Murine hematopoietic blast colony-forming cells and their progeny have distinctive membrane marker profiles

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Contributed by Donald Metcalf, September 15, 2009 (sent for review August 27, 2009)

Two distinct bone marrow-derived blast colony-forming cells can generate colonies of lineage-restricted progenitor cells in agar cultures of murine bone marrow. Both cell types selectively had a Kit$^+$ ScaI$^+$ phenotype distinguishing them from most lineage-restricted progenitor cells. Multicentric blast colony-forming cells stimulated by stem cell factor plus interleukin-6 (IL-6) (BL-CFC-S) were separable from most dispersed blast colony-forming cells stimulated by Flt3 ligand and IL-6 (BL-CFC-F) using CD34 and Flt3R probes. Multicentric BL-CFC-S cofractionated with colony-forming units, spleen (CFU-S) supporting the possibility that the 2 cells may be identical. The colony populations generated by BL-CFC-S were similar in their phenotype and proliferative capacity to progenitor cells in whole bone marrow but the progeny of BL-CFC-F were skewed with an abnormally high proportion of Kit$^-$ Flt3R$^+$ cells whose clonogenic cells tended to generate only macrophage progeny. Both blast colony populations had a high percentage of GR1$^+$ and Mac1$^+$ cells but BL-CFC-F colonies also contained a significant population of B220$^+$ and IL-7R$^+$ cells relevant to the superior ability of BL-CFC-F colony cells to generate B lymphocytes and the known dependency of this process on Flt3 ligand and IL-7. The commitment events and phenotypic changes during the generation of differing progenitor cells in blast colonies can now be clonally analyzed in a convenient in vitro culture system.

blast colonies | lineage commitment | hematopoietic stem cells

Hematopoietic stem cells and their early progeny, the spleen colony-forming cells (units) (CFU-S) are both multipotential and capable of self-generation (1, 2). However, the assay of both types of cell requires analysis of the consequences of injecting these cells into lethally-irradiated recipients. Both assays are skewed in favor of cells able to survive and proliferate in lethally-irradiated recipients and neither assay permits an accurate assessment of self-generation, the documentation of heterogeneity amongst these populations, or an analysis, at the individual cell level, of lineage commitment events during the generation of multiple lineages of maturing cells.

There is therefore an urgent need for the development of an efficient in vitro clonal culture technique able to support the clonal proliferation of multipotential, self-generating cells with properties similar to those of stem cells or, more likely, CFU-S.

Previous studies documented that 2 types of cells can be cultured from murine marrow that are able to generate colonies composed of blast cells, many of which are committed progenitor cells in various lineages (3). Multicentric blast colony-forming cells stimulated by stem cell factor (SCF) plus IL-6 (BL-CFC-S) were largely separable by fluorescence-activated cell sorting (FACS) sorting from dispersed blast colony-forming cells stimulated by Flt3 ligand (FL) plus IL-6 (BL-CFC-FL). After 7 days of incubation, BL-CFC-S blast colonies contained larger cell numbers and had a higher content of granulocyte-committed progenitor cells than did blast colonies generated by BL-CFC-FL. Conversely, although both blast colony populations could generate large numbers of macrophage-committed progenitors, dendritic cells, and T and B lymphocytes, BL-CFC-FL had a substantially higher capacity to produce both T and B lymphocytes (3). Both types of blast colony-forming cell also exhibited a low, but definite, capacity for self-generation.

In short, both types of blast colony-forming cells represent cells whose properties are similar to those of CFU-S if not to stem cells themselves. As a consequence, colony formation by blast colony-forming cells provides opportunities for analyzing the manner in which lineage commitment occurs during the generation of their progeny. The in vitro clonal cultures also allow surface marker characteristics of initiating cells and their progeny to be determined. With this information, analyses can then be performed on various mutations in hematopoietic precursor cells that result in skewing of commitment pathways with the consequent abnormal production of mature cells.

In the present baseline study on normal mice, the phenotypes of cells generating the 2 types of blast colonies were analyzed. Analyses were also performed to determine both whether the progeny produced within blast colonies reproduced the phenotype and the proliferative capacity of lineage-committed progenitors in whole mouse bone marrow.

Results

Preliminary analysis of FACS fractions of adult C57BL bone marrow showed that the lineage-positive fraction (B220$^+$, Ter119$^+$, CD4$^+$, CD8$^+$, GR-1$^+$, and Mac1$^+$) contained no clonogenic lineage-committed or blast colony-forming cells detectable in agar cultures. Fractionation of lineage-negative cells using Kit and ScaI as markers showed (Fig. 1) that approximately 10% of cells were Kit$^+$ ScaI$^-$ and only 2% of cells were Kit$^+$ ScaI$^+$. Strikingly, in cultures stimulated by SCF+ interleukin-3 (IL-3)+EPO, virtually no clonogenic cells were observed in the more numerous Kit$^-$ ScaI$^-$ or Kit$^-$ ScaI$^+$ fractions. Further, in 3 replicate culture experiments, using this same combined stimulus, a notable segregation was observed between lineage-committed progenitor cells (88% of which were Kit$^+$ ScaI$^-$) and blast colony-forming cells (78% of which were Kit$^+$ ScaI$^+$) (Fig. 1, Table 1).

Further studies on blast colony-forming cells, therefore, concentrated on lineage-negative ScaI$^+$ Kit$^+$ (LSK) populations. Although such populations contained most murine blast colony-forming cells and a very high percentage of the cells had a uniform morphology of blast cells, the populations were by no

Author contributions: D.M., A.P.N., and S.J.L.L. designed research; D.M., A.P.N., S.J.L.L., L.D.R., and S.M. performed research; D.M., A.P.N., S.J.L.L., B.P., and G.K.S. analyzed data; and D.M. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0910354106/DCSupplemental.

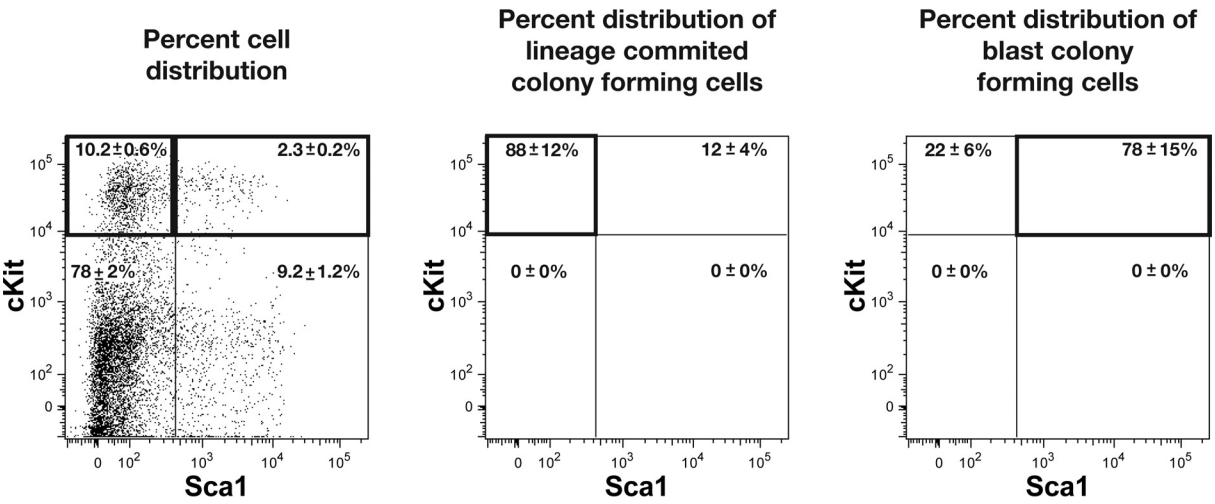

Fig. 1. FACS analysis of C57BL lineage$^-$ bone marrow cells showed that all lineage-restricted and blast colony-forming cells were restricted to Kit$^+$ fractions. However, in cultures stimulated by SCF+IL-3+EPO, calculation of the absolute number of colony-forming cells from the frequency data in Table 1 and the absolute number of cells in Fig. 1 shows that blast colony-forming cells were dominantly Scal$^+$ whereas lineage-restricted cells were Scal$^-$. Mean data ± standard deviations from 3 separate experiments.

means homogeneous and, as shown in Table 1, contained a miscellany of colony-forming cells. Furthermore, only one third of the cells were clonogenic in assays using a variety of stimuli (Table 1).

In previous studies, when LSK fractions were further subdivided using CD34 and Flt3R as markers, 3 subsets were described; (*a*) a CD34$^-$, Flt3R$^-$ population having essentially all observed long-term repopulating activity, (*b*) CD34$^+$ Flt3R$^-$ cells, highly enriched for short-term repopulating CFU-S, and (*c*) CD34$^+$ Flt3R$^+$ cells which were mutlipotential but had no significant in vivo repopulating capacity (4). In 6 replicate fractionation experiments using LSK cells, and the protocol of Yang et al. (4), multicentric blast cloning-forming cells were found to be present in all 3 fractions but were at their highest frequency in CD34$^+$ Flt3R$^-$ fractions (3) (Table 2). As previously noted, dispersed blast colony cells responding to FL plus IL-6 were selectively segregated in the CD34$^+$ Flt3R$^+$ fraction. However, as shown in Table 2, all 3 fractions also contained additional lineage-committed progenitor cells and, furthermore, the total number of detected colony-forming cells was fewer than 50% of the cells cultured, even in the more active fractions, indicating the presence in all 3 fractions of additional unidentified cells.

Because most lineage-committed progenitors segregate in Lin$^-$ Kit$^+$ ScaI$^-$ fractions, these fractions have been extensively studied (5). Separation studies using CD34 and FcγRII/III divide this population into 3 subpopulations [common myeloid progenitors (CMP); granulocyte-macrophage progenitors (GMP) and megakaryocyte/erythroid progenitors (MEP)], with evidence (5) suggesting that CMP are ancestral to GMP and MEP populations. When C57BL marrow cells were separated according to this protocol, the results of 4 replicate experiments (supporting information (SI) Table S1) indicated that GMP fractions did indeed contain a high percentage of lineage-restricted myeloid progenitor cells and MEP fractions a high percentage of megakaryocyte progenitors cells. Interestingly, CMP fractions had a lower overall content of clonogenic cells but did contain small numbers of blast colony-forming cells, despite the derivation of these fractions from a population having only 22% of the blast colony-forming cells in mouse marrow (Table 1). While CMP fractions are likely to contain GMP and MEP cells (6), the blast colony-forming cell content of CMP fractions supports other evidence (5) that cells in this fraction can generate both GMP and MEP cells. It should be noted however, that again most of the cells in these 3 fractions were not clonogenic in agar culture and the additional cells present were not identified.

From previous studies on the properties of blast colony-forming cells, both types of cell are likely to be early members of the multipotential hemopoietic family tree (3). In the absence of FACS separation protocols able to generate pure populations of CFU-S or blast colony-forming cells, the possibility remains unresolved whether these 2 cells share a common ancestor or indeed are identical. It is of interest, however, that in the fractionation of LSK subsets using CD34 and Flt3R, there was an obvious relationship between CFU-S and multicentric blast colony-forming cells (Fig. 2). The CD34$^+$Flt3R$^-$ LSK fraction demonstrated the greatest CFU-S potential (Table 2, p_{adj} = 4e-6, Tukey multiple comparison of means) in agreement with previous reports (4) and demonstrated the highest content

Table 1. Colony formation by fractions of lineage$^-$ C57BL bone marrow cells

Fractions	Blast Multi	Blast Disp	G	GM	M	EO	Meg/E
Lin$^-$ Kit$^+$ Sca1$^+$	73 ± 14	23 ± 4	8 ± 3	17 ± 8	24 ± 10	0 ± 0	16 ± 5
Lin$^-$ Kit$^+$ Sca1$^-$	4 ± 2	2 ± 1	35 ± 6	11 ± 5	22 ± 2	1 ± 0.5	36 ± 9
Lin$^-$ Kit$^-$ Sca1$^+$	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Lin$^-$ Kit$^-$ Sca1$^-$	0 ± 0	0 ± 0	0 ± 0	0.2 ± 0.3	0 ± 0	0 ± 0	0 ± 0

Mean colony counts in cultures of 500 cells ± standard deviations from three replicate experiments using a combined stimulus of stem cell factor, IL-3, and erythropoietin. Blast multi, multicentric blast; Blast disp, dispersed blast; G, granulocyte; GM, granulocytic-macrophage; M, macrophage; Eo, eosinophil; Meg/E, megakaryocyte (with or without erythroid cells) colonies.

Table 2. Colony formation by LSK subfractions of C57BL bone marrow

Cell Fraction	Stimulus	Blast Multi	Blast Disp	G	GM	M	Eo	Meg	CFU-S Per 100 Cells
$CD34^-$ $Flt3R^-$ LT HSC % of LSK cells = 15 ± 3	SCF + G-CSF	8 ± 4	5 ± 2	1 ± 1	1 ± 2	0 ± 0	0 ± 0	2 ± 4	1.3 ± 0.3
	FL + IL-6	5 ± 4	8 ± 6	0 ± 0	0.4 ± 0.9	0 ± 0			
	SCF + IL-3 + EPO	7 ± 7	9 ± 3	0 ± 0	0 ± 0	1 ± 1	0 ± 0	4 ± 3	
$CD34^+$ $Flt3R^-$ ST HSC % of LSK cells = 35 ± 7	SCF + G-CSF	28 ± 11	4 ± 2	4 ± 5	3 ± 2	1 ± 0.4	0 ± 0	0.5 ± 0.6	3.6 ± 0.4
	FL + IL-6	14 ± 3	8 ± 4	2 ± 2	2 ± 1	4 ± 3			
	SCF + IL-3 + EPO	22 ± 7	10 ± 4	4 ± 5	3 ± 3	3 ± 4	0 ± 0	6 ± 2	
$CD34^+$ $Flt3R^+$ MPP % of LSK cells = 50 ± 17	SCF + G-CSF	6 ± 3	4 ± 1	1 ± 0	1 ± 1	2 ± 2	0 ± 0	0 ± 0	0.12 ± 0.1
	FL + IL-6	6 ± 3	31 ± 9	0 ± 0	1 ± 1	2 ± 1			
	SCF + IL-3 + EPO	6 ± 4	2 ± 2	1 ± 1	1 ± 2	3 ± 3	0 ± 0	0 ± 0	

Mean colony counts in cultures of 100 cells ± standard deviations from six replicate fractionation experiments of LSK cells from C57BL marrow cells. G, granulocyte; GM, granulocyte-macrophage; M, macrophage; Eo, eosinophil; Meg, megakaryocyte; Blast multi, multicentric blast colony; Disp, dispersed blast colony; LT HSC, long-term repopulating hematopoietic stem cells; ST HSC, short-term repopulating cells; MPP, multipotential progenitor cells.
CFU-S data represent colony numbers per 100 injected cells and no seeding factors have been applied.

of multicentric blast colony-forming cells (Fig. 2, Table 2, p_{adj} = 0.013 SCF+IL-6, p_{adj} = 0.014 FL+IL-6, Tukey multiple comparison of means). This indicates that both were coenriched by the fractionation procedure and suggests that the 2 cell types could be identical. In sharp contrast, cells forming dispersed blast colonies differed from cells forming multicentric blast colonies in showing a clear inverse relationship with CFU-S frequency.

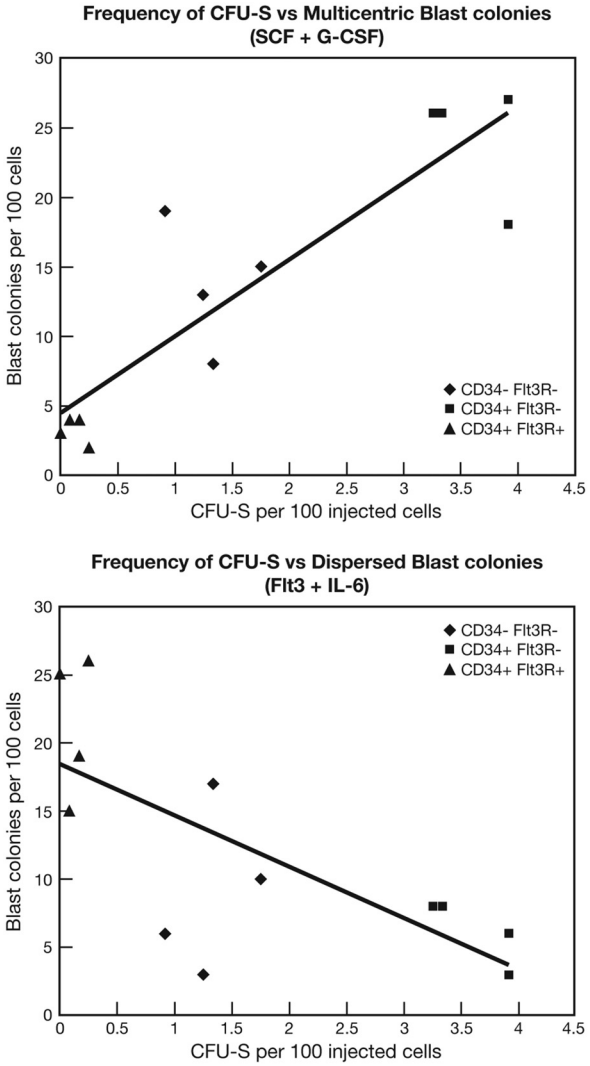

Fig. 2. FACS fractionation of LSK subsets showed a correlation in the distribution of CFU-S and multicentric blast colonies. Dispersed blast colonies showed no such correlation. Data shown are mean data from 4 replicate experiments.

Composition of Blast Colonies. To examine how closely the progeny of blast colony-forming cells recapitulate bone marrow populations, analyses were performed on pools of blast colonies grown from LSK fractions of C57BL marrow that had been stimulated either by SCF+IL-6 or FL+IL-6.

Fractionation, using Kit and ScaI markers, showed that the cellular composition of SCF-stimulated multicentric colonies was reasonably similar of that of $lineage^-$ bone marrow populations (Fig. 3). However, pooled dispersed blast colonies stimulated by FL+IL-6 differed radically in containing a much higher percentage of $ScaI^+$ cells, the majority of which were further atypical in being Kit^-.

The mean number of cells in the 7-day multicentric SCF-

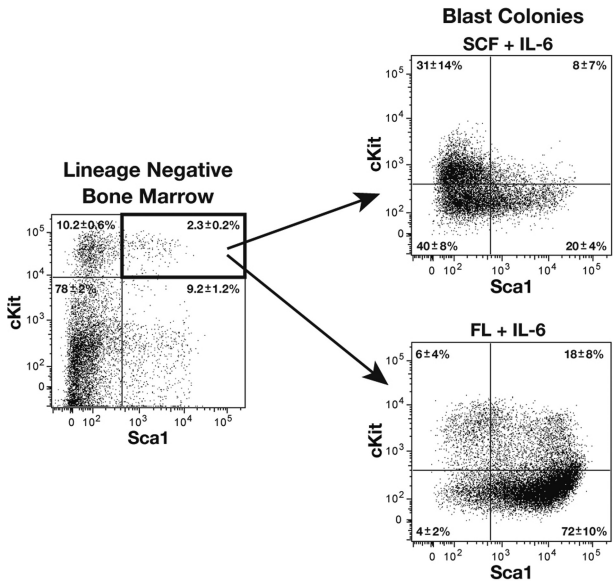

Fig. 3. FACS analysis of 7-day blast colony cells stimulated by SCF+IL-6 showed similarity to $lineage^-$ bone marrow cells when separated by Kit and ScaI. However, colony cells stimulated by FL+IL-6 showed excessive proportions of $ScaI^+$ cells, both Kit^+ and Kit^-. Mean data ± standard deviations from 3 separate experiments.

Table 3. Colony formation by fractionated blast colony cells

	GM-CSF			M-CSF			SCF + IL-3 + EPO				
Cells	G	GM	M	G	GM	M	Blast Multi	Blast Disp	G	GM	M
SCF + IL6											
Kit^+ $Sca1^+$	2 ± 2	4 ± 2	20 ± 12	0 ± 0	0 ± 0	42 ± 11	0.3 ± 0.6	0.3 ± 0.6	8 ± 7	2 ± 3	26 ± 13
Kit^+ $Sca1^-$	9 ± 2	7 ± 3	16 ± 15	3 ± 2	1 ± 1	24 ± 3	0.2 ± 0.3	0 ± 0	18 ± 9	6 ± 3	3 ± 1
Kit^- $Sca1^-$	1 ± 2	0.1 ± 0.2	8 ± 3	0 ± 0	0 ± 0	16 ± 4	0 ± 0	0 ± 0	4 ± 4	0.5 ± 0.5	1 ± 2
Kit^- $Sca1^+$	0 ± 0	0 ± 0	5 ± 4	0 ± 0	0 ± 0	16 ± 7	0.1 ± 0.2	0 ± 0	0.1 ± 0.2	0 ± 0	1 ± 1
FL + IL-6											
Kit^+ $Sca1^+$	0.1 ± 0.2	0.3 ± 0.6	12 ± 7	0.2 ± 0.2	0 ± 0	26 ± 14	1 ± 1	0.1 ± 0.2	1 ± 2	2 ± 2	3 ± 2
Kit^+ $Sca1^-$	1 ± 1	2 ± 3	25 ± 3	0 ± 0	1 ± 1	55 ± 17	0 ± 0	0 ± 0	5 ± 7	8 ± 9	9 ± 2
Kit^- $Sca1^-$	0 ± 0	0 ± 0	4 ± 2	0 ± 0	0 ± 0	11 ± 5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Kit^- $Sca1^+$	0 ± 0	0 ± 0	0.6 ± 0.4	0 ± 0	0 ± 0	9 ± 3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.2

Mean colony counts in cultures of 100 cells in 3 replicate experiments ± standard deviations. No eosinophil or megakaryocyte colonies were observed in any cultures.

stimulated colonies used in the present studies was 3,900 ± 590 cells versus the mean number in dispersed FL-stimulated colonies of 2,310 ± 700 cells. The content of lineage-committed progenitor cells in blast colonies is highly variable (3), but in previous studies on C57BL multicentric colonies ranged from 10 to 15% according to the stimulus used in the secondary cultures ($n = 35$) and varied from 5 to 11% in dispersed colonies ($n = 25$) (3). Blast colony cells, fractionated using Kit and ScaI markers, were cultured using various stimuli to establish whether the colony cells that were clonogenic lineage-committed progenitors had a particular phenotype. The results (Table 3, combined with Fig. 3) showed that blast colony cells differed in a number of respects from uncultured $lineage^-$ bone marrow cells. In multicentric SCF-stimulated colonies, approximately 75% of clonogenic cells that were Kit^+ were also $ScaI^-$, a situation similar to the 88% in $lineage^-$ bone marrow. However, unlike the absence of clonogenic cells from populations negative for Kit in marrow cells, from calculations based on absolute cell numbers in the various fractions, approximately 45% of clonogenic cells in blast cell populations were Kit^-, including 15% that were also $ScaI^+$. With dispersed FL-stimulated blast colony populations, again using a similar absolute calculation, approximately 45% of clonogenic cells were Kit^- and 43% were also $ScaI^+$, possibly reflecting the disproportionate number of cells with this phenotype in these blast colonies or changes in Kit membrane expression during culture.

For both types of colony, the aberrant Kit^- clonogenic cells were predominantly macrophage-committed progenitors responding particularly to stimulation by macrophage colony stimulating factor (M-CSF). This bias was less prominent in Kit^+ fractions, particularly those from SCF-stimulated multicentric colonies. The analysis also indicated that prefractionated Kit^+ $ScaI^+$ blast colony-forming cells, did generate some progeny that remained Kit^+ $ScaI^+$. Potentially such cells could have included progeny of self-generative divisions and it was of interest that some progeny blast colony-forming cells were indeed present in this population.

The study documented that many of the clonogenic progeny of blast colony-forming cells had an atypical phenotype compared with uncultured marrow cells. Possibly, this abnormal phenotype was a consequence of the in vitro cultures.

To further determine the expression of membrane markers on blast colony cells before secondary culture, pools of 7-day C57BL blast colonies were prepared from cultures stimulated by either SCF+IL-6 or FL+IL-6. Blast colony cells failed to exhibit the erythroid marker Ter119. However, blast colony populations of both types contained cells positive for Mac1 and GR1 (Fig. 4). In view of the superior capacity of dispersed blast colony cells to generate B-lymphocytes in underlayer cultures containing FL and IL-7 (3), it was of interest that these colonies also had significant subpopulations positive for B220 and IL-7R.

Because none of these lineage markers is present on blast colony-forming cells in LSK fractions it is clear that major

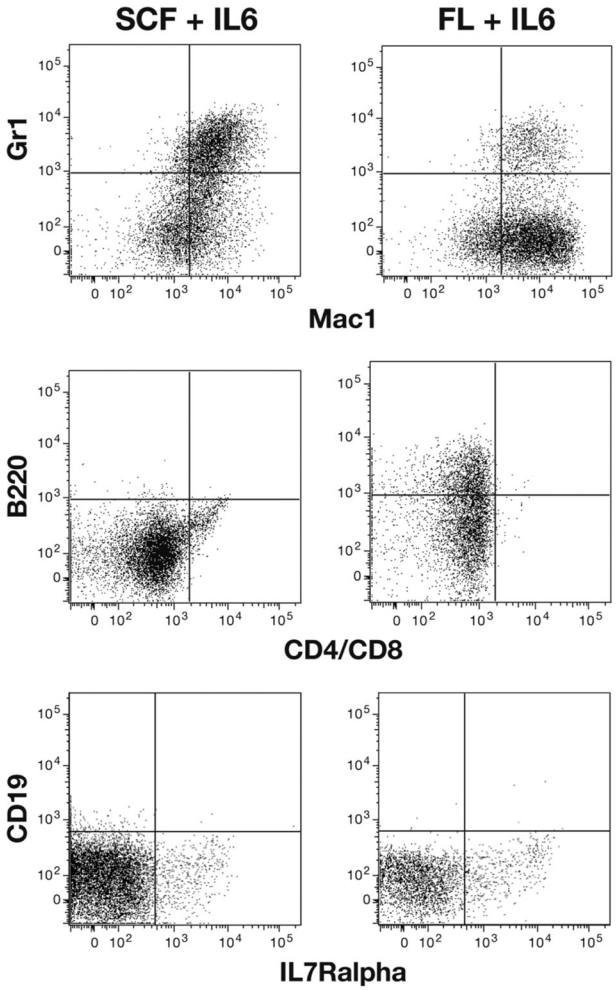

Fig. 4. FACS analysis of 7-day blast colony cells stimulated either by SCF+IL-6 or FL+IL-6 showed cells with markers of myeloid and lymphoid cells. Note that both types of colony contained $GR1^+$ and $Mac1^+$ cells but that only the FL+IL-6 colonies contained significant numbers of $B220^+$ and $IL\text{-}7R^+$ cells.

lineage commitment events with consequent changes in membrane marker expression occur in vitro during the formation of blast colonies and that these are linked to the development of lineage-restricted progenitor cells in these colonies.

Discussion

The present experiments were undertaken to further characterize hematopoietic blast colony-forming cells in the mouse. The 2 types of such colony-forming cells—multicentric and dispersed—are candidates for cells that are assayable in vivo as CFU-S or, less likely, repopulating stem cells but blast colony-forming cells have the overwhelming advantage of being able to be grown clonally in vitro and then to be subject to detailed scrutiny at the individual cell level. Cell separation procedures can enrich for long-term repopulating (stem) cells or for short-term repopulating cells (colony-forming units spleen, CFU-S) (4, 5) but, as shown in the present study, the current methods for fractionation still result in populations that are clearly heterogeneous for clonogenic cells, and this situation is not helped by the imprecision of current in vivo assay systems. As a consequence, while the present study showed that multicentric blast colony-forming cells were significantly coenriched in fractions enriched for CFU-S, this remains merely a correlative observation falling short of positively identifying these blast colony-forming cells as being CFU-S.

In keeping with the profound heterogeneity of apparently identical subsets of hematopoietic cells (7), it was noteworthy that blast CFC were in fact detected in fractions reported to be the exclusive source of long-term repopulating cells and, conversely, also in multipotential progenitor cells (MPP) fractions having limited repopulating capacity (4, 8). The blast colony-forming cells located in these different cell populations appeared to produce similar colonies, but it might repay future studies to determine whether there are fraction-based differences in self-generative capacity or capacity to generate progenitor cells and the lineage restriction of such cells. Conversely, attention needs to be paid to the nature of the lineage-committed progenitors also found in stem cell and CFU-S fractions. These cells appeared to be unusual as shown by their dependency on 2 or more stimuli before proliferation (Table 2).

Analysis of blast colonies by a secondary culture of blast colony cells has already revealed an extreme degree of heterogeneity between colonies. Despite this, mean data from pooled blast colonies reproduced fairly well the content of various progenitor cells in the marrow (3). The present analysis of surface markers on pooled blast colonies showed differences in these populations from those on normal marrow cells and also documented a consistent sharp difference between multicentric and dispersed colonies, particularly in the content in the latter colony type of unusual cells with surface markers Kit^- $ScaI^+$. In normal marrow, loss of ScaI marks the transition between $ScaI^+$ immature stem cells, CFU-S, and blast colony-forming cells on one hand and more mature lineage-committed progeny of these cells that lack ScaI on the other. Conversely, retention of Kit^+ appears to be mandatory in murine marrow cells if they are to remain clonogenic, at least in the myeloid lineages (4, 5) (Table 1).

Both types of blast colonies contained cells that differ from those in normal marrow in that many colony cells had lost expression of Kit before losing ScaI and, more unusual, could remain clonogenic even having lost expression of Kit. Loss of Kit, and therefore of responsiveness to SCF, may well account for the frequent inability of SCF+IL-3+EPO to stimulate secondary colony-formation by colony cells that are demonstrably highly clonogenic when stimulated by M-CSF or granulocyte-macrophage colony stimulating factor (GM-CSF). An example of this is evident in Table 3 where few SCF-responsive cells were noted in Kit^- fractions of blast colony cells.

Some apparent anomalies in the maturation pattern of blast colony cells may be due to their culture in vitro or to the age of blast colonies at sampling although the loss of Kit1 before ScaI is not an anomaly that would appear to be correctable if the cells were to remain clonogenic progenitor cells.

It was notable that B220 expression in colony cells was only unequivocal in dispersed colonies stimulated by Flt3 ligand and IL-6. Flt3 ligand is known to be a requirement for the generation of B lymphocytes (9, 10) and previous studies showed that dispersed colonies were clearly superior to multicentric colonies as sources of B lymphocytes in secondary culture (3).

Although the stimuli used in the present experiments appeared to be the most effective so far tested, as judged by blast colony numbers and size, they may not be optimal for certain types of differentiation commitment or may not duplicate cytokine conditions in bone marrow. In particular, if stromal cells play an important role in self-renewal and commitment events in early hematopoietic cells, the present clonal cultures lack input from such stromal cells. Given the frequency of blast colony-forming cells in bone marrow (approximately 10–20% of all progenitor cells) and the in vivo existence of the agents used to stimulate the proliferation in vitro of blast colony cells—SCF, IL-6, Flt3 ligand and granulocyte colony stimulating factor (G-CSF) —it seems likely that blast CFC would contribute substantially, and perhaps even dominantly, to the production of various cell lineages in vivo.

The present data provide a useful set of parameters for normal blast colony-forming cells and their clonal progeny and further document commitment events occurring during blast colony development. This information will be of value in identifying abnormalities or differences when analyses are performed on blast colony-forming cells from stressed or regenerating animals or in mice with various hematopoietic defects based on known genetic lesions.

Experimental Procedures

Mice. All mice were produced in the animal facility of The Walter and Eliza Hall Institute and all studies were approved by the Animal Ethics Committee of this Institute.

Culture. Marrow (25, 000) cells were cultured in 35 mm Petri dishes containing 1 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing 20% modified newborn calf serum and 0.3% agar. Blast colony formation was stimulated either by the use of a final concentration of 100 ng per ml murine stem cell factor (SCF) plus 10 ng per ml of human granulocyte colony-stimulating factor (G-CSF) or 100 ng per ml murine interleukin-6 (IL-6) or by murine Flt3 ligand (FL) 500 ng per ml plus murine IL-6 100 ng per ml. G-CSF was purchased from Amgen and all other cytokines were produced in this laboratory. After 1 week of incubation at 37 °C in a fully-humidified atmosphere of 10% $C0_2$ in air, cultures were scored at 35× magnifications then fixed with 1 ml of 2.5% glutaraldehyde. After 4 h, cultures were floated intact onto glass slides, allowed to dry, then stained for acetylcholinesterase and then with Luxol Fast Blue and hematoxylin. Final colony counts and typing were then performed on the entire cultures at 50× or 100 magnifications (11).

Reculture of Blast Colonies. Individual 7-day blast colonies were removed using a fine sterile pipette, resuspended in 8 ml of agar-medium, then cultured in duplicate for a further 7 days using 10 ng per ml of murine GM-CSF, 10 ng per ml of murine M-CSF or 100 ng per ml of murine SCF plus 10 ng per ml of murine IL-3 and 4 IU erythropoietin. Secondary colonies were processed and scored as above.

Mass Harvesting of Blast Colonies. 7-day blast colonies are removed using a fine sterile pipette and pooled in 7 ml volume of 5% serum saline. Colony cells were resuspended by repeated pipetting and washed 3 times by centrifugation at 500g. To remove remaining agar fragments, each cell suspension was passed through a sterile 5 ml polystyrene cell-strainer (BD Falcon).

Cell Sorting and Staining. For agar culture experiments using 7–12 week C57BL bone marrow, cells were stained with biotinylated antibodies specific for lineage markers CD4 (GK1.5), CD8 (53–6.7), B220 (6B2), Gr-1 (8C5), TER119,

and Mac1 (M1/70) with secondary staining using streptavidin conjugated AlexFluor 700. The lineage negative population was fractionated using APC-conjugated cKit (2B8), PeCy7-conjugated ScaI (D7), FITC-conjugated CD34 (RAM34), and PE-conjugated FcγRII/III (2.4G2) on a FACSAria (BD Biosciences). Pooled blast colonies were stained with fluorochrome conjugated anti-mouse antibodies to cKit (2B8), ScaI (D7), CD34 (RAM34), Flt3R (CD135 A2F10.1), or fluorophore-conjugated antibodies to lineage antigens, including antihuman CD41a (HIP8), and the cells were sorted by flow cytometry on a FACSAria (BD Biosciences) for secondary culture or analyzed on an LSR II (BD Biosciences).

Colony-Forming Unit-Spleen Assays. Recipient mice 7–12 weeks of age were administered 11Gy gamma-irradiation using a ^{137}Cs source (Atomic Energy) at a dose rate of 30cGy/minute split over 2 doses up to 4 hours apart. LSK subpopulations were fractionated on a FACS-Aria instrument (BD Biosciences) as described, and 400 cells injected each of LT-HSC, ST-HSC, and MPP into separate irradiated recipients. Recipient mice were maintained on oral antibiotic after transplantation (neomycin sulfate, Sigma). Spleens were harvested from recipients at 8 days following transplantation and fixed in Carnoy's solution (60% (vol/vol) ethanol, 30% (vol/vol) chloroform, 10% (vol/vol) acetic acid) and macroscopic colonies were counted.

ACKNOWLEDGMENTS. This work was supported by the Carden Fellowship Fund of the Cancer Council, Victoria, the National Health and Medical Research Council, Canberra (Program 461219) and the National Institutes of Health, Bethesda (Grant CA22556). A.N. and S.J.L. were recipients of Australian Postgraduate Awards.

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