# Differences in the Frequency of Normal and Clonal Precursors of Colony-Forming Cells in Chronic Myelogenous Leukemia and Acute Myelogenous Leukemia

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**Acute myelogenous leukemia (AML) is a clonal disease that is heterogeneous with respect to the pattern of differentiative expression of the leukemic progenitors. In some patients, the involved stem cells manifest pluripotent differentiative expression, whereas in others, the involved progenitors manifest differentiative expression mainly restricted to the granulocytic pathway. This is in contrast to chronic myelogenous leukemia (CML) which is a clonal disease known to arise in a pluripotent stem cell. Therefore, we tested whether these leukemias could be distinguished with respect to their involvement of immature precursors by studying colony-forming cells (CFC) and their precursors from four glucose-6-phosphate dehydrogenase (G6PD) heterozygous patients with AML and five patients with CML. CFC were separated from their precursors by FACS for expression of CD33 and CD34 followed by growth in a long-term culture (LTC) system. The vast majority of CFC express both the CD33 and CD34 antigens, but their less mature precursors, detected by their ability to give rise to CFC in LTC, express only CD34. In three of the four patients with AML, the $CD33^-CD34^+$ cells produced CFC in LTC that appeared to be predominantly or completely normal (ie, nonclonal) in origin. In the fourth patient, a significant enrichment of nonclonal progenitors was obtained in the $CD33^-CD34^+$ population, but these cells may also have included significant numbers of clonal cells. In contrast, in four of five patients with CML, cultures of both the $CD33^-CD34^+$ and $CD33^+CD34^+$ populations produced CFC in LTC that were almost entirely clonal in origin, whereas in the fifth patient a substantial number originated from nonclonal stem cells. These data indicate that granulocyte/monocyte progenitors are predominantly clonally derived in CML and AML. In CML, their precursors are also predominantly clonal, but in some cases of AML they are not. These findings may have implications for understanding the success or failure of current therapies of AML and CML.**


THE MYELOID LEUKEMIAS are clonal diseases that are heterogeneous with respect to differentiative expression of the stem cell of origin. This heterogeneity has been demonstrated in studies of women with leukemia who are heterozygous for the X-chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD).[1-6] During early embryogenesis, one of the two G6PD genes is randomly inactivated in somatic cells of females. Thus, in a woman heterozygous for the usual G6PD gene ($Gd^B$), and a variant such as $Gd^A$, normal tissues are composed of two cell populations: Some cells express only type A enzyme, and the remaining cells express only type B G6PD. In contrast, only one enzyme type is found in tumors derived from a single cell.

Studies with G6PD or the Philadelphia chromosome (Ph) as markers demonstrate that chronic myelogenous leukemia (CML) involves stem cells that are pluripotent precursors of granulocytes, monocytes, erythrocytes, platelets, and B-lymphoid cells.[4-6] G6PD studies of patients with acute myelogenous leukemia (AML) indicate that it is heterogeneous with respect to the differentiative expression of the involved stem cells.[2] In six older patients, the same single-enzyme G6PD type observed in the blasts was also observed in erythrocytes and platelets, indicating that the leukemias originated in a multipotent stem cell. In contrast, in 16 other, predominantly younger patients, the erythrocytes and platelets were mainly derived from normal stem cells. In three other cases, an intermediate pattern of differentiative expression was noted: mature erythrocytes were not derived from the abnormal clone, but many BFU-E were apparently clonally derived, suggesting that the leukemic stem cells gave rise to erythroid progenitors in vivo, but not to mature erythrocytes.

These results indicate that in some patients with AML, the leukemias originate in a highly immature pluripotent stem cell, whereas in others, the differentiative expression of the involved stem cells is mainly limited to the granulocytic pathway. The latter pattern could be explained if the leukemia originated in: (a) a pluripotent stem cell that proliferates in an uncontrolled fashion but whose differentiative expression leads to clonal dominance only in the granulocytic lineage because proliferation in the erythrocytic and megakaryocytic lineages is ineffectual or suppressed; (b) one pluripotent stem cell whose growth is normally regulated and does not display clonal dominance, but whose progeny on maturation into CFU-GM display unregulated growth and become the dominant precursors of granulocytes and monocytes; or (c) a more mature progenitor committed to a specific lineage, eg, CFU-GM. If one of the latter two explanations is correct, in patients with differentiative expression of the leukemia largely restricted to the G/M lineage, progenitors less mature than CFU-GM would be either completely of nonclonal origin or would contain only a rare clonal precursor. In contrast, if the first

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explanation were correct, these immature precursors would be mainly clonally derived, a finding expected in patients with a pluripotent disease such as CML.

To test this hypothesis, we studied G6PD heterozygous patients with AML or CML with methods that we developed to separate colony-forming cells (CFC) from their precursors.[7] The precursor cells express the CD34 antigen but not the CD33 antigen and are detected by their ability to give rise to CFC in a long-term culture (LTC) system. In contrast, CFC, including CFU-GM, BFU-E and CFU-GEMM, express both CD33 and CD34.[7] In previous studies, we showed that in four of seven patients with AML, CFC and their progenitors that remained after lysis of cells with anti-CD33 antibody and complement were predominantly or completely of nonclonal origin,[8] (I. Bernstein, J. Singer, P. Fialkow, unpublished observations). In the present study, we directly isolated $CD33^+CD34^+$ CFC and $CD33^-CD34^+$ precursors of CFC from G6PD heterozygous females with AML or CML and determined whether they were derived from the leukemic clone or from normal stem cells.

## MATERIALS AND METHODS

*Patients.* Clinical and some culture data for three of the patients have been reported (patients 13, 16, and 26 in ref 2; patients 13 and 16 are patients 1 and 3 in ref 8; and patient 26 is reported in ref 9). CML patients 1, 2, 3, and 5 were $Ph^+$ and were studied during the chronic phase of leukemia. CML patient 4 is $Ph^-$ and was studied during blast crisis. After we obtained informed consent from all patients, bone marrow (BM) or peripheral blood (PB) samples were drawn in heparin (10 U/mL) at the referring institutions and air-expressed to Seattle. With the exception of CML patient 3, light-density PB cells were separated with Ficoll-Hypaque (density 1.077) and studied after freezing in liquid nitrogen in 90% newborn calf serum and 10% dimethyl sulfoxide (DMSO). In the case of CML patient 3, BM cells were used and the separated cells were studied fresh.

*Antibody preparation and purification.* The anti-CD34 antibody, 12-8 (murine monoclonal IgM),[10] was partially purified from ascites fluids by boric acid precipitation as described previously.[7] The anti-CD33 antibodies p67-5 and p67-6 [IgG2a and IgG1 mouse monoclonal antibodies (MoAbs), respectively] were generated and purified as described previously.[7] These MoAbs competitively inhibit binding of L4F3 antibody to the CD33 antigen[11] (I. Bernstein, R. Andrews, unpublished observations). As isotype controls for staining, anti-mouse Thy-1.1 MoAbs 1A14 (IgG2a), 31A (IgG1), and anti-mouse Thy1.2 MoAb H12C12 (IgM) were used.

*Staining and cell sorting.* Cells were examined with indirect immunofluorescence antibody-staining techniques and separated with FACS as described previously.[7] All staining was performed at concentrations of $10^7$ cells/mL in sterile phosphate-buffered saline (PBS) supplemented with 2% human AB serum (Irvine Scientific, Irvine, CA). For two-color staining, untreated cells were incubated with both 12-8 (20 μg/mL) and p67-5 in ascites (1:$10^3$ dilution), or p67-6 in purified form (10 μg/mL). Control cells were incubated with (a) H12C12 (20 μg/mL) and 1A14 ascites (1:$10^3$) or purified 31A antibody (10 μg/mL), (b) H12C12 and p67-5 or p67-6, or (c) 12-8 and 1A14 or 31A. Control and experimental cells were incubated with the primary antibodies for 30 minutes at 4°C and then washed twice with PBS containing 2% human AB serum. All samples were then incubated with a mixture of biotin-conjugated goat anti-mouse IgM antisera (1:80 dilution, μ-chain specific, Tago, Inc, Burlingame, CA) and FITC-conjugated goat anti-mouse IgG (1:20 dilution, γ-chain specific, Southern Biotech, Birmingham, AL) for 30 minutes at 4°C, and then washed twice. Finally, all cells were incubated with phycoerythrin-conjugated avidin (1:20 dilution, Becton-Dickinson, Oxnard, CA) for 30 minutes at 4°C, washed once, and sorted with a FACS-440 or a FACS-II (Becton-Dickinson). Cells were collected into RPMI–10% fetal bovine serum. They were considered positively stained if the fluorescent antibody-stained cells were greater than 96% to 98% of control-stained cells. In studies of patients 13 and 26, the $CD33^\pm$ subset of $CD34^+$ cells displayed fluorescence intensity greater than 70% and 34% of control (31A or 1A14)-stained $CD34^+$ cells, respectively.

*In vitro cultures.* After sorting, cell aliquots were assessed for colony growth in the presence of medium conditioned by phytohemagglutinin (PHA)-stimulated T cells as described previously.[12] The remaining cells were placed in culture tubes ("Ambitube" Lux Plastics, Naperville, IL) containing allogeneic irradiated (1,100 cGy) marrow adherent-cell layers from 3- to 4-week-old LTCs from normal donors as reported earlier.[13] In one experiment, recombinant human Steel factor (c-*kit* ligand) was added at 20 ng/mL (supplied by S. Gillis, Immunex, Seattle, WA). The number of cells placed in each tube depended on the number of cells obtained after cell sorting. In the $CD33^-CD34^+$ group, fewer than $10^4$ cells/tube were used in most experiments. Each week the cultures were vigorously agitated by pipetting the supernatant medium several times to remove one half of the medium. Cells from each Ambitube were harvested separately, washed twice and, without counting, cultured for CFU-GM growth in a single tissue culture dish. The LTCs were maintained for up to 6 weeks.

After enumeration, colonies were individually harvested and tested for G6PD by cellulose-acetate electrophoresis.[12] All detectable colonies on a plate, or on a predefined portion of the plate, were analyzed. Occasional colonies that had both G6PD types were excluded from analysis. Randomly selected colonies were individually plucked and stained with Wright-Giemsa or myeloperoxidase to verify their granulocytic lineage.

## RESULTS

The age, diagnosis, and ratio of G6PD enzyme types in normal tissue and leukemic cells are shown in Table 1. These data were reported previously for three of the AML patients.[2] Normal tissues were obtained from each patient except patient 36 and were shown to express both G6PD types, whereas leukemic blasts from each patient expressed only one enzyme type. The leukemic progenitors in at least three of the patients with AML had differentiative expression mainly restricted to the granulocyte lineage. We could not determine this for patient 36 owing to transfusions of erythrocytes and platelets and the unavailability of normal tissue. As anticipated, the leukemic stem cells in the five CML patients had pluripotent differentiative expression.

*AML.* Cells from the AML patients were separated by two-color immunofluorescence into populations with the phenotypes $CD33^-CD34^+$, $CD33^+CD34^+$, and $CD34^-$. In some cases, $CD34^+$ cells, when stained with anti-CD33 antibody, displayed low-intensity fluorescence. The cells were collected and designated $CD33^\pm CD34^+$. Sorted and unsorted cells were cultured over irradiated marrow adherent-cell layers. Cells in the supernatant were assayed weekly for colony formation in semisolid medium.

In studies of patient 13, the cumulative G6PD A/B ratio

**Table 1. G6PD Findings in Normal Tissue, Blood Cells, and Cultured CFU-GM and BFU-E From Four Patients With AML and Five Patients With CML**

| | | | | G6PD* | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Disease† | Patient | FAB | Age (yr) | Normal Tissue‡ | Mononuclear Cells | RBC | Platelets | CFU-GM | BFU-E |
| AML | 13 | M4 | 34 | 3:2 | 1:0 | 2:1 | 3:2 | 25:0 | 2:3 |
| AML | 16§ | M4 | 20 | 1:2 | 0:1 | 0:1‖ | 1:4 | 11:106 | 9:29 |
| AML | 26 | M2 | 8 | 1:3 | 1:0 | 2:3 | NT | 35:16 | 3:4 |
| AML | 36 | M3 | 1 | NT | 1:0 | 3:2‖ | NE | 5:1 | NT |
| CML | 1 | | 36 | 1:1 | 1:0 | 1:0 | 1:0 | NG | 25:0 |
| CML | 2 | | 56 | 2:1 | 1:0 | 1:0 | 1:0 | 46:1 | 39:3 |
| CML | 3 | | 65 | 1:1 | 1:0 | 1:0 | 1:0 | 19:0 | 23:0 |
| CML | 4 | | 73 | 1:1 | 1:0 | 1:0 | 1:0 | 59:0 | NG |
| CML | 5 | | 43 | 3:1 | 1:0 | 1:0 | 1:0 | 47:0 | 48:0 |

NT, not tested; NG, no growth; NE, not evaluable.

*Ratio of variant type G6PD to type B G6PD for normal tissue and blood cells; number of colonies showing variant type G6PD: number showing type B from CFU-GM and BFU-E.

†Karyotype analysis of patients with AML showed 52,XXX,+4,+5,+8,+8,+19; 46, XX, t(8;21) (q22;q22); and 46,XX,t(15;17)(q22;q11.2) in patients 16, 26, and 36, respectively (not performed in patient 13). Four of the CML patients are $Ph^+$. CML patient 4 is $Ph^-$ (BCR/ABL studies were not performed in this patient). All AML patients expressed the CD33 antigen with all cells positive for patients 13 and 16 and 37% and 90% of cells positive in patients 26 and 36.

‡Skin from patient 13 and the 5 CML patients, pooled T-cell colonies from patient 16, and both T-cell colonies and cultured marrow fibroblasts from patient 26.

§Studied in first relapse.

‖Erythrocytes were not studied before transfusion. Data are for RBC studied from 4 hours to 15 days after the last transfusion.

of colonies derived from the LTC of $CD33^-CD34^+$ cells was 75:64 (Table 2). This ratio of A/B type CFC was less than the 3:2 ratio observed in normal tissues from this patient, however, suggesting that all or nearly all type A G6PD was normally derived. This is in contrast to results from cultures seeded with $CD33^\pm$ $CD34^+$ cells, in which only type A G6PD CFC were found, the same G6PD type present in leukemic blasts. Moreover, the numbers of these colonies decreased after week 1, whereas increasing numbers of colonies were grown from $CD33^-CD34^+$ CFC. The colonies grown from LTC seeded with unsorted cells were predominantly G6PD type A ($P < .0001$ in comparison with the 75:64 ratio of G6PD A/B colonies grown from LTC seeded with $CD33^-CD34^+$ cells). Cultures of $CD34^-$ and $CD33^+CD34^+$ cells failed to generate CFC (data not shown).

The results from studies of the two sorted cell populations from patient 26 were similar. Only cultures of $CD33^-CD34^+$ cells produced colonies after week 2 in LTC, and the ratio of A/B colonies was 6:52, presumably representing cells of nonclonal origin, since the leukemic clone in this patient displayed G6PD type A. Cultures established with $CD33^\pm CD34^+$ cells failed to produce colonies at 4 and 6 weeks, but did produce 23 ± 6 CFC after 2 weeks that displayed a G6PD A/B ratio of 15:2. Similarly, isolated $CD33^+CD34^+$ cells gave rise to 35 ± 17 CFC with a G6PD A/B ratio of 10:0 after 2 weeks of LTC, and failed to give rise to CFC after 4 and 6 weeks of LTC (data not shown). These findings for both the $CD33^\pm CD34^+$ and $CD33^+CD34^+$ cells are consistent with their having a predominantly leukemic cell origin ($P < .0001$ when either group is compared with colonies from $CD33^-CD34^+$ cultures). The results, however, differ from those observed in patient 13 in that unsorted cells, tested after 4 weeks of culture, gave rise only to type B colonies and were therefore derived from normal stem cells. Expression of normal CFC in LTC of AML cells was observed previously.[9,14]

Similar results were obtained with sorted cells from patient 36. Whereas only type A G6PD was detected in the mononuclear cells present in the cell sample, an approximately equivalent number of CFU-GM expressing type A and B G6PD were derived from LTC initiated with $CD33^-CD34^+$ cells. Notably, substantial numbers of CFC were obtained from the single flask containing recombinant human Steel factor (c-*kit* ligand). Although these CFC included significant numbers of normal progenitors expressing the G6PD type not present in the abnormal clone, we could not determine the extent to which they may have included clonal CFC because we could not compare it with the G6PD ratio in normal tissues since such tissues were unavailable. Furthermore, although a ratio of 3:2 was observed in RBC, the sample was obtained 24 hours after the patient received an RBC transfusion. Cultures of $CD33^+CD34^+$, $CD33^+CD34^-$, or $CD33^-CD34^-$ cells failed to give rise to significant numbers of CFU-GM (data not shown).

Patient 16 was studied in first relapse. Her leukemic clone expressed G6PD type B. All but 1 of 40 CFC generated in LTC of unsorted marrow were of type B G6PD. In addition, the $CD34^-$ cells from this patient gave rise to a small number of CFU-GM in LTC after 2, 3, and 4 weeks of culture ($2 \pm 1$ to $5 \pm 1/10^6$ cells), and all 80 colonies tested expressed type B G6PD (data not shown). In contrast, 21 of 110 colonies derived from LTC of sorted $CD33^-CD34^+$ cells expressed type A G6PD. Because this patient's normal tissue expressed G6PD in an A/B ratio of 1:2, we can assume that all 21 of the type A CFC and approximately twice that number (ie, 42 of type B CFC) were of probable normal origin. Thus, a substantial number

**Table 2. Studies of AML: G6PD Analysis of Colonies From Two-Stage, LTMC After Separation of Blood Mononuclear Cells by Two-Color Fluorescence-Activated Cell Sorting**

| Patient/ Duration of LTC (wk) | $CD33^-$ $CD34^+$ | | $CD33^\pm$ $CD34^+$ | | Unsorted | |
|---|---|---|---|---|---|---|
| | No. Col.* | G6PD† | No. Col.* | G6PD† | No. Col.* | G6PD† |
| Pt 13 | | | | | | |
| 0 | 2 | NT | 123 ± 29 | 37:0 | 140 ± 29 | 37:0 |
| 1 | 1,365 ± 611 | 21:12 | 62 ± 43 | 35:0 | 132 ± 45 | 39:1 |
| 2 | 487 ± 249 | 21:12 | 4.7 ± 4 | 8:0 | NG | — |
| 3 | 2,522 ± 649 | 24:17 | 7 ± 4 | 7:0 | 6 ± 3 | 23:5 |
| 5 | 1,033 ± 385 | 9:23 | NG | — | 3 ± 1 | 7:2 |
| Total | | 75:64 | | 87:0 | | 106:8 |
| Pt 16‡ | | | | | | |
| 0 | NT | — | ND | — | NT | — |
| 2 | 693 ± 472 | 4:24 | ND | — | 8 ± 3 | 1:24 |
| 3 | 293 ± 206 | 12:31 | ND | — | 1 ± 0 | 0:15 |
| 4 | 104 ± 61 | 5:20 | ND | — | NT | — |
| 5 | 178 | 0:14 | ND | — | NG | — |
| Total | | 21:89 | | | | 1:39 |
| Pt 26 | | | | | | |
| 0 | NT | — | NT | — | NG | — |
| 2 | 42 ± 20 | 3:5 | 23 ± 6 | 15:2 | NG | — |
| 4 | 24 ± 14 | 0:11 | NG | — | NG | — |
| 6 | 229 ± 333 | 3:36 | NG | — | 84 | 0:13 |
| Total | | 6:52 | | 15:2 | | 0:13 |
| Pt 36 | | | | | | |
| 3 | 88,254§ | 9:10 | NG | — | NG | — |
| 4 | 15,556 | 6:4 | NG | — | NG | — |
| 4 | 103,492§ | 10:11 | NG | — | NG | — |
| 5 | 4,762§ | 7:4 | NG | — | NG | — |
| Total | | 32:29 | | | | |

For patients 13, 26, 16, and 36, percentages of sorted cells that were $CD33^-$ $CD34^+$ were 0.2%, 14.3%, 0.44%, and 0.02%, respectively; 1.9%, 12.8%, 0.04%, and 0.08% were $CD33^+$ $CD34^+$, respectively; for patients 13 and 26, and 80.4% and 30.9% of cells were $CD33^\pm$ $CD34^+$.

Abbreviations: ND, not done; other abbreviations as in Table 1.

*Mean number of colonies grown from cultures in Ambitubes or T-25 tissue culture flask ±1 SD normalized for $1 \times 10^5$ starting cells. SD not shown indicates that only a single culture was established.

†The number of G6PD type A/G6PD type B colonies.

‡In studies of patient 16, only cells with low 90° scatter were evaluated.

§CFC derived from a single LTC established with recombinant human Steel factor (c-*kit* ligand) at 20 ng/mL.

(~63 of the 110 colonies) probably were nonclonal, presumably normal, progenitors. Moreover, the ratio of A/B type CFC (21:89) was significantly different from that obtained using unsorted marrow cells (1:39) ($P < .01$, Fisher's exact test), indicating that isolation of $CD33^-CD34^+$ cells allowed a significant enrichment of nonclonal progenitors.

*CML.* In contrast to the results in AML, FACS-separated $CD33^-CD34^+$ cells from CML patients 1, 2, 4, and 5 gave rise to CFC that expressed essentially only the G6PD type of the CML clone after 4 weeks of LTC (Table 3). Similarly, $CD33^+CD34^+$ cells from three of these patients also gave rise to CFU-GM, almost all of which expressed the G6PD type of leukemic clone. After 4 weeks of culture, unsorted cells from patient 1 gave rise to colonies of apparent normal derivation (G6PD A/B ratio 5:11).

After 2 to 4 weeks in culture, $CD33^-CD34^+$ cells from CML patient 3, whose leukemic clone was G6PD type A, gave rise to appreciable numbers of CFC derived from normal stem cells. At week 4, the G6PD A/B ratio was 6:8, suggesting that the tested CFC were derived predominantly from normal progenitors. The G6PD A/B ratio of 8:14 for colonies grown from $CD33^+CD34^+$ cells after 4 weeks in LTC suggests that these cells were also derived from normal progenitors (normal tissue A/B ratio was 1:1). Surprisingly, however, even after 4 weeks in LTC, unsorted marrow cells continued to give rise only to G6PD type A CFC, the G6PD type associated with the CML clone.

**Table 3. Studies of CML: G6PD Analysis of Colonies From Two-Stage, LTC After Separation of Blood Mononuclear Cells by Two-Color Fluorescence-Activated Cell Sorting**

| Patient/ Duration of LTC (wk) | $CD33^-$ $CD34^+$ | | $CD33^+$ $CD34^+$ | | Unsorted | |
|---|---|---|---|---|---|---|
| | No. of Col.* | G6PD† | No. of Col.* | G6PD† | No. of Col.* | G6PD† |
| Pt 1‡ | | | | | | |
| 0 | — | — | — | — | — | — |
| 2 | 46 ± 59 | 9:1 | 24 ± 23 | 6:0 | 234 | 11:2 |
| 3 | 72 ± 6 | 23:0 | 31 | — | NG | — |
| 4 | 130 ± 55 | 23:2 | 13 ± 7 | 4:0 | 1,847 | 5:11 |
| Total | | 65:3 | | 10:0 | | 16:13 |
| Pt 2 | | | | | | |
| 0 | NT | — | NT | — | 24 ± 7 | 42:0 |
| 3 | 3,920 | 23:2 | 14,857 | 14:1 | NG | — |
| 4 | 23,760 | 26:1 | 7,400 | 13:0 | NG | — |
| 5 | 13,760 | 28:1 | 2,400 | 15:0 | NG | — |
| 6 | 39,520 | 27:0 | 1,000 | 15:0 | NG | — |
| 7 | 7,200 ± 1,526 | 42:0 | 30 | NT | NG | — |
| 8 | 19,920 ± 2,150 | 24:0 | 171 ± 81 | 9:0 | NG | — |
| 9 | 11,520 | 33:0 | 100 ± 61 | 7:0 | NG | — |
| Total | | 203:2 | | 73:1 | | 42:0 |
| Pt 3 | | | | | | |
| 0 | NT | — | NT | — | 10 ± 2 | 26:1 |
| 1 | NT | — | NT | — | 12 ± 5 | 13:0 |
| 2 | 3,759 ± 810 | 26:12 | 104 | NT | 13 ± 3 | 30:0 |
| 3 | 648 ± 589 | 22:3 | 404 ± 132 | 7:1 | 3 ± 1 | 28:0 |
| 4 | 167 ± 56 | 6:8 | 263 ± 223 | 8:14 | 2 ± 2 | 11:0 |
| Total | | 54:23 | | 15:15 | | 108:1 |
| Pt 4 | | | | | | |
| 0 | 1,000 | 8:0 | NG | — | 16 ± 6 | 23:5 |
| 2 | 140 ± 54 | 25:0 | NG | — | 1 ± 0 | 14:0 |
| 3 | 172 ± 80 | 26:0 | NG | — | 2 ± 1 | 29:0 |
| 4 | 95 ± 30 | 13:1 | NG | — | NG | — |
| Total | | 72:1 | | | | 66:5 |
| Pt 5 | | | | | | |
| 0 | 350 | 15:0 | 1,500 | 26:0 | 49 ± 6 | 15:0 |
| 2 | 867 ± 700 | 22:1 | 2,116 ± 1,471 | 22:1 | 21 ± 11 | 26:0 |
| 4 | 150 ± 144 | 19:2 | 47 ± 39 | 13:0 | 1 ± 0 | 14:3 |
| Total | | 56:3 | | 61:1 | | 55:3 |

Abbreviations as in Table 1.

*Mean number of colonies grown from cultures in Ambitubes ±1 SD normalized for $1 \times 10^6$ starting cells. SD not shown indicates that only a single culture was established.

†The ratio of G6PD type A/G6PD type B colonies.

‡In studies of patient 1, $CD33^+$ $CD34^+$ cells were also collected. These cells gave rise to 20, 459, and 289 CFC per $10^5$ cells input after 2, 3, and 4 weeks, respectively, in LTMC. All colonies showed type A G6PD.

## DISCUSSION

CFC from normal subjects are characterized by expression of the CD34 and the CD33 antigens, but the less mature precursors of the CFC detectable by LTC are $CD33^-CD34^+$.[7] These findings suggested that $CD33^+CD34^+$ cells could be separated from their less mature $CD33^-CD34^+$ precursors in patients with AML, thereby allowing determination of whether the precursors of CFC originate from the leukemic-cell clone. In a previous study, we showed that lysis of leukemic cells with an anti-CD33 antibody and complement allows residual normal progenitors to grow in LTC from cells of patients with AML.[8] The present investigation expands our previous study. By selecting isolated $CD33^-CD34^+$ cells and $CD33^+CD34^+$ cells, we were able to compare the clonal involvement of progenitors that expressed the CD33 antigen directly with their precursors that were $CD33^-$.

In studies of three of four patients with AML (patients 13, 36, and 26), cells with a $CD33^-CD34^+$ phenotype contained precursors of CFC that were predominantly nonclonal. However, the presence of some clonal progenitors cannot be excluded for patient 36 because a comparison with normal tissues was not possible. The data do suggest that patient 16 had some clonal progenitors, although, as with the other three patients, a portion, or perhaps most of the $CD33^-CD34^+$ CFC precursors were nonclonal.

These findings demonstrate that in some patients with AML, immature hematopoietic precursors distinguished by their $CD33^-CD34^+$ phenotype circulate in blood and are mainly of nonleukemic origin. This observation and the finding that the more mature $CD33^+CD34^+$ precursor pool is predominantly clonally derived is consistent with the hypothesis that in some patients with AML the leukemic cells develop from a clone of mature progenitors or that the leukemia involved occasional less mature $CD33^-$ stem cells that expand in an uncontrolled manner only after maturation to a $CD33^+$ CFC. Because of the heterogeneous nature of AML, the extent to which primitive precursors are involved in the leukemic process might be determined when larger numbers of patients are tested. With the development of methods for evaluating X-linked DNA polymorphisms in a large group of informative female patients, such a study should now be possible.

The findings in AML contrast with the observations in CML. In the latter disease, substantial numbers of $CD33^-CD34^+$ cells are derived from the abnormal clone. In studies of four of the five patients with CML, $CD33^-CD34^+$ cells, as well as $CD33^+CD34^+$ cells gave rise to CFC that were derived predominantly from the CML clone. In patient CML-3, in whom BM rather than blood was studied, after 2 to 4 weeks in LTC, the $CD33^-CD34^+$ as well as $CD33^+CD34^+$ cells gave rise to substantial numbers of nonclonally derived colonies, as evidenced by the number of the colonies expressing the G6PD type not found in the malignant clone. Findings of nonclonal progenitors after LTC of unsorted marrow cells from some patients with CML has been reported.[15] Unseparated marrow cells from this patient gave rise only to clonally derived CFC; however, we did not use enzymatic treatment of the adherent layer to release progenitors with a high proliferative potential that may be preferentially normal. In addition, expression of normal progenitors in LTC from unseparated cells was noted after 4 weeks of culture of cells from Patient CML-1.

The expression of clonally derived precursors of CFC in CML is consistent with results of direct cell lineage studies demonstrating that this leukemia involves pluripotent stem cells.[4-6] The apparent lack of involvement of similar immature precursors in some cases of AML may account for the ability to eradicate the leukemic clone by combination chemotherapy in some patients. The presence of $CD33^-CD34^+$ cells derived from normal stem cells in AML may also underlie the ability to cure some patients with myeloablative therapy followed by autologous BM transplant. Evidence suggesting that the $CD33^-CD34^+$ population contains the cells which repopulate the BM includes observations that isolated $CD34^+$ BM cells reconstitute the BM after BM ablative therapy[16-18] and that hematopoiesis can be reconstituted with BM depleted of $CD33^+$ cells.[19] If this hypothesis is correct, the presence of clonally derived $CD33^-CD34^+$ precursors may be correlated with a poorer long-term response to treatment in AML. Addressing this question, however, will require assessment of large numbers of patients, including patients whose AML involves stem cells with pluripotent differentiative expression.

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