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The Effect of Recombinant Mast Cell Growth Factor on Purified Murine Hematopoietic Stem Cells

By Peter de Vries,* Kenneth A. Brasel,* June R. Eisenman,* Alan R. Alpert,‡ and Douglas E. Williams*

*From the *Departments of Experimental Hematology and ‡Biochemistry, Immunex Corporation, Seattle, Washington 98101*

Summary

Pluripotent hematopoietic stem cells (PHSC) are very rare cells whose functional capabilities can only be analyzed indirectly. For a better understanding and possible manipulation of mechanisms that regulate self-renewal and commitment to differentiation of PHSC, it is necessary to purify these cells and to develop assays for their growth in vitro. In the present study, a rapid and simple, widely applicable procedure to highly purify day 14 spleen colony-forming cells (day 14 CFU-S) is described. Low density bone marrow cells ($\rho \leqslant 1.078$ g/cm^3) were enriched by two successive light-activated cell sorting procedures. In the first sort, cells within a predetermined light scatter (blast cell) window that are wheat germ agglutinin/Texas Red (WGA/TxR) positive and mAb 15-1.4.1/fluorescein isothiocyanate negative (granulocyte-monocyte marker) were selected. In the second sort, cells were selected on the basis of retention of the supravital dye rhodamine 123 (Rh123). Cells that take up little Rh123 (Rh123 dull cells) and those that take up more Rh123 (Rh123 bright cells) were 237-fold and 132-fold enriched, respectively, for day 14 CFU-S.

Both Rh123 fractions were cultured for various time periods in vitro in the presence of mast cell growth factor (MGF), with or without interleukin 3 (IL-3) or IL-1α. Both Rh123 fractions proliferated in response to MGF alone as determined by a [^{3}H]TdR assay or by counting nucleated cells present in the cultures over time. MGF also acted synergistically with both IL-3 and IL-1α to promote stem cell proliferation. Stimulation of both Rh123 fractions with MGF alone did not result in a net increase of day 14 CFU-S. Stimulation with MGF + IL-3 or MGF + IL-α resulted in a 4.4- or 2.6-fold increase of day 14 CFU-S in the Rh123 dull fraction, and an 11.6-fold or 2.6-fold increase of day 14 CFU-S in the Rh123 bright fraction, respectively.

The data presented in this paper indicate that in vitro MGF acts on primitive hematopoietic stem cells by itself and also is a potent synergistic factor in combination with IL-3 or IL-1α.

Hematopoiesis is a dynamic process by which large numbers of mature blood cells with very specific functions and limited life spans are generated (reviewed in reference 1). All the different blood cell types originate from a common population of pluripotent hematopoietic stem cells (PHSC)[1] found predominantly in the bone marrow (BM) of mammals. PHSC are normally quiescent and are characterized by their capacity to renew themselves and to generate committed progenitors of the erythroid, megakaryocytic, myeloid, or lymphoid lineages (1, 2).

A number of procedures based on physiochemical and immunochemical characteristics have been developed for the purification of murine PHSC (3–11). The late spleen colony-forming cells (day 12–14 CFU-S) have long been regarded as the PHSC (12–14) and the purification of day 12–14 CFU-S has often been used as an end point in many of the above purification procedures. However, it has been noted that the day 12–14 CFU-S content of a BM graft does not adequately predict the long term hematopoietic reconstitutive capacity (the ultimate test for PHSC) of the graft (11, 15–17). The use of the mitochondrial dye rhodamine 123 (Rh123) revealed a functional heterogeneity among day 12–14 CFU-S and has attributed to the notion that only a subpopulation of day 12–14 CFU-S is closely related to if not identical with PHSC (7, 9–11, 18, 19).

In vitro analysis of the growth and proliferation requirements of purified stem cell fractions may well lead to a better understanding of the factors involved in the regulation of self-renewal and differentiation. Multiplication of purified stem

[1] *Abbreviations used in this paper:* BM, bone marrow; CFU-S, spleen colony-forming cells; FLS, forward light scatter; HGF, hematopoietic growth factor; LD, low density; MGF, mast cell growth factor; PHSC, pluripotent hematopoietic stem cells; Rh 123, rhodamine 123; Sl, Steel locus; SSC, side light scatter; TxR, Texas Red; W, white spotting; WGA, wheat germ agglutinin.

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cells would also be beneficial for BM transplantation and potentially for gene therapy. Thus far, attempts to culture (purified) day-12 CFU-S in vitro have not resulted in the net in vitro production of these cells (20, 21).

Recently a new hematopoietic growth factor (HGF) has been described called mast cell growth factor (MGF), stem cell growth factor, or kit ligand (22–24). The gene encoding this factor maps to the Steel (Sl) locus of the mouse (24, 25) and is the ligand for the c-kit proto-oncogene, the product of the dominant white spotting (W) locus (26). It has long been appreciated that mutations at the Sl and W loci cause a severe impairment of PHSC development. The defect in W is intrinsic to the hematopoietic stem cells themselves (27, 28) while that in Sl is in the stromal microenvironment (27, 29, 30). Considering the seriousness of these defects it is of interest to study the effect of MGF on normal hematopoietic stem cells.

In the present study we describe a relatively rapid and simple procedure to purify hematopoietic stem cells (day-14 CFU-S). This procedure was used to examine the effect of rMGF either alone or in combination with other HGFs on the proliferation and growth of purified stem cell fractions in vitro. We demonstrate that MGF in combination with IL-3 or IL-1α in suspension cultures causes a net production of day-14 CFU-S.

Materials and Methods

Mice. Throughout this study 6- to 8-wk-old female B6C3 (C57Bl/6 × C3H/HeN)$_{F1}$ mice (Charles River Breeding Laboratories, Wilmington, MA) were used as BM donors or as BM transplantation recipients in the CFU-S assay. The mice were housed in conventional animal quarters.

Preparation of Cell Suspensions. BM cell suspensions were obtained by flushing the femoral and tibial shafts with 1 ml ice cold Hepes buffered (10 mM, pH 6.9) HBSS without phenol red (HH; Gibco Laboratories, Grand Island, NY). After repeated pipetting, the cells were passed through a 88 μm pore size Nitex filter (Small Parts Inc., Miami, Florida), washed and counted in a hemocytometer. Cells were kept on ice unless stated otherwise.

Spleen Colony Forming Assay (CFU-S Assay). To determine the CFU-S content of BM cell suspensions, the spleen colony forming assay was used (12). Unfractionated and sorted BM cells were transplanted intravenously (lateral tail vein) into lethally irradiated recipients (7–10 per group). The mice were exposed to 950 rad of gamma irradiation from a ^{137}Cs source (Mark 1 irradiator; Shepard and Associates, Glendale, CA) at a dose rate of 245 rad per min, and were transplanted 1–4 h after irradiation.

14 d after transplantation the mice were killed and their spleens were removed and fixed in Teleyesniczki's solution. The macroscopically visible spleen colonies (day 14 CFU-S) were counted.

Immunofluorescence Staining for Stem Cell Purification. BM cells were separated on a discontinuous density gradient of metrizamide (Nycomed, Oslo, Norway) as previously described (3). During the centrifugation (1,000 *g*, 10 min, 15°C) the cells were stained with Texas Red conjugated wheat germ agglutinin (WGA/TxR: 1 μg/ml; Molecular Probes Inc., Eugene, OR). The low density (LD) cells ($\rho \leq 1.078$ g/cm^3) were collected, washed once in HH, resuspended in HH containing 5% heat-inactivated FCS (Rehatuin, Purchase, NY) and 0.2% sodium azide (HSA; HH + Serum + Azide) and stained with FITC conjugated mAb 15-1.4.1 (15-1.4.1/FITC). mAb 15-1.4.1 is directed against mature murine BM monocytes and granulocytes and their immediate precursors, but does not react with day 14 CFU-S (31). After 30 min on ice the cells were washed once with HSA. The cells were resuspended in HH + 5% FBS (HS: HH + Serum) at a concentration of 2–4 × 10^6 cells/ml and analyzed and sorted.

Sorted cells were subsequently stained with Rh123 (Eastman Kodak Co., Rochester, NY) and at the same time WGA/TxR was removed from the cells by incubating the cells at 37°C for 20 min in 0.2 M *N*-acetyl-D-glucosamine + 5% FBS and 0.1 μg/ml Rh123 (9). After two washes with HH the cells were resuspended in HS, analyzed and resorted.

Light Activated Cell Sorting. Stained cells were analyzed and sorted with an unmodified dual laser equipped FACStar® Plus flow cytometer (Becton-Dickinson and Co., San Jose, CA) in the following way: first, the WGA/TxR positive cells were selected by setting electronic gates around these cells using the FACStar® software package (Becton Dickinson and Co.). Second, the "blast cells" were selected by gating out the WGA positive (WGA$^+$) cells with intermediate to high forward light scatter (FLS) and low to intermediate side light scatter (SSC) intensities (3). Finally, the 15-1.4.1 negative WGA$^+$ blast cells were selected. The selected WGA$^+$/15-1.4.1$^-$ LD blast cells were sorted (normal-R mode). During the sort the cells were kept at 4°C.

After removal of WGA/TxR conjugates and staining with Rh123 (see above) the cells were run through the sorter again. Cells in the same FCS/SSC window as during the first sort were sorted on basis of their Rh123 fluorescence intensity into a Rh123 dull and Rh123 bright population.

The first laser was operated at 488 nm and 200 mW and generated FLS, SSC, FITC, and Rh123 signals. The FLS signal was used to trigger the electronics of the system. FLS and SSC were amplified linearly. FITC and Rh123 signals were measured through a DF 530/30 filter and were amplified logarithmically. TxR fluorescence was generated by the second laser operating in all lines mode at 2W and used to pump a dye laser filled with Rhodamine 6G to generate light of 590 nm and 75 mW. TxR signals were measured through a DF 630/22 filter and amplified logarithmically.

Liquid Cultures. Sorted Rh123 dull and Rh123 bright stem cells were seeded into 24-well tissue culture plates (Costar, Cambridge, MA) and cultured in the presence or absence of HGF(s) at a final volume of 2 ml/well. Cells were cultured in McCoy's 5A medium (Gibco Laboratories) supplemented with 20% heat inactivated FBS, 50 μM 2-ME, essential + nonessential amino acids, sodium-pyruvate, vitamins, glutamine, penicillin, and streptomycin. Cells were incubated at 37°C in a fully humidified atmosphere of 6.5% CO_2 and 7% O_2 in air. After the indicated number of days the cells were harvested, washed once with HH, counted (trypan blue), and injected into lethally irradiated recipients for determination of the day 14 CFU-S content.

Proliferation Assays. Proliferation of purified stem cells was assessed in [^{3}H]TdR incorporation assays. Cells were cultured in supplemented McCoy's 5A medium in the presence or absence of HGF(s) in 96-well flat-bottom tissue culture plates (Costar) at a final volume of 200 μl/well. Cells were incubated at 37°C in a fully humidified atmosphere of 6.5% CO_2 and 7% O_2 in air for 96 h. Subsequently, the cells were pulsed with 2 μCi per well of [^{3}H]TdR (81 Ci/mmol; Amersham Corp., Arlington Heights, IL) and incubated for an additional 16 h. Samples were harvested onto glass fiber filters and counted using Geiger-Müller gas ionization (Packard Matrix 96 counter; Packard Instrument Company, Meriden, CT).

Hematopoietic Growth Factors. Murine rIL-3 and human rIL-1α were used at a final concentration of 250 ng/ml. Murine rMGF

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was used at a concentration of 285 ng/ml, unless stated otherwise. Murine rMGF was purified from yeast supernatant as described (32). All hematopoietic growth factors were manufactured by Immunex Corp. (Seattle, WA).

Statistical Analysis. The SD of the CFU-S counts were calculated using Poisson distribution statistics. Production of day 14 CFU-S in suspension cultures was tested for statistical significance using the Wilcoxon rank-sum test.

Results

Purification of Hematopoietic Stem Cells. A new purification procedure for the enrichment of day 14 CFU-S was developed that is relatively fast and simple and consists of a density cut followed by two cell sorting runs. In the first sort the cells were selected on the basis of their light scatter characteristics (FLS and SSC), WGA/TxR, and mAb 15-1.4.1/FITC-fluorescence intensities (Fig. 1, *A–C*). In the second sort the cells were selected on the basis of their Rh123 fluorescence intensity (Fig. 1, *D*). With this purification procedure high enrichments of day 14 CFU-S could be obtained (Table 1). The Rh123 dull/WGA$^+$/15-1.4.1$^-$/LD blast cells (Rh123 dull cells) were on average, 237-fold enriched for day 14 CFU-S, 1.8 times more enriched than the Rh123 bright WGA$^+$/15-1.4.1$^-$/LD blast cells (Rh123 bright cells). Rh123 bright cells are generally considered to be less primitive than the Rh123 dull cells (7–11, 18, 19). In the present study we did not determine the long term reconstitutive capacity and marrow and thymus repopulating ability of the sorted Rh 123 fractions. Previously the Rh 123 dull fraction, enriched similarly for day 12–14 CFU-S using a number of different purification protocols, has been shown to contain stem cells with long term reconstituting capacity and marrow and thymus repopulating ability, whereas the Rh123 bright fraction predominantly consisted of more mature in vivo and in vitro clonogenic stem cells (7–11, 18, 19, P. de Vries, unpublished data).

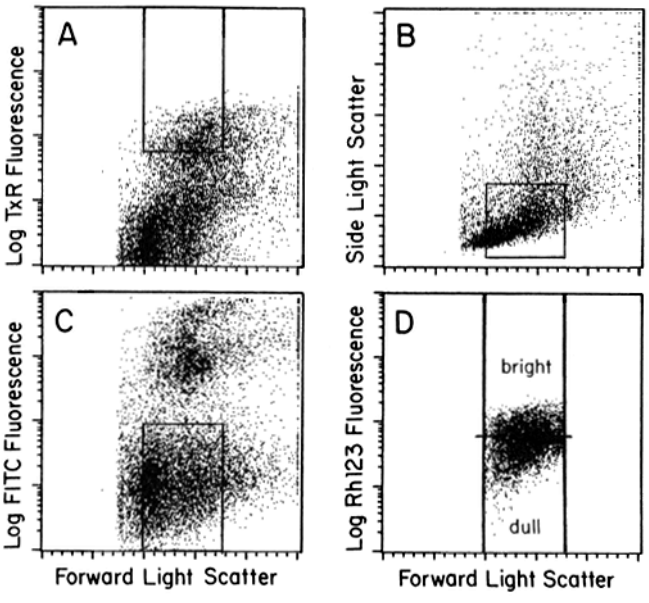

Figure 1. Flow cytometric selection of hemopoietic stem cells. (*A*) selection of WGA/TxR$^+$ cells. (*B*) selection of "blast" cells on the basis of FLS and SSC intensities. (*C*) selection of 15-1.4.1/FITC$^-$ cells. (*D*) Rh123 fluorescence distribution of the cells selected in steps (*A*) (*B*), and (*C*). The dot plots show only nucleated low density mouse BM cells. A FLS threshold was set to electronically gate out erythrocytes.

Table 1. *Frequency and Enrichment of Day 14 CFU-S in Sorted Cell Populations*

Cell suspension	Percent of nucleated BM cells	Day 14 CFU-S per 10^5 cells	Enrichment factor
Unseparated bone marrow	100.00	35.4 ± 3.1	1
Rh123 dull cells	0.03	8,400 ± 864	237
Rh123 bright cells	0.03	4,666 ± 950	132

The figures represent the mean ± SD of 15 experiments in which 150 sorted Rh123 dull and 150 Rh123 bright cells or 2.5–3 × 10^4 unseparated nucleated BM cells were injected into groups of 10 lethally irradiated syngeneic recipients.

MGF Stimulates the Proliferation of Purified Stem Cell Fractions. We studied the effect of MGF on the purified stem cell suspensions in a [^{3}H]TdR assay. The results of studies in which Rh123 dull and Rh123 bright fractions were cultured in the presence of different concentrations of MGF are depicted in Fig. 2. It is apparent that both the Rh123 dull and Rh123 bright stem cells proliferated in the presence of MGF alone. The Rh123 bright stem cells were more responsive to MGF than the Rh123 dull stem cells. The dose response curves indicated that maximal proliferation of Rh123 bright and Rh123 dull stem cells was obtained with the same concentration of MGF. However, the ^{3}H-cpm maximum of Rh123 bright cells was almost four times higher than that of Rh123 dull cells (Fig. 2).

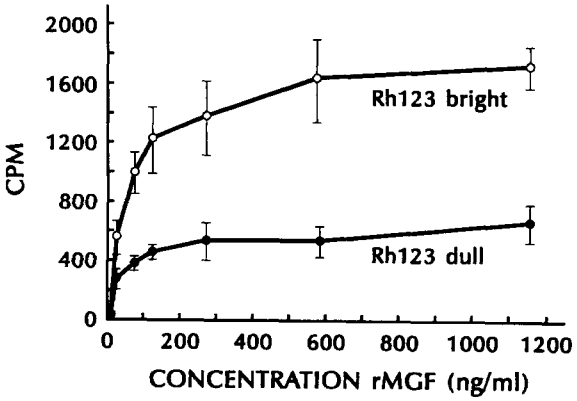

Figure 2. rMGF stimulates the proliferation of purified stem cell fractions in a dose dependent way. Cell input at the initiation of the experiment: 705 Rh123 dull and 728 Rh123 bright cells per well. Each point represents the mean number of cpm from four wells ± SD.

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MGF Acts Synergistically with IL-3 and IL-1α. We showed that MGF alone could stimulate the proliferation of purified stem cell suspensions. We were interested to see if such stimulation by MGF could be enhanced by the addition of other HGFs. We therefore compared the [^{3}H]TdR incorporation of purified stem cell fractions when cultured with MGF, IL-3, or IL-1α either alone or in combination.

Rh123 bright cells were more responsive than Rh123 dull cells when stimulated with any of the factors alone, particularly in the case of IL-3 stimulation (Table 2). The proliferation induced by MGF alone in both cell fractions was less than that induced by IL-3 alone. MGF and IL-3 in combination resulted in a greater than additive [^{3}H]TdR incorporation compared with cultures with either factor alone. By comparing the expected cpm for MGF and IL-3 alone with that obtained for the combination, the synergistic effect of MGF and IL-3 in the Rh123 dull fraction was about five times greater than in the Rh123 bright fraction (Table 2). Similarly, MGF and IL-1α also acted synergistically on both Rh123 fractions. The synergistic effect of MGF and IL-1α was approximately two times greater on Rh123 dull stem cells than on Rh123 bright stem cells. The synergistic effect of MGF with IL-3 and IL-1α was identical for the Rh123 dull cells, whereas in the Rh123 bright fraction the synergy between IL-1α and MGF was 2.5 times greater than between MGF and IL-3.

MGF Causes the Production of Nucleated Cells. Purified stem cell fractions were cultured for various times in MGF, IL-3, or IL-1α alone, or in MGF + IL-3 or IL-1α. At the indicated times (after 4, 7, 11, and 14 d) the cells were collected, counted and injected into lethally irradiated syngeneic recipients to determine the day 14 CFU-S content (see below).

MGF alone significantly stimulated the proliferation of Rh123 dull and Rh123 bright cells (Fig. 3) as was also observed in the [^{3}H]TdR assay. The production of viable cells in the Rh123 bright fraction was higher than in the Rh123 dull fraction. This is in agreement with the [^{3}H]TdR incorporation data and suggests that Rh123 bright cells may be more sensitive to MGF alone than Rh123 dull cells.

Table 2. *rMGF Alone and in Combination with rIL-3 or IL-1α Stimulates Proliferation of Purified Stem Cell Fractions*

	cpm	
Growth stimulus	Rh123 dull	Rh123 bright
None	15 ± 2	10 ± 5
IL-1α	20 ± 5	31 ± 13
MGF	537 ± 139	1,384 ± 229
IL-3	1,917 ± 260	12,186 ± 1,736
MGF + IL-3	51,781 ± 8,722	53,151 ± 5,812
MGF + IL-1α	13,104 ± 4,008	14,158 ± 2,024

Each point represents the mean ^{3}H-cpm of four wells ± SD. rMGF was used at a concentration of 285 ng/ml, rIL-3 and rIL-1α were used at 250 ng/ml. Cell input: 705 Rh123 dull and 728 Rh123 bright cells per well.

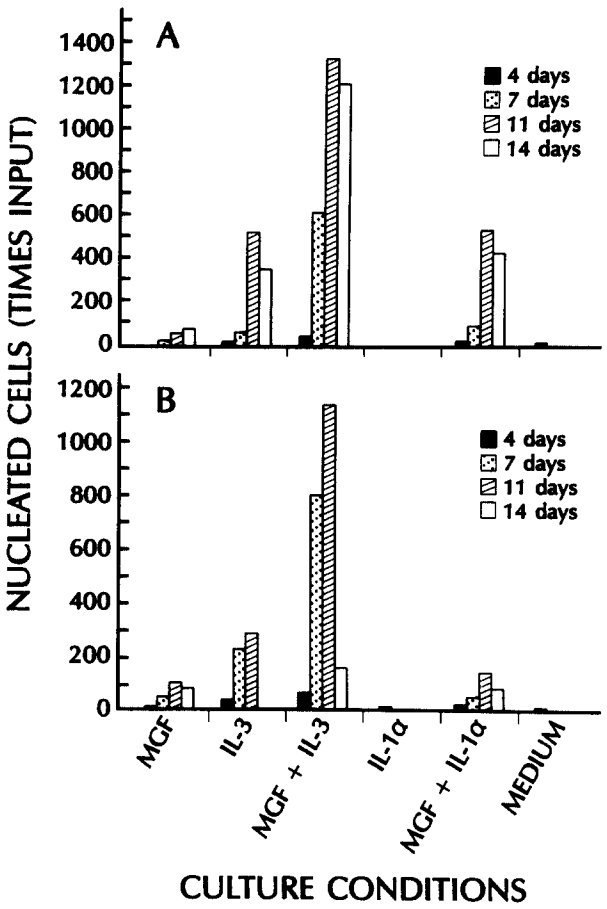

Figure 3. rMGF stimulates the production of nucleated BM cells in liquid culture by purified stem cell fractions. (*A*) Rh123 dull cells; 1277 cells seeded per well at day 0. (*B*) Rh123 bright cells; 1592 cells seeded per well at day 0.

A synergistic effect on nucleated cell production was observed between MGF + IL-3 and MGF + IL-1α. The synergistic effect was greater on Rh123 dull cells than on Rh123 bright cells, in agreement with the results obtained with the [^{3}H]TdR assay (Table 2). The production of nucleated cells in cultures with MGF alone or with MGF plus IL-3 or IL-1α started after day 4 and reached a maximum after 11 d, except in the Rh123 dull fraction cultured with MGF alone where the number was highest after 14 d (Fig. 3).

Production of Day-14 CFU-S In Vitro. Cells from the same cultures as described above were injected into lethally irradiated syngeneic recipients to determine the day 14 CFU-S content of these cultures and to find out if day 14 CFU-S were produced during in vitro culture. Culture of sorted cells with MGF alone over a 14-d culture period did not stimulate the net production of day 14 CFU-S in vitro from either Rh123 dull of Rh123 bright stem cells. However, MGF alone promoted the survival of day 14 CFU-S in vitro for up to 14 d (Fig. 4).

Culture of sorted cells with IL-3 alone did not stimulate a statistically significant net production of day 14 CFU-S in the Rh123 dull or Rh123 bright fraction. Culture of sorted stem cells with MGF + IL-3, did not result in net produc-

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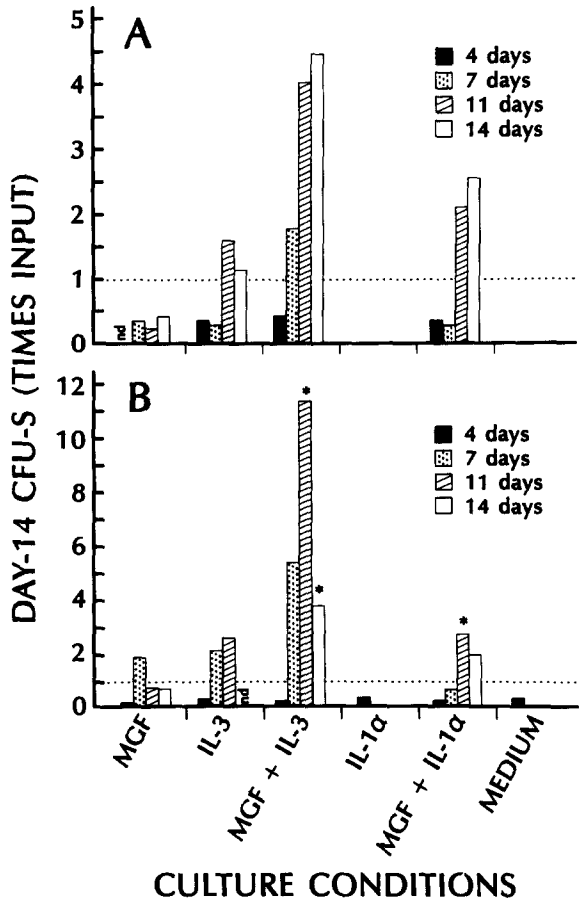

Figure 4. Effect of rMGF on day 14 CFU-S numbers in Rh123 dull and bright fractions after several days in liquid culture. At the start of the culture 116 Rh123 dull (*A*), and 83 Rh123 bright (*B*) day 14 CFU-S were seeded per well. For determination of day 14 CFU-S contents recipient mice (7 to 10 per group) were injected with 5,000 cultured cells. The number of day 14 CFU-S in the bars indicated by an asterisk (*) were significantly different from the input numbers of day 14 CFU-S at the 5% level using the Wilcoxon rank-sum test. nd; not done.

tion of day 14 CFU-S in the Rh123 dull and bright fractions 4 d after the onset of the cultures. However, from day 4 onward, day 14 CFU-S were produced in a synergistic fashion. In the Rh123 dull fraction, a 4.4-fold maximum increase in day 14 CFU-S was observed after 14 d in culture, however, this production was not statistically significant. In the Rh123 bright fraction, a larger and statistically significant production (11.6-fold maximum increase after 11 d) of day 14 CFU-S was observed in cultures containing MGF and IL-3 (Fig. 4).

MGF and IL-1α also acted synergistically in the production of day 14 CFU-S in both Rh123 fractions. The production of day 14 CFU-S with this factor combination was first observed after 11 d in culture, 4 d later than was observed in cultures with MGF + IL-3 indicating a heterogeneity in the sorted cell suspensions. After 11 and 14 d of culture the day 14 CFU-S numbers in MGF + IL-1α containing cultures in the Rh123 dull and Rh123 bright fraction were 2 to 2.5 times higher than the input numbers at the start of the cultures (Fig. 4). The increase in the number of day 14 CFU-S in the Rh123 bright fraction after 11 d in culture with MGF and IL-1α was statistically significant, whereas the production after 14 d and in the Rh123 dull fractions appeared not to be statistically different from the number of day 14 CFU-S at the start of the cultures. Thus, the Rh123 bright cells appeared to be more responsive to MGF in combination with IL-3 or IL-1α than the Rh123 dull cells with respect to their capacity to generate day 14 CFU-S, at least during the 14-d period of these experiments. However, the synergistic effect of MGF in combination with IL-3 or IL-1α was greater in the Rh123 dull fraction. It should be noted, that in the Rh123 dull fraction, despite the fact that the increase in day 14 CFU-S was not statistically significant, the trend was continuing upward even at day 14 in culture. Thus, longer term cultures may be needed to observe production of day 14 CFU-S in the Rh123 dull population. This would be in keeping with the generally assumed more primitive nature of Rh 123 dull day 14 CFU-S than the Rh 123 bright day 14 CFU-S.

Discussion

In this report we demonstrate a net production of day 14 CFU-S grown in liquid cultures of purified hematopoietic stem cells (day 14 CFU-S). It can be seen from our data that in order to achieve this production of day 14 CFU-S, at least two HGFs were required which act in a synergistic fashion (Fig. 4). One of these factors was MGF, which is encoded by the S1 locus on mouse chromosome 10. Two other necessary HGFs for production of day 14 CFU-S in vitro appeared to be either IL-3 or IL-1α. It has been reported that IL-3 does not act on cells in the G_0 phase of the cell cycle, and therefore is not capable of acting directly on the most primitive stem cells, which are normally quiescent cells (33–36). IL-1α on its own has no direct stimulatory effects on the proliferation of primitive hematopoietic cells (37, 38, this paper), but may promote responsiveness to other later acting hematopoietins.

MGF acted synergystically with IL-3 and IL-1α on the proliferation and net production of day 14 CFU-S (Figs. 3 and 4, Table 2). The effects of MGF, certainly early after the onset of the cultures, were most likely directly on the purified stem cells themselves, because of the low numbers of cells seeded into these cultures and because cells such as endothelial cells, fibroblasts, macrophages, and T lymphocytes, known to produce HGFs, were removed by the sorting procedure (see Fig. 1).

A 237-fold mean enrichment of Rh123 dull day 14 CFU-S and an 132-fold enrichment for Rh123 bright day 14 CFU-S was obtained with the purification procedure described here (Table 1). If the number of day 14 CFU-S in unseparated BM is considered, the enrichment factors obtained are in good agreement with previously published procedures (3–5, 7, 39). With the new separation procedure it is possible to purify hematopoietic stem cells (day 14 CFU-S) without requirement for any fluorescent probes or magnetic-bead conjugated antibodies bound to the cell surface. Using the technique described in this report loss of stem cells due to opsonization

by cells in the reticuloendothelial system is avoided (40). In addition, the new procedure is widely applicable and not restricted to cells of mouse strains with certain specific haplotypes (31), in contrast to other procedures which are either MHC class-1 or Ly-6^b haplotype restricted (3–5, 7).

In this study we did not determine the spleen seeding efficiency (41) of the sorted stem cell fractions neither before nor after in vitro culture. If we assume like others (3, 5) a spleen seeding efficiency of 10% it can be calculated from Table 1 that the Rh 123 dull fraction averaged 84% day 14 CFU-S and the Rh123 bright fraction 47% day 14 CFU-S. It has been demonstrated before, but not in this study, that at least a proportion of the Rh123 dull cells are capable of reconstituting the complete hematopoietic system (myeloid and lymphoid) of lethally irradiated recipients and are therefore considered to be PHSC (7, 10, 11, 15), whereas the Rh123 bright fraction consists predominantly of less primitive committed stem and progenitor cells. Ploemacher and Brons (11) and others (7, 18) have proposed the existence of a more primitive stem cell than the day 12–14 CFU-S present in the Rh123 dull fraction. This cell (pre–CFU-S) does not form spleen colonies within 2 wk after transplantation, but is considered to be the precursor for day 14 CFU-S (16). In the Rh123 dull fraction no statistically significant production of day 14 CFU-S was observed with MGF + IL-3 or with MGF + IL-1α, although an increased production of day 14 CFU-S was observed with time for both HGF combinations (Fig. 4). The lack of a statistically significant production of day 14 CFU-S might be partially due to the rather large variation in some of the day 14 CFU-S numbers in the Rh123 dull cultures. In addition, the lack of a significant production of day 14 CFU-S might be explained by the assumption that a more primitive hematopoietic stem cell type like the pre–CFU-S is present in the Rh123 dull fraction. It may take longer than 14 d in culture for such a primitive cell to produce progeny capable of giving rise to day 14 CFU-S. It is not known if the observed production of day 14 CFU-S in the studies reported here was due to self-renewal of day 14 CFU-S or to maturation divisions of pre–CFU-S.

Rh123 bright cells were more responsive to MGF alone than the Rh123 dull cells (Table 2, Figs. 2–4). In contrast, Rh123 dull cells were more responsive to the synergistic effects of MGF in combination with IL-3 or IL-1α (Table 2, Figs. 3 and 4). These findings, together with the notion that cells responsive to synergistic factors are more primitive than cells that do not (34), suggest that the Rh123 dull stem cells are more primitive cells than the ones in the Rh123 bright fraction. To determine whether MGF acts on the ultimate PHSC, or on a more mature stem cell type like the day 14 CFU-S, cultured cells from both Rh123 fractions need to be assayed in vivo for their long-term reconstitutive capacity.

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Address correspondence to Dr. Peter de Vries, Department of Experimental Hematology, Immunex Corporation, 51 University Street, Seattle, WA 98101.

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