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A novel *in vitro* assay for murine haematopoietic stem cells

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Summary Study of the biology of haematopoietic stem cells is crucially dependent on the availability of suitable *in vitro* assays. Existing assays have suffered from the fact that they detect small subcompartments of the total stem cell compartment. This limits experiments where it is required to assay a high proportion of stem cells, e.g. the enumeration of stem cell numbers under varying conditions or the identification and purification of stem cell regulators. We describe an *in vitro* assay which shows macroscopic colony formation and limited self-renewal capacity *in vitro*. The detected cell (CFU-A) has a low cycling status in normal bone marrow (NBM) and responds to known stem cell regulators. The incidence (100–200 per 10^5 in NBM), the proliferative characteristics under stress and some of the physical properties are similar to stem cells detected by colony formation after transplantation into lethally irradiated recipients (CFU-S). These data indicate that our assay detects a high proportion of haematopoietic stem cells *in vitro*. This will facilitate experiments on stem cell behaviour which have previously been difficult to conduct.

In haematopoiesis, pluripotent stem cells give rise to different types of lineage-restricted progenitor cells which form all types of differentiated blood cells (Quesenberry, 1983). Stem cells are primarily involved in some of the major disorders of proliferation, including chronic myelogenous leukemia (Fialkow *et al.*, 1977), acute nonlymphocytic leukemia (Fialkow *et al.*, 1987) and in some cases of acute lymphoblastic leukemia (Tachibana *et al.*, 1987). It is therefore important to understand the control mechanisms underlying stem cell behaviour. In order to dissect the intricate regulatory network of haematopoiesis the general approach has been to identify extracellular regulatory mediators and to analyze their effects upon different subsets of cells. To this end the use of *in vitro* assays for the growth and proliferation of haematopoietic progenitor cells has proved invaluable and has allowed the identification of a number of specific regulatory molecules (Metcalf, 1986; Clark & Kamen, 1987). The search for regulators affecting stem cells has not been equally successful which is mainly due to the lack of suitable *in vitro* assays. A number of *in vivo* and *in vitro* assays have been described which detect cells with properties of haematopoietic stem cells. An *in vivo* assay (CFU-S) (Till & McCulloch, 1961) has given valuable information about the organisation of the stem cell compartment but is somewhat cumbersome for the identification and characterisation of stem cell regulators. Several *in vitro* assays (Johnson & Metcalf, 1977; Metcalf *et al.*, 1979; Bradley & Hodgson, 1979; Humphries *et al.*, 1981; Nakahata & Ogawa, 1982) also detect cells with stem cell properties but the efficiency is ten- to hundredfold lower compared with the *in vivo* assay (CFU-S) implying the detection of only small subcompartments of the total stem cell compartment.

Here we describe an *in vitro* assay detecting a cell (CFU-A) which gives rise to at least four different blood cell lineages (monocytic, granulocytic, erythroid, megacaryocytic) and shows limited self-renewal capacity *in vitro* (Pragnell *et al.*, 1988). It is affected by regulators previously shown to be specific for CFU-S (Lord *et al.*, 1976; Lord *et al.*, 1977) and shows a frequency in normal bone marrow comparable with CFU-S. The assay employs two defined growth factors, granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF, CSF-1) (Pragnell *et al.*, 1988). We report on experiments characterizing the proliferative behaviour of the CFU-A *in vivo* and *in vitro* and on some physical characteristics which may be useful for the purification of CFU-A. The possible applications of this assay are discussed.

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Materials and methods

Cell suspensions

Bone marrow or spleen cells were obtained from 8- to 12-week-old NIH/01a mice kept in the animal facility of the Beatson Institute. Femurs were flushed either with Hank's salt solution (HBSS) containing 5% FCS or with alpha-modified Eagle's medium (alpha-MEM). Cells were then washed, counted and kept on ice until use. To obtain spleen cell suspensions, spleens were taken out, minced with scissors and pressed through a 100 μm sieve. Cells were washed twice, counted and kept on ice.

CFU-A assay

The assay was performed as described previously (Pragnell *et al.*, 1988). In brief, appropriate numbers of cells to give 10 to 20 colonies (e.g. 10^4 for normal bone marrow) were plated in 4 ml alpha-MEM with 25% FCS, 2 mM glutamine and 0.3% agar on top of 4 ml of the same medium containing 0.6% agar, 10% conditioned medium (CM) of the cell line L-929 (as a source of M-CSF, CSF-1) and 10% CM of the cell line AF1-19T (as a source of GM-CSF) into a 60 mm plastic dish. Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO_2, 5% O_2, 85% N_2 for 11 days. These conditions were found to facilitate the growth of mixed colonies. Since preliminary experiments had shown that the incubation in 5% CO_2 in air gave the same number and size of CFU-A colonies under different conditions we used these conditions for some experiments where mixed colony formation was not required. Colonies with a diameter $\geq$2 mm were scored as CFU-A, as described by Pragnell *et al.* (1988). The number of CFU-A colonies was linearly related to the number of cells seeded, suggesting that the assay is clonal (Pragnell *et al.*, 1988). Experiments using recombinant GM-CSF (kindly donated by Dr J. De Lamarter, Biogen, Geneva) instead of AF1-19T CM gave the same number and slightly smaller colonies under varying conditions and the addition of antiserum against CSF-1 (a gift of Dr E.R. Stanley, New York) abolished colony formation almost completely (Pragnell *et al.*, 1988). These observations suggest that two defined growth factors (GM-CSF, CSF-1) are sufficient to allow colony formation of CFU-A *in vitro*.

Irradiation and CFU-S assays

Mice were lethally irradiated with 9.5 Gy gamma-rays using a Cobalt-60 source. For repopulation studies and CFU-S assays appropriate numbers of cells were injected in 0.5 ml

aliquots. For CFU-S assays spleens were taken out 10–12 days after transplantation and fixed in Bouin's fluid (Till & McCulloch, 1961). Colony counts in animals without transplant were below one colony per spleen. The seeding factor (f) was determined as published previously (Lahiri *et al.*, 1970). In short, normal bone marrow cells were injected into lethally irradiated mice and, in parallel experiments, the number of CFU-S and CFU-A was determined in these cell suspensions. Twenty-four hours later the number of CFU-S and CFU-A in a fraction of the recipient spleen was measured and the f-factor calculated.

Progenitor assays

Granulocyte-macrophage progenitor cells (GMCFC) were assayed by culturing 5×10^4 cells in 30 mm plastic dishes containing 1 ml of 0.3% agar in alpha-MEM supplemented with 25% FCS and 2 mM glutamine. CM from L-929 cells as a source of M-CSF at a final concentration of 10% or recombinant GM-CSF (Biogen, Geneva) at a final concentration of 400 pg/ml was used as colony-stimulating activity. The cultures were incubated at 37°C in 5% CO_2 in air and counted on day 7.

Density gradients

Using two stock solutions of Percoll (Pharmacia, Uppsala, Sweden) in HBSS (300 mosmol kg^{-1}, pH 6.7) containing 5% FCS of 1.050 g cm^{-3} and 1.085 g cm^{-3} a linear density gradient was created with a two-chamber gradient former. 4×10^7 bone marrow cells were suspended in 4 ml of the lighter stock solution, given on top of the gradient and subjected to centrifugation (1,200 *g*, 20 min). About twenty fractions were collected from the top, washed once and the total cells were counted. Equal numbers of each fraction were then assayed for CFU-A, CFU-S and GM-CFC.

Flow cytometry and cell sorting

A slightly modified FACS II (Becton Dickinson, Mountain View, California, USA) was used. The laser was tuned at 488 nm (0.4 W). Cells suspended in HBSS (300 mosmol kg^{-1}, pH 6.7) containing 1% FCS, 60 $\mu g\,ml^{-1}$ penicillin and 100 $\mu g\,ml^{-1}$ streptomycin at $5 \times 10^6\,ml^{-1}$ were analyzed and sorted into 5 ml tubes containing HBSS with 20% FCS and antibiotics. For binding studies, wheat germ agglutinin (WGA-FITC, Sigma, Poole, UK) was added to a final concentration of 2 $\mu g\,ml^{-1}$.

Results

Haematopoietic stem cell properties of CFU-A

Haematopoietic stem cells are operationally defined as cells which give rise to more than 3 different blood cell lineages and which show self-renewal capacity. Using the conditions described under **Materials and methods** we cultured CFU-A cells and picked individual colonies between day 6 and 9 and examined them morphologically on stained cytospins. We found macrophages, granulocytes, early erythroid cells, megakaryocytes and cells with a blast-like morphology in varying proportions which shows the ability of CFU-A to form several blood cell lineages. To test for self-renewal capacity, we picked individual day 7 CFU-A colonies and replated them into new dishes. A high proportion of cells from primary colonies (0.2%–0.6% of all plucked cells, 5%–60% of all plucked colonies) gave rise to secondary colonies of which about 10% were of CFU-A type and the rest of progenitor type, showing that CFU-A do self-renew under these conditions (Pragnell *et al.*, 1988). Another important feature of stem cells is their low cycling status in normal bone marrow. We measured the fraction of CFU-A in S-phase using a suicide technique with cytosine arabinoside. We found that a low proportion of ~8–15% of CFU-A are in S-phase in normal bone marrow.

Table I summarizes different properties of CFU-A, CFU-S and GM-CFC. Several regulators have been described which affect exclusively CFU-S and not GM-CFC (Lord *et al.*, 1976; Lord *et al.*, 1977). We found that CFU-A respond to these regulators in a manner similar to CFU-S (Pragnell *et al.*, 1988). The frequency of CFU-A in normal bone marrow is about 150 to 200 per 10^5 cells. Assuming a seeding efficiency for CFU-S of ~7%, we find about 200 to 300 CFU-S per 10^5 in normal bone marrow. In conclusion, the data show that CFU-A have properties in common with haematopoietic stem cells and represent a cell compartment which overlaps largely with the cells detected in the CFU-S assay.

Proliferative characteristics in vivo *and* in vitro

In order to characterize the behaviour of the CFU-A under conditions of proliferative stress, we have transplanted different numbers of normal bone marrow cells into lethally irradiated syngenic recipients and determined the number of CFU-A in the spleen at different times after transplantation (Figure 1). The number of CFU-A increases exponentially over the first 14 days and then reaches a level which equals the preradiation level of normal mice. The apparent doubling time is 17.7 h, 18.3 h and 16.9 h for a graft size of 10^6, 10^5 and 3×10^4 cells, respectively, which lies within the range which has been reported for CFU-S, ranging from 16 h to 25 h depending on the source of CFU-S (Schofield, 1970). For a given time after transplantation the contents of CFU-A in the spleen is roughly proportional to the number of CFU-A injected. In the first 14 days of repopulation the exponential increase is independent of the graft size as shown previously for CFU-S (Schofield & Lajtha, 1969), which makes it likely that the proliferative stimulus is not mediated by the transplanted cells but by the microenvironment of the host. From these results we cannot tell whether the exponential increase in CFU-A reflects self-renewal or proliferation and differentiation of a more primitive cell, or the balance between self-renewal and differentiation of the CFU-A itself. It seems reasonable to assume that some self-renewal of CFU-A may take place *in vivo* as we have shown some self-renewal under *in vitro* conditions which seem to favour differentiation over self-renewal.

We have also studied the proliferation kinetics of cells deriving from CFU-A *in vitro*. Normal bone marrow cells were seeded into the CFU-A assay and colonies were collected 5, 7 and 10 days after initiation of the culture. The number of cells per colony was determined for each single colony (colonies on day 5 were pooled due to the low number of cells). Two groups of colonies can be distinguished on day 10, one group ranging from $1–6 \times 10^4$ cells per colony and the other group ranging from $1–4 \times 10^3$ cells per colony (data not shown). The former group represents the CFU-A, the latter group are likely to represent progenitor cell colonies. Assuming that there is no proliferative lag after initiation, and that proliferation slows down late in culture, we calculated the doubling time by fitting a straight through the number of cells per CFU-A colony by least squares from initiation of the culture to day 7. The resulting doubling time was 12.5 h.

Density properties

To determine the buoyant density of the CFU-A a linear density gradient was created ranging from 1.055 g cm^{-3} to 1.085 g cm^{-3} and normal bone marrow cells were subjected to isopycnic centrifugation. Fractions were collected and assayed for the total number of cells and the number of CFU-A, CFU-S and GM-CFC (Figure 2). The majority of CFU-A ranges in density from 1.061 g cm^{-3} to 1.073 g cm^{-3}, with peak values at 1.064 g cm^{-3}. While the total cells range from 1.052 g cm^{-3} to 1.080 g cm^{-3} ~80% of them have a density higher than 1.070 g cm^{-3}. The CFU-S show a similar profile to the CFU-A with a peak at 1.064 g cm^{-3}. The progenitor cells have a density distribution which is slightly

Table I Comparison of CFU-A, CFU-S and GM-CFC

	CFU-A	*CFU-S*[a]	*GM-CFC*
Frequency per 10^5:			
bone marrow	148	27	156
spleen	21	7	18
Seeding efficiency	7.8%	8.4%	1.0%
Colony type	M, G, E, Meg	M, G, E, Meg, B	M, G
Self-renewal	*in vitro*	*in vivo*	no
Cells in S-phase[b]:			
normal BM	8%	6%	30%
normal BM + S	49%	34%	37%
PH − BM	28%	34%	31%
PH − BM + I	11%	4%	27%

[a]The differentiation potential (Till & McCulloch, 1961; Lala & Johnson, 1978) and the self-renewal capacity (Siminovitch *et al.*, 1963) of CFU-S have been shown by others; [b]Details of the cycling experiments are published elsewhere (Pragnell *et al.*, 1988).

Abbreviations: M = Macrophage, G = Granulocyte, E = Erythrocyte, Meg = Megacaryocyte, B = B-Lymphocyte, BM = Bone marrow, PH-BM = Bone marrow after phenylhydrazine treatment, S = Stem cell stimulator (Lord *et al.*, 1977), I = Stem cell inhibitor (Lord *et al.*, 1976).

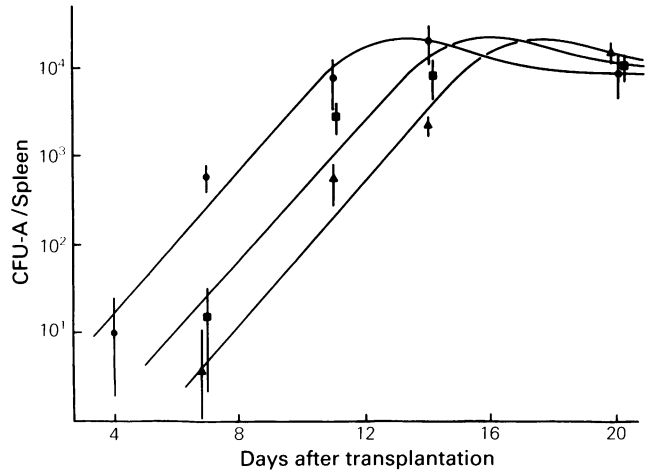

Figure 1 Repopulation of CFU-A. Lethally irradiated mice were injected with 10^6 (circles), 10^5 (squares) or 3×10^4 (triangles) normal bone marrow cells. Different times after transplantation the number of CFU-A in the spleens were determined. Five spleens were pooled for each point. Bars represent s.e.m. of the counts of 4 dishes. The straight parts of the curves were fitted by least squares.

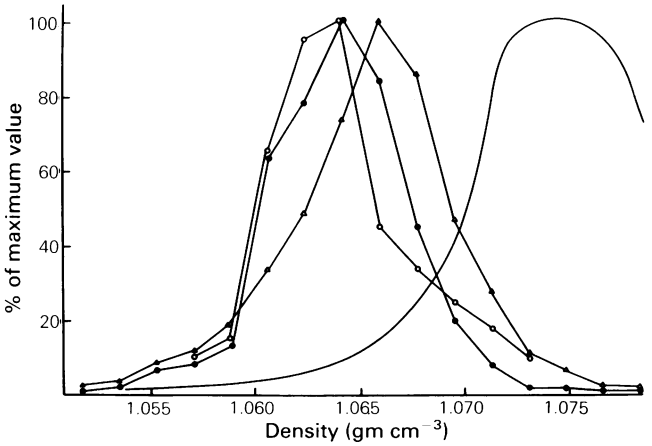

Figure 2 Density properties. Normal bone marrow cells were subjected to centrifugation on a density gradient and fractions of increasing density were obtained. Total cells (drawn line) were determined and the fractions were assayed for CFU-A (closed circles), CFU-S (open circles) and GM-CFC (open triangles). Values represent number of CFC per fraction and are given as percentage of the maximum value.

broader and shifted to a higher density with a peak value at 1.066 g cm^{-3}, as has been shown by others (Bol *et al.*, 1977). For purification experiments of CFU-A we have chosen to use a discontinuous gradient with a cut-off value of 1.070 g cm^{-3} which leaves ~80% of CFU-A but just 20% of total cells giving an enrichment factor of 4–5 (data not shown).

Flow cytometric properties

Cell sorters are now routinely used to obtain highly enriched populations of specific cell types. We have determined some flow cytometric properties of the CFU-A which can be used for further purification studies. The forward light scatter (FLS) is regarded as a measure for cell diameter (Engh & Visser, 1979). Figure 3a shows the distribution of total cells and of CFU-A for the FLS. The CFU-A have a high FLS (Ch 120–Ch 240) and are mainly found in the third peak containing preferentially granulocytes and cells with a blast-like morphology. The perpendicular light scatter (PLS) is thought to be a measure of the internal structure of a cell (Engh & Visser, 1979). Figure 3b shows that the CFU-A have a low to medium PLS. Since CFU-S have a high number of receptors for wheat germ agglutinin (WGA) Visser *et al.*, 1984) we determined the binding of WGA to CFU-A. In Figure 3c it can be seen that the CFU-A are found in the high binding fraction of cells. In comparison with the published data on CFU-S and GM-CFC (Engh & Visser, 1979; Visser *et al.*, 1984; Lord & Spooncer, 1986) the CFU-A appears not to be different in FLS and PLS to either CFU-S or GM-CFC. CFU-A might have, in common with CFU-S, a slightly higher number of WGA-receptors. Taking together all three flow cytometric parameters we have been able to enrich about 5 to 10-fold for CFU-A in the first instance. When a density cut and two rounds of sorting (Lord & Spooncer, 1986) were used as additional procedures we obtained a 40-fold enrichment (cloning efficiency 4%). In future, we hope to identify the CFU-A with more specific surface markers. In conjunction with DNA content measurements of these cells and the employment of multiparameter analysis (Hollander *et al.*, 1988) this approach could be used as a quick assay for the purification of regulators affecting stem cell proliferation.

Discussion

Several *in vitro* assays have been described which detect subcompartments of the whole stem cell compartment. Some of these assays, like the blast cell colonies (Nakahata &

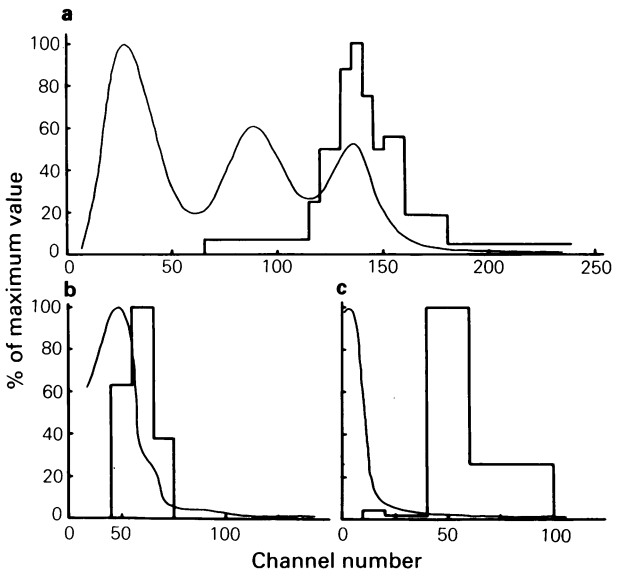

Figure 3 Flow cytometric properties. Normal bone marrow cells were sorted on a light-activated cell sorter (FACS II) according to forward light scatter (a), perpendicular light scatter (b) or binding of WGA-FITC (c). Fractions of increasing intensity were obtained, the number of total cells (drawn line) and of CFU-A (bars) in each fraction were determined. The values are given as percentage of maximum value.

Ogawa, 1982) or the high proliferative potential colony-forming cells (HPP-CFC) (Bradley & Hodgson, 1979) detect cells which are possibly more primitive than the cell detected in our assay. These assays are useful in order to characterize the detected cell within the hierarchy of stem cells and to gain knowledge about the organisation of the stem cell compartment. But they are seriously limited in questions concerning the majority of stem cells, like the enumeration of stem cells under varying conditions or the identification of stem cell regulators. It is possible to approach these questions using our assay which seems to detect a high proportion of the heterogeneous stem cell compartment.

For example, we have been interested in the pathogenesis of diseases caused by different murine leukemia viruses. The malignant histiocytosis sarcoma virus (Franz *et al.*, 1985) causes a disseminated histiocytosis and a severe anaemia. We have measured the levels of stem cells (CFU-A and CFU-S) in infected mice and have found a serious ablation of stem cells in the final stages of the disease (Eckmann & Pragnell, 1987). We are currently investigating if stem cells are primarily affected by determining the differentiation pattern of mixed colonies *in vitro* (CFU-A) and *in vivo* (CFU-S) and trying to relate the results to expression of viral proteins. Since CFU-A colonies contain a fairly high number of cells (ca. 5×10^4 per colony) it should be possible to determine molecular changes in individual colonies on a clonal basis and to relate these to altered differentiation potential.

A different area of application is the identification of stem cell regulators. We have tested two established regulators of stem cells (Lord *et al.*, 1976; Lord *et al.*, 1977; Pragnell *et al.*, 1988) and found that their action can indeed be monitored with our assay. Recently, we have identified a cell line which is secreting an inhibitor of haematopoietic stem cells into the supernatant and we are currently purifying the protein by using the CFU-A assay (Graham *et al.*,, unpublished results). The purification of such a protein provides new insight into the control of stem cell proliferation and might also in the long-term lead to therapeutic applications in humans. As it is very likely that there is a similar protein in humans (Wright *et al.*, 1980) it is conceivable that human haematopoietic stem cells could be taken out of cell cycle which would make them relatively more resistant to cytostatic drug treatment during cancer chemotherapy.

In contrast to studies on murine CFU-S, the identification of human haematopoietic stem cells has been relatively more difficult. We have recently applied the conditions of the murine CFU-A assay to human bone marrow and find the same sort of colonies having mixed character, showing limited self-renewal *in vitro* and having a low cycling status *in vivo* (Freshney *et al.*, manuscript in preparation). This shows that the human counterpart of the CFU-A is likely to be derived from a haematopoietic stem cell. Since the frequency of this cell is comparable with human GM-CFC and murine CFU-A and CFU-S it is likely that a cell of considerable relevance within the stem cell compartment is detected by the human assay. This allows us to enumerate stem cell numbers in different conditions of disease and during drug treatment. It would also allow the identification of regulator molecules affecting proliferation and frequency of stem cells *in vitro* and, possibly, *in vivo*, leading to new therapeutic approaches.

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