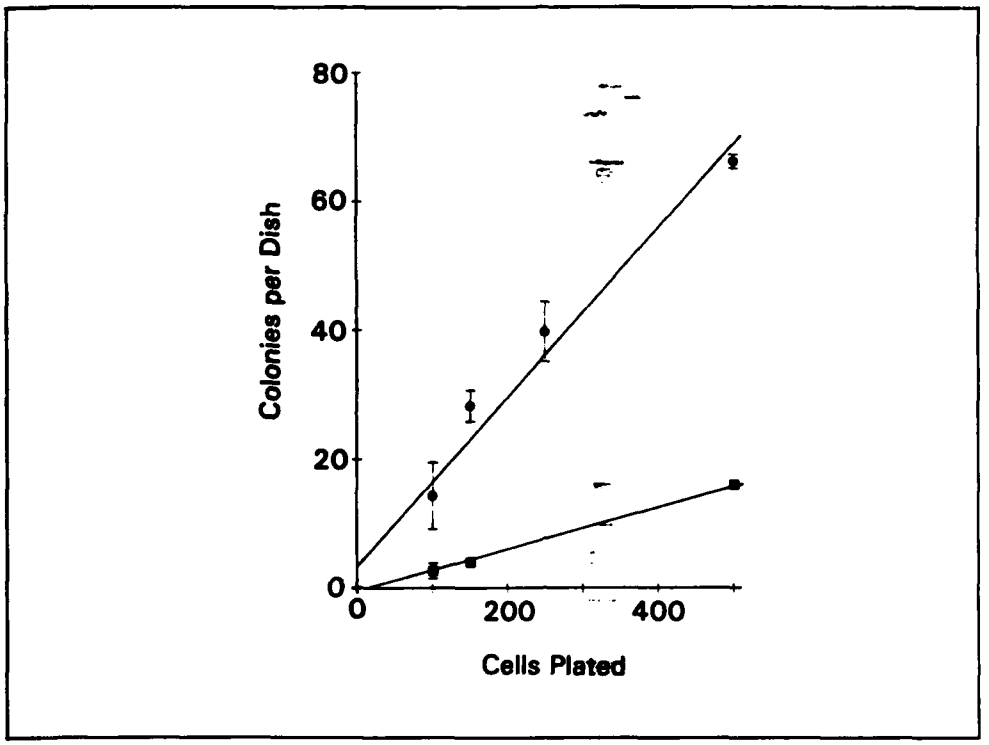

Fig 3. CFC dose-response curves for enriched $Ox7^{20}$, $Ox22^{-}$, and $W3/13^{d}$ (●—●) and $W3/13^{b}$ (■—■) cells.

Figure 5 shows that the $Ox7^{20}$ and $Ox22^{-}$ cells were slightly more Rhl23 positive than most marrow cells. Within this population, CFU-s were predominantly Rhl23 dull.

The Indo-1 violet/blue fluorescence ratio of normal marrow and the $Ox7^{20}$, $Ox22^{-}$, and $W3/13^{d}$ were found to be nearly identical (Figs. 6A and B). Titering Indo-1-loaded marrow cells with increasing amounts of ionomycin at 8°C to a final concentration of 105 μM ionomycin showed that marrow lymphocytes were the most sensitive to ionomycin; myelocytes were less sensitive, and what appeared to be predominantly erythroid precursors were the least sensitive. Intermediate between lymphocytes and myelocytes were the $Ox7^{20}$, $Ox22^{-}$, and $W3/13^{d}$ or $W3/W13^{b}$ cells. At 35 μM ionomycin, the resulting histogram for marrow cells was trimodal (Fig. 6A). As shown in the correlated plot(s) of PhyB-Ox22 Fab' (or APC-Ox7 Fab' or FITC-W3/13 IgG) fluorescence versus Indo-1 violet/blue fluorescence ratio (Fig. 7), the origin of the 3 peaks appeared to be cell lineage specific. In contrast, the $Ox7^{20}$, $Ox22^{-}$ and $W3/13^{d}$ gated population appeared to be heterogeneous (Fig. 6B).

Indo-1 (0.3 μg/ml) and ionomycin (35 μM) were not toxic for CFU-s. These cells were found to be relatively resistant, as compared with marrow lymphocytes, to ionomycin-induced increases in cytosolic Ca^{++} levels (Fig. 6A). Efforts to induce cytosolic Ca^{++} fluxes in the cells found within the $Ox7^{20}$, $Ox22^{-}$, and $W3/13^{d}$

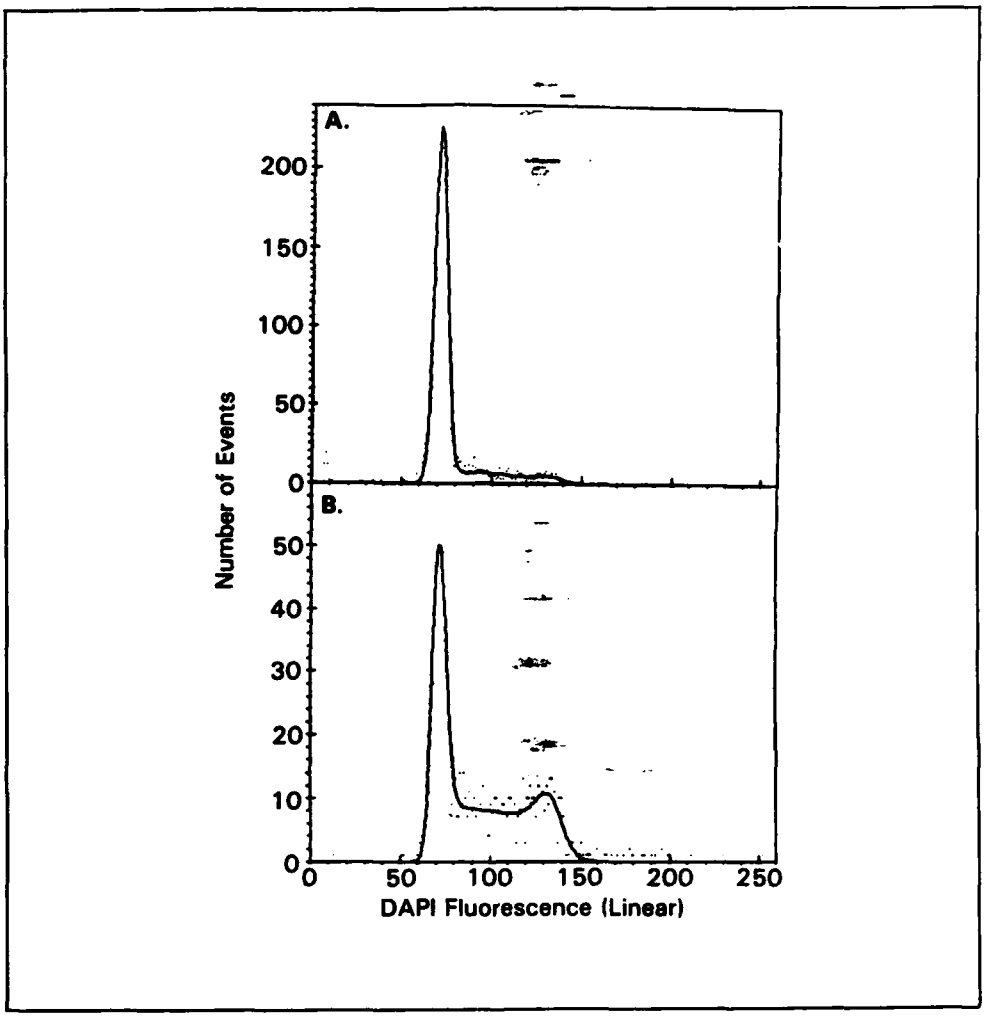

Fig 4. DNA histograms. A) APC-Ox7^{20}, PhyB-Ox22^{-}, and FITC-W3/13^{d} cells. B) APC-Ox7^{20}, PhyB-Ox22^{-}, and FITC-W3/13^{b} cells. Points are actual data points while the solid lines are computer-fitted lines generated by the Cotfit program based on the method of *Fox* [6].

population with recombinant G-CSF, PWCM and IL-1, IL-2, and IL-3 at either 8°C or 37°C were not successful.

Discussion

By single laser excitation of FITC, PhyB, and APC, it was possible to perform three-parameter immunofluorescence sorts for rat CFU-s with an enrichment of 468-fold being achieved. This enrichment is an improvement over the 320-fold purification reported by *Goldschneider et al.* [7] using anti-Thy-1 and light scatter properties of CFU-s from cortisone pre-treated rats. The cortisone treatment diminished marrow lymphocytes and separated them from cortisone-resistant CFU-s. Starting with marrow from normal rats, *Goldschneider et al.*

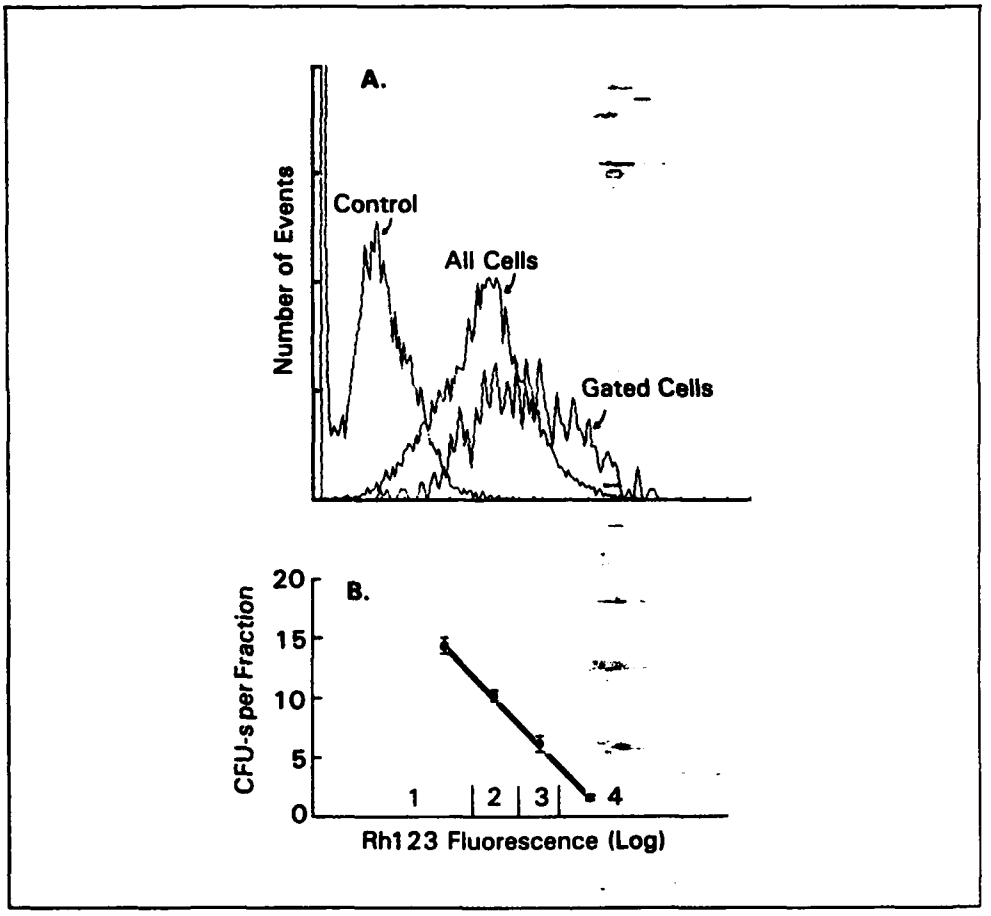

Fig 5. A) Rh123 green fluorescence of APC-Ox7^{20} and PhyB-Ox22^{-} labeled marrow cells. The green fluorescence of double-labeled cells without Rh123, the green fluorescence of double-labeled cells previously incubated with Rh123 and the green fluorescence of gated APC-Ox7^{20} and PhyB-Ox22^{-} cells are illustrated. B) CFU-s content of fractions sorted only on the Rh123 fluorescence. The number of CFU-s is expressed as the number of colonies per fraction from 1 × 10^{6} sorted normal cells.

[7] achieved a CFU-s enrichment of 151-fold. Using the present purification protocol, marrow lymphocytes were gated out of the sort by the Ox22 monoclonal antibody, and the cortisone pretreatment as well as density gradient pre-purification steps [8] could be omitted.

The size of the Ox7^{20}, Ox22^{-} and W3/13^{d} population was determined to be 0.213% of the total marrow population. If all rat CFU-s are characterized by this phenotype, then the maximum enrichment of CFU-s that could be achieved by this sorting protocol would be 469-fold. Depending upon whether normal marrow CFU-s concentration is determined from the CFU-s dose-response curve for normal marrow (Fig. 2A) or from the controls for the results graphed in Figure 2B, the maximum CFU-s concentration of the "purified" CFU-s population would be between 12,006 to 14,070 CFU-s per 1 × 10^{6} cells. The observed purity of the enriched CFU-s population was found to be 11,500 CFU-s per 1 × 10^{6} cells

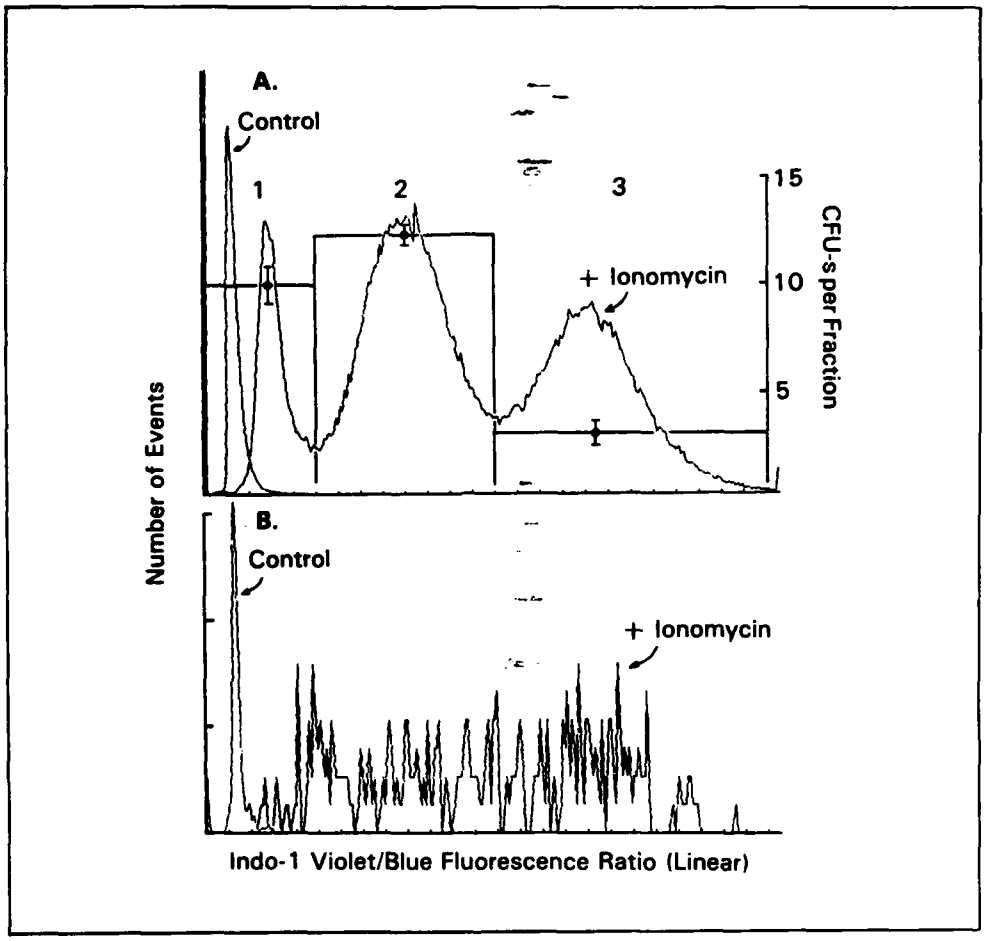

Fig 6. A) Indo-1 violet/blue fluorescence ratio of normal and ionomycin-incubated (35 μM at 8°C) marrow cells. CFU-s content of fractions sorted only on Indo-1 violet/blue fluorescence ratio. Results are expressed as CFU-s per fraction, based on a sort of 1×10^6 cells. B) The Indo-1 violet/blue fluorescence ratio histograms of the APC-Ox7^{20}, PhyB-Ox22$^-$, and FITC-W3/13^d gated cells in the presence and absence of ionomycin are shown for comparison.

indicating that 82 to 96% of all rat CFU-s are defined by this phenotype.

Mouse CFU-s have been enriched 441- to 613-fold by cell sorting [9-11]. The present enrichment of rat CFU-s was 468-fold. In order to calculate the absolute purity of the enriched CFU-s populations, it is necessary to adjust for the number of CFU-s injected i.v. and the number that actually "seed" the spleen and form colonies. This correction factor is commonly referred to as "f." Unfortunately, there are a variety of ways to calculate f; the 2 h, 24 h, 24 h post-irradiated recipient, dip, and extrapolation methods have all been used and generate estimates for f between a high of 30% and a low of 0.2% [12]. The 2 h and 24 h f values are similar in the rat and mouse, and if used, indicate that mouse FACS-enriched CFU-s populations [9-11] are approximately 10-fold more pure than rat-enriched CFU-s population. However, the "extrapolation" method for determining f indicates a

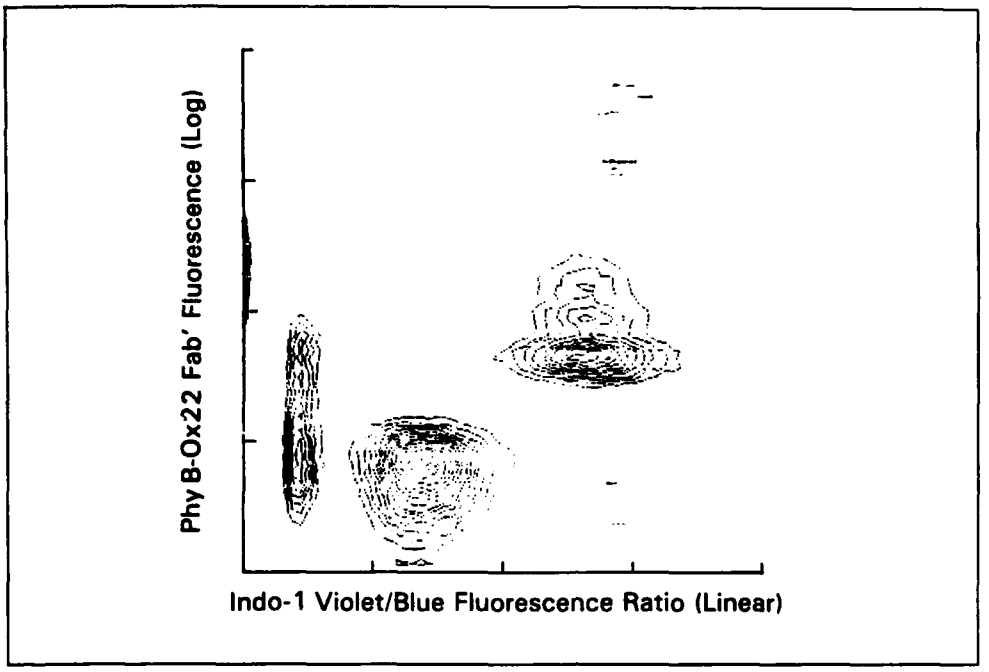

Fig 7. A correlated plot of Indo-1 violet/blue fluorescence ratio versus PhyB-Ox22 fluorescence following incubation with ionomycin (35 μM at 8°C).

5- to 7-fold lower f for rat CFU-s as compared to mouse CFU-s. If the extrapolated f's are used, it can be calculated that the FACS-enriched rat and mouse CFU-s populations are of the same relative purity. The extrapolated f for mouse CFU-s is 5%; for the BN rat CFU-s (uncorrected marrow CFU-s concentration approximately 30 to 50 CFU-s per 1×10^6) it is 0.9% [12, 13]. Using the BN rat CFU-s seeding efficiency for the Lewis rat CFU-s, slightly greater than a 100% (111%) purity of the highly enriched Lewis rat CFU-s was calculated.

Because *van Bekkum's* studies [12] demonstrated different splenic homing characteristics for rat and mouse CFU-s, it is interesting to note that the mouse CFU-s population can be subdivided into 8-day Rh123 bright, 12-day Rh123 bright, and 12-day Rh123 dull CFU-s subpopulations [14-16]. The mouse CFU-s subpopulations are also phenotypically distinct [9, 17]. In the rat CFU-s assay, only 12-day CFU-s is observed [13], and it is primarily, but not exclusively, an Rh123 dull CFU-s (Fig. 5). Efforts to demonstrate phenotypically distinct subpopulations within the rat 12-day CFU-s population have not yet been successful [3]. Therefore, there is the possibility (with the reservations noted in the last paragraph) that the mouse CFU-s seeding efficiency is the average seeding efficiency of several CFU-s subpopulations, some of which are not detected in the rat CFU-s assay. Further, the mouse 12-day Rh123 dull CFU-s is Thy-1 dim [18], wheat germ agglutinin (WGA) very bright [10], while the rat CFU-s is Thy-1 very bright [7], WGA dim [19]. These differences in phenotype may account, in part, for differences in the homing of rat and mouse CFU-s to the spleen.

It might be questioned whether the rat CFU-s develops more slowly than the mouse CFU-s, so that the rat 12-day CFU-s is more representative of the mouse 8-day CFU-s than the mouse 12-day CFU-s. Results to date do not support such a concept. The rat 12-day CFU-s is primarily Rh123 dull, and like the mouse 12-day CFU-s [20], appears to be relatively resistant to the cytotoxic compound 5-fluorouracil (studies in progress).

An alternative hypothesis would be that both the rat and mouse have the same absolute number of CFU-s, but because the amount of rat marrow is ten times greater than that found in the mouse, the concentration of rat marrow CFU-s is tenfold less than that determined for the mouse. Such a hypothesis is contradicted by the facts that A) the concentration of Thy-1-positive, lineage-negative cells (phenotype of both rat and mouse CFU-s) in rat and mouse marrow is approximately the same, i.e., between 0.2 to 0.4% of the total marrow cellularity [1, 2, 9, 21]; B) one of seven of these mouse- or rat-enriched marrow cells are capable of generating an in vitro CFC colony, and C) the amount of normal marrow or Thy-1- positive, lineage-negative marrow required for a successful bone marrow transplantation on a Kg body-weight basis is nearly identical for both rat and mouse [2, 12, 21]. However, our preliminary work using irradiated long-term chimeric rats to assay hematopoietic stem cells by the limiting dilution technique appears to support the alternative hypothesis, as does the observation that the concentration of rat marrow prothymocytes assayed by direct intrathymic injection—seeding efficiency 30 to 100% [22, 23]—is approximately one-tenth of that found for mice [23].

The $Ox7^{20}$, $Ox22^{-}$, and $W3/13^{d}$ population is a slowly proliferating cell population (20% in the S phase of cell cycle) as compared to the $W3/13^{b}$ population, which has 56% of its cells in the S phase of cell cycle. This finding was consistent with CFU-s having been characterized as non-proliferating cells [24]. The hematological function of the $Ox7^{20}$, $Ox22^{-}$, and $W3/13^{b}$ is, at the present time, not known.

The $Ox7^{20}$, $Ox22^{-}$, and $W3/13^{d}$ cells were heterogeneous in their response to elevation of cytosolic Ca^{++} levels by ionomycin. CFU-s found within this limited marrow population also share this trait as shown by sorting for CFU-s based on their ionomycin-perturbed Indo-1 violet/blue fluorescence ratios. Efforts to relate differences in intracellular Ca^{++} management to CFU-s differentiation and/or proliferation by challenge with ILs or CSFs were unsuccessful. This was unexpected, for the cells within the enriched CFU-s population can differentiate in the thymus (unpublished results) into thymocytes and, in the in vitro CFC culture to myelocytes, suggesting these cells are multipotent.

The apparent conflict in the observations of a) a restricted rat CFU-s phenotype limited to less than 0.213% of the cells found within rat marrow and b) the extreme physiological heterogeneity of cells within this subpopulation including CFU-s, as shown by challenge with ionomycin and to a lesser extent by Rh123 uptake, cannot be considered proof of heterogeneity within the rat CFU-s compartment. As has been noted by *Wilson et al.* [25], flow cytometric analysis underestimates intracellular Ca^{++} levels of individual cells if cytosolic Ca^{++} levels are being generated by frequency modulation of the Ca^{++} signaling system.

Although we have no evidence that CFU-s are capable of supporting Ca^{++} spiking and prolonged Ca^{++} oscillations, the postulated heterogeneity within the CFU-s populations based on the results of cell sorting experiments using intracellular fluorescent probes might be questioned. The CFU-s population is certainly an oscillating system with regard to G_o, G_1 transition [26], and ATP production may fluctuate between glycolysis and mitochondrial respiration [27, 28]. The interpretation of flow cytometry, intracellular fluorescent probe studies is critically dependent upon understanding the nature of the probe-cell interaction. One must also consider that analysis of cellular physiology by flow cytometry is a one-time point measurement made on individual cells. If these cells constitute a randomly fluctuating cell population, the results might suggest greater heterogeneity within the CFU-s population than that which actually exists [29].

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