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**Research Article**

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# Heterogeneity of Clonogenic Cells in Acute Myeloblastic Leukemia

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## Abstract

The expression of differentiation-associated surface antigens by the clonogenic leukemic cells from 20 patients with acute myeloblastic leukemia (AML) was studied with a panel of seven cytotoxic monoclonal antibodies (anti-Ia, -MY9, -PM-81, -AML-2-23, -Mo1, -Mo2, and -MY3). The surface antigen phenotypes of the clonogenic cells were compared with the phenotypes of the whole leukemic cell population, and with the phenotypes of normal hematopoietic progenitor cells. In each case the clonogenic leukemic cells were found within a distinct subpopulation that was less "differentiated" than the total cell population. Clonogenic leukemic cells from different patients could be divided into three phenotype groups. In the first group (7 of 20 cases), the clonogenic cells expressed surface antigens characteristic of the normal multipotent colony-forming cell (Ia, MY9). These cases tended to have "undifferentiated" (FAB M1) morphology, and the total cell population generally lacked expression of "late" monocyte antigens such as MY3 and Mo2. A second group (seven cases) of clonogenic cells expressed surface antigens characteristic of an "early" (day 14) colony-forming unit granulocyte-monocyte (CFU-GM), and a third group (six cases) was characteristic of a "late" (day 7) CFU-GM. The cases in these latter two groups tended to have myelomonocytic (FAB M4) morphology and to express monocyte surface antigens. These results suggest that the clonogenic cells are a distinct subpopulation in all cases of AML, and may be derived from normal hematopoietic progenitor cells at multiple points in the differentiation pathway. The results further support the possibility that selected monoclonal antibodies have the potential to purge leukemic clonogenic cells from bone marrow in some AML patients without eliminating critical normal progenitor cells.

## Introduction

Acute myeloblastic leukemia (AML)<sup>1</sup> is a clonal malignant disorder of myeloid progenitor cells that results in the progres-

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1. Abbreviations used in this paper: AMoL, acute monocytic leukemia; AML, acute myeloblastic leukemia; AMML, acute myelomonocytic leukemia; CFU-GM, colony-forming unit granulocyte-monocyte; DMSO, dimethyl sulfoxide; IMDM, Iscove's modified Dulbecco's

sive accumulation of leukemic cells that fail to differentiate normally (1). A small subset of leukemic cells from many patients with AML has the ability to form a colony in vitro in semisolid medium (2, 3). These clonogenic cells, leukemic colony-forming cells (L-CFC), have several properties that are not shared by the majority of leukemic cells: a high fraction of L-CFC is in S-phase (4), the cells have the ability to divide five or more times in vitro, and at least a subset of L-CFC has self-renewal capability (5). L-CFC share these properties with normal myeloid progenitor cells, and it has been suggested that the L-CFC act in vivo as progenitor cells to maintain the rest of the leukemic cell population (4, 6). However, despite having properties of a progenitor cell, the relationships between L-CFC and the total leukemic population have not been defined.

We have previously demonstrated that differentiation-associated myeloid surface antigens detected by monoclonal antibodies could be used to identify L-CFC as a distinct subpopulation of cells in some patients with acute myelomonocytic leukemia (AMML) (7). It was shown that L-CFC expressed antigens characteristic of very immature cells (Ia, MY7) but lacked MY4, an antigen characteristic of later monocytic cells, despite the fact that a majority of the leukemic cells in AMML patients express MY4. These preliminary observations thus suggested that the highly proliferative MY4 negative L-CFC may indeed function as progenitor cells, giving rise to MY4 positive cells that have minimal proliferative potential, a situation analogous in some respects to normal myelopoiesis.

In this study, we have investigated the surface antigens of L-CFC in 20 patients with AML with the goals of (a) comparing L-CFC with the majority of the leukemic cells in each patient; (b) comparing L-CFC from different AML patients of different morphologic subtypes, and (c) comparing L-CFC with normal bone marrow colony-forming cells. The results demonstrate that L-CFC are a distinct subpopulation of leukemic cells in virtually all cases of AML and can be distinguished from more differentiated leukemic cells by their surface antigen phenotype. Three different "stages" of L-CFC were defined by the progressive acquisition of surface antigens in a sequence analogous to normal granulocyte-monocyte progenitor cell maturation. Finally, the degree of in vivo differentiation of leukemic cells in individual AML patients is at least partially dependent on the stage of L-CFC present in that patient.

## Methods

**Monoclonal antibodies.** Production and characterization of murine monoclonal antibodies anti-MY3, anti-MY9, anti-AML-2-23, and anti-PM-81 have been previously described (8-11). Anti-Mo1 and anti-

medium; L-CFC, leukemic colony-forming cells; MEM, minimal essential medium; CFU-Mix, colony-forming unit-mixed granulocyte/erythrocyte; NSE, nonspecific esterase; SE, specific esterase.

Mo2 antibodies (12) were provided by Dr. Robert Todd (Dana-Farber Cancer Institute) and anti-I2 (anti- $\text{Ia}$ ) (13) by Dr. Lee Nadler (Dana-Farber Cancer Institute). Anti-PM-81, Mo1, Mo2, and MY3 are of the IgM subclass; anti-MY9 and anti-AML-2-23 are of the IgG2b subclass; and anti-I2 is of the IgG2a subclass. Anti-MY3 recognizes the same antigen determinant (55,000 mol wt) recognized by anti-MY4 (which is not lytic). All antibodies were lytic with rabbit complement at titers of greater than 1:1,000 dilution of ascites.

**Leukemic samples.** Peripheral blood or bone marrow aspirate cells were obtained under sterile conditions from untreated patients with AML after they gave informed consent. Mononuclear cells were prepared by Ficoll-Hypaque sedimentation (1.077 g/cm<sup>3</sup>). Leukemic cells were cryopreserved in 10% dimethyl sulfoxide (DMSO) in the vapor phase of liquid nitrogen and later thawed using DNase (100 µg/ml, Worthington Diagnostic Systems, Freehold, NJ) in minimal essential medium with 2.5% human AB serum. Wright's stained cytocentrifuge smears were examined to determine the percentage of leukemic blasts. All patients had >54% blasts in the analyzed sample and usually >75%.

The diagnosis of AML using FAB classification criteria (14) was established by standard techniques using Wright-Giemsa stained smears, alpha-naphthylacetate esterase (nonspecific esterase, NSE), and naphthol AS-D chloroacetate esterase (specific esterase, SE) staining patterns. FAB types M1 and M2 showed no monocytoid features and were generally SE+ and NSE-. Acute myelomonocytic leukemias (AMML, FAB type M4) showed morphological features of partial monocytic differentiation and tended to be NSE+ and SE+. Acute monocytic leukemia (AMoL, FAB type M5) tended to be NSE+ and SE-.

In some experiments, bone marrow cells were obtained from normal volunteers and similarly processed. Studies on normal cells were performed on fresh (noncryopreserved) samples.

**Colony assays for normal and leukemic progenitor cells.** L-CFC (7, 15, 16) and normal granulocyte-monocyte progenitor cells (CFU-GM) (17) were assayed by plating 0.25–1.0 × 10<sup>5</sup> cells/ml in 0.5 ml cultures in Iscove's modified Dulbecco's medium (IMDM, Gibco Laboratories, Grand Island, NY) containing 20% fetal calf serum (FCS, Flow Laboratories, Inc., Rockville, MD), 10% GTC conditioned medium (Gibco) (18), and 0.3% agar (Agar Noble, Difco Laboratories, Detroit, MI) over an underlayer containing 0.5% agar in the same medium. After 7–10 d, the agar overlayers were removed from the underlayers, dried on glass slides, fixed in methanol, and stained for SE and NSE activity and counter-stained with hematoxylin as previously described (19). All patients' colonies stained positively for either SE or NSE, or both. The cells in all colonies were morphologically blast cells, confirming their leukemic origin. Colonies composed of normal appearing granulocytes were rarely observed in this study. This assay system did not appear to support the growth of T cell colonies (7, 9). Aggregates of greater than 20 blast cells were enumerated as leukemic colonies (4, 5). Normal bone marrow or peripheral blood CFU-GM were assayed in the same manner. CFU-GM were enumerated on day 7 (clusters of 4–39 cells plus colonies of 40 or more cells) and day 14 (colonies of 40 or more cells). Mixed colonies (colony forming unit-mixed granulocyte/erythrocyte; CFU-Mix) were cultured in IMDM containing 0.9% methylcellulose, 30% FCS, 2 × 10<sup>-5</sup> M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 10% phytohemagglutinin-stimulated leukocyte conditioned medium (20). Erythropoietin (1 U/ml, Step III, Connaught Laboratories, Swiftwater, PA) was added on day 4. Marrow cells were plated at 1 × 10<sup>5</sup>/ml and mixed lineage colonies counted after 14–16 d.

**Complement lysis.** To determine the surface antigen phenotype of the CFU-Mix, CFU-GM, and L-CFC, aliquots of 1 × 10<sup>6</sup> cells suspended in RPMI 1640 (Gibco Laboratories) with 10% FCS were incubated with monoclonal antibodies (1:250 dilution of ascites) for 30 min at 4°C. After one wash step, the cells were suspended in rabbit complement (3–4-wk-old rabbits, Pel-Freeze Biologicals, Rogers, AR) at a dilution of 1:6. The complement had been previously adsorbed with fresh normal bone marrow mononuclear cells and had no demonstrable toxicity against CFU-GM or L-CFC. After 90 min at 37°C the cells

were washed once and resuspended in IMDM containing 20% FCS for colony assay. Negative controls included treatment with complement alone, and treatment with a nonbinding IgG monoclonal antibody (8A7) plus complement. Anti-beta-2-microglobulin (supplied by Dr. L. Nadler, Dana Farber Cancer Institute) was used as a positive control, and routinely produced lysis of >95% of L-CFC or CFU-GM.

**Immunofluorescence assays.** Antibody reactivity with the whole leukemic cell population cells was determined by indirect immunofluorescence (8). Aliquots of 0.5–1.0 × 10<sup>6</sup> cells were incubated (4°C, 30 min) with 100 µl of antibody (ascites fluid diluted 1:250 in minimal essential medium (MEM) containing 2.5% human AB serum, washed two times, and further incubated with fluorescein-conjugated goat anti-mouse Ig (Tago Laboratories, Burlingame, CA). After two additional wash steps, fluorescent antibody-coated cells were detected by a flow cytometer (FC200/4800A, Ortho Instruments, Westwood, MA). Background fluorescence, determined by using a nonreactive monoclonal IgG antibody, was subtracted. For the experiments described in Table IV, a positive reaction was considered to be >20% of cells more fluorescent than background.

## Results

**AML patient population.** Samples from 113 patients were tested for in vitro colony formation according to the condition described in Methods. The morphological subtypes of these patients using the FAB classification were M1, 24%; M2, 10%; M3, 8%; M4, 44%; and M5, 14%. 53 patients (47%) displayed no in vitro growth, 34 (30%) formed clusters of 3–19 cells, and 26 (23%) formed colonies composed of more than 20 cells. Sufficient cells were available from 20 of these latter cases for repeated testing, and an analysis of these samples forms the basis of this report. The diagnosis of AML was established in all cases by standard morphologic and cytochemical criteria (14), and confirmed by surface marker analysis (21).

Table I. Patient Characteristics

| Patient number | Age | Sex | FAB classification | Sources* of cells | Percent blasts |
|----------------|-----|-----|--------------------|-------------------|----------------|
| 1              | 65  | M   | M4                 | BM                | 95             |
| 2              | 52  | F   | M1                 | PB                | 99             |
| 3              | 82  | M   | M2                 | PB                | 54             |
| 4              | 29  | M   | M1                 | PB                | 90             |
| 5              | 24  | F   | M1                 | PB                | 90             |
| 6              | 1   | M   | M1                 | BM                | 80             |
| 7              | 33  | M   | M5                 | PB                | 90             |
| 8              | 27  | F   | M4                 | BM                | 85             |
| 9              | 37  | M   | M4                 | BM                | 95             |
| 10             | 10  | M   | M4                 | PB                | 99             |
| 11             | 67  | F   | M4                 | PB                | 90             |
| 12             | 53  | F   | M5                 | BM                | 90             |
| 13             | NA‡ | M   | M4                 | PB                | 75             |
| 14             | 2   | F   | M4                 | BM                | 100            |
| 15             | 29  | M   | M4                 | PB                | 90             |
| 16             | 4   | F   | M4                 | PB                | 90             |
| 17             | 51  | F   | M4                 | PB                | 85             |
| 18             | NA  | F   | NA                 | PB                | 95             |
| 19             | 83  | F   | M4                 | PB                | 95             |
| 20             | 83  | F   | M2                 | BM                | 90             |

\* Bone marrow (BM) or peripheral blood (PB).

‡ Information not available.

A summary of clinical characteristics of the patient population is shown in Table I. The patients ranged in age from 1 to 83 yr (median, 43 yr). There were 9 males and 11 females. 20% of patients' cells were classified as FAB type M1, 10% as M2, 55% as M4, and 10% as M5. The cells of one patient (number 18) were not classifiable by FAB criteria and are listed as "unclassified." 65% of samples studied were peripheral blood, and 35% were bone marrow. Peripheral blood samples contained 54–99% blasts (median, 90%) and bone marrow samples contained 80–100% blasts (median, 90%).

**Surface antigen phenotype of normal CFU-GM.** The monoclonal antibody panel was selected to provide maximum ability to discriminate among different stages of granulocyte-monocyte precursor cells ranging from the normal "mixed" colony forming cell to the mature monocyte and granulocyte. The reactivities of anti-Ia (anti-I2), MY9, PM-81, AML-2-23, Mo1, Mo2, and MY3 with normal mature and immature bone marrow myeloid cells determined primarily by cell sorting experiments have previously been reported (8–13) and are summarized in Fig. 1. To precisely compare the surface antigens of L-CFC with corresponding normal progenitor cells, the reactivity of this panel of antibodies with normal bone marrow CFU-GM and CFU-Mix from a series of normal donors was determined by complement lysis in experiments performed simultaneously with L-CFC lysis experiments (Table II).

As shown in Table II, CFU-Mix cells express Ia and MY9 antigens, but lack PM-81, AML-2-23, Mo1, Mo2, and MY3. The day 14 CFU-GM express Ia and MY9, and in addition

express PM-81 (on 59% of cells). The most mature clonogenic cell, the day 7 CFU-GM, expresses Ia, MY9, and PM-81, and now also expresses AML-2-23 antigen. As shown in Table II, the acquisition of these differentiation-associated antigens during normal hematopoiesis may be "gradual," particularly in the cases of PM-81 and AML-2-23 antigens as summarized schematically in Fig. 1. Further, the data indicate that the antigens Ia, MY9, PM-81, AML-2-23, Mo1, Mo2, and MY3 are added sequentially during normal monocyte development from CFU-Mix cells, and thus can be used to phenotypically "order" different stages of hematopoietic cells; i.e., a cell expressing Ia, MY9, and PM-81 is more mature than a cell expressing only Ia and MY9.

**Surface antigen phenotype of L-CFC.** A comparison of the surface antigens expressed by the total AML cell population (determined by indirect immunofluorescence) and the L-CFC (determined by complement lysis) is shown in Table III. The plating efficiency of the leukemic samples ranged from  $44 \pm 8$  to  $2,992 \pm 80$  colonies per  $10^5$  cells. In those cases where preliminary studies revealed colony overgrowth at  $5 \times 10^4$  cells/culture, cells were plated at  $1.25 \times 10^4$  cells/culture. The relationship of the number of colonies obtained to number of cells plated was determined by plating leukemic cells from cases 5, 8, 9, and 16 at concentrations from  $1.5 \times 10^3$  to  $5 \times 10^4$  cells per culture. Fig. 2 shows a linear relationship between the number of cells plated and the number of colonies observed within this range.

90% of the samples tested expressed Ia, MY9, and PM-81 on >20% of the total cell population, while AML-2-23 was positive on 84%, Mo1 on 85%, Mo2 on 37%, and MY3 on 40%. As described, different surface antigen phenotypes correlate with certain morphologic features (21). FAB M1 and M2 leukemias tend to express antigens of early myeloid cells such as MY9, and lack antigens of later monocytoïd cells such as MY3 and Mo2, while myelomonocytic leukemias (FAB M4) tend to express the monocyte markers MY3 and Mo2. In the present series, eight of nine cases that expressed Mo2, MY3, or both were thus classified as AMML (FAB M4), and only one of six cases of M1 or M2 leukemia expressed either of these two monocyte antigens on >20% of leukemic cells. It is noteworthy that both of the M5 cases (classified independently by morphology and cytochemistry) lacked expression of Mo2 and MY3, an uncommon finding in monocytic leukemia. Of the three M1 leukemia cases evaluated for expression of all the antigens, all three expressed Ia, MY9, and Mo1 (low antigen density of Mo1 in each case), two expressed PM-81, and no cases expressed AML-2-23, Mo2, or MY3.

The phenotypes of the L-CFC were frequently different from the phenotypes of the majority cell populations (Table III). Ia antigen was expressed on 42–100% of L-CFC (median 90%), MY9 on 21–99% (median 91%), PM-81 on 0–96% (median 66%), AML-2-23 on 0–96% (median 20%), Mo1 on 0–61% (median 2%), Mo2 on 0–34% (median 0%), and MY3 on 0–32% (median 6%). A two-sided sign test (22) was used to determine if there were any significant differences between the percentages of total cells expressing each antigen, and the percentage of L-CFC expressing that antigen. Ia and MY9 were expressed on a higher percentage of L-CFC than total cells ( $P = 0.02$  and  $0.001$ , respectively), while Mo1 and Mo2 were expressed on a higher percentage of total cells than

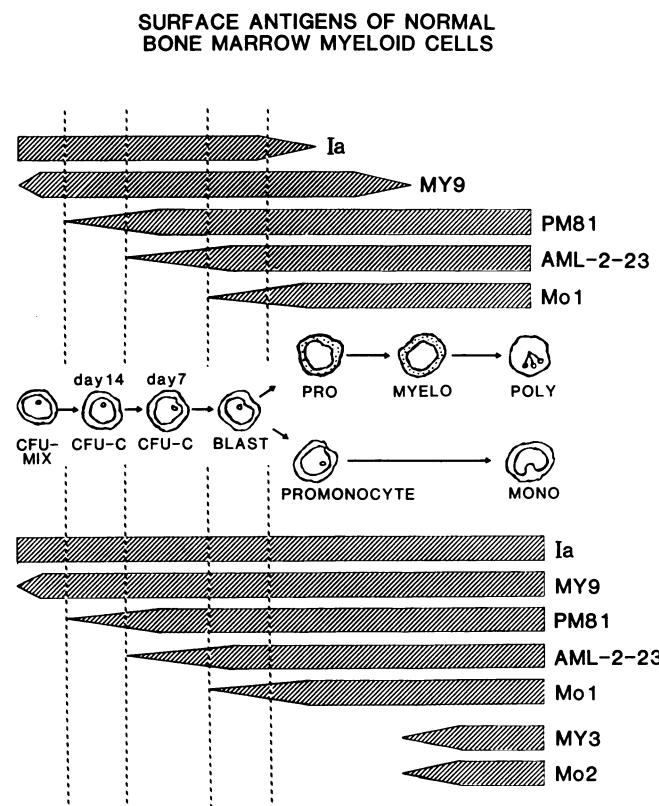


Figure 1. Schematic representation of the distribution of surface antigens on normal bone marrow myeloid cells.

*Table II. Surface Antigens of Normal Bone Marrow Colony Forming Cells*

| Antigen  | Percent cells* positive | Percent positive colonies† |       |                  |      |          |       | CFU- Mix | P |
|----------|-------------------------|----------------------------|-------|------------------|------|----------|-------|----------|---|
|          |                         | Day 7<br>CFU-GM            | P§    | Day 14<br>CFU-GM | P    | CFU- Mix | P     |          |   |
| Ia       | 21±4                    | 87±11                      | NS    | 95±8             | NS   | 91±12    | 0.006 |          |   |
| MY9      | 40±13                   | 85±14                      | NS    | 78±15            | 0.03 | 65±16    | 0.006 |          |   |
| PM-81    | 29±8                    | 71±19                      | NS    | 59±19            | 0.03 | 7±3      | NS    |          |   |
| AML-2-23 | 56±27                   | 54±24                      | 0.016 | 29±15            | 0.03 | 10±18    |       |          |   |
| Mo1      | 38±7                    | 10±9                       |       | 12±10            |      | 0        |       |          |   |
| Mo2      | 11±3                    | 9±9                        |       | 8±9              |      | 0        |       |          |   |
| MY3      | 12±2                    | 11±2                       |       | 9±8              |      | 0        |       |          |   |

\* Percent of total bone marrow mononuclear cells positive by indirect immunofluorescence (10,000 cells analyzed, three samples, mean±SD).

† Percent decrease in colonies after treatment with antibody and complement, compared to treatment with negative control antibody and complement, mean±SD of six experiments with triplicate cultures in each.

§ P values based on one-sided sign test. NS, not significant.

L-CFC ( $P = 0.01$  and  $0.02$ , respectively). Most importantly, in virtually every case there were one or more “late” antigens expressed on the majority of leukemic cells that were not

detected on L-CFC. In cases where clusters were found in addition to colonies, the phenotype of clusters was similar to the phenotype of colonies (data not shown). Two antigens, Ia

*Table III. Comparison of Surface Antigens of the Total AML Cell Population and the L-CFC*

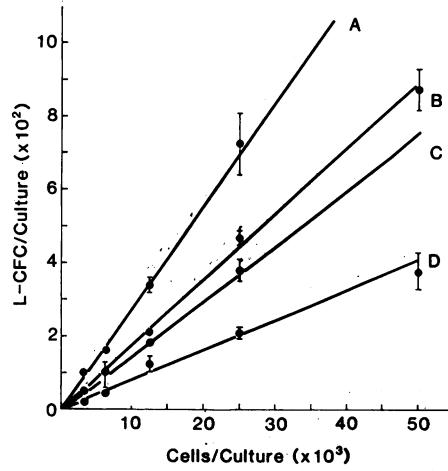
| Patient number               | L-CFC/ $10^5$ cells* | Percent antigen positive cells |        |       |       |       |       |          |       |       |       |       |       |       |       |
|------------------------------|----------------------|--------------------------------|--------|-------|-------|-------|-------|----------|-------|-------|-------|-------|-------|-------|-------|
|                              |                      | IA                             |        | MY9   |       | PM-81 |       | AML-2-23 |       | Mo1   |       | Mo2   |       | MY3   |       |
|                              |                      | Total‡                         | L-CFC§ | Total | L-CFC | Total | L-CFC | Total    | L-CFC | Total | L-CFC | Total | L-CFC | Total | L-CFC |
| 1                            | 768±176              | 79                             | (95)   | 5     | (31)  | 45    | (18)  | 71       | (29)  | 34    | (0)   | 7     | (0)   | 24    | (0)   |
| 2                            | 536±56               | 97                             | (100)  | 72    | (96)  | NA    | (10)  | NA       | (5)   | 3     | (21)  | 1     | (15)  | 1     | (6)   |
| 3                            | 110±8                | 54                             | (100)  | 74    | (91)  | 35    | (0)   | 49       | (0)   | 54    | (28)  | NA    | (NA)  | 46    | (15)  |
| 4                            | 169±9                | 93                             | (93)   | 86    | (99)  | 64    | (25)  | 13       | (20)  | 42    | (0)   | 10    | (0)   | 10    | (14)  |
| 5                            | 740±66               | 60                             | (99)   | 37    | (98)  | 18    | (24)  | 10       | (6)   | 21    | (0)   | 6     | (0)   | 6     | (11)  |
| 6                            | 126±18               | 13                             | (42)   | 65    | (79)  | 29    | (0)   | 22       | (0)   | 38    | (0)   | 17    | (0)   | 17    | (23)  |
| 7                            | 252±18               | 82                             | (62)   | 72    | (78)  | 96    | (20)  | 96       | (0)   | 76    | (0)   | 4     | (0)   | 2     | (0)   |
| 8                            | 2,992±80             | 85                             | (84)   | 86    | (99)  | 92    | (93)  | 64       | (49)  | 22    | (11)  | 9     | (17)  | 6     | (7)   |
| 9                            | 2,208±196            | 97                             | (76)   | 53    | (78)  | 92    | (80)  | 75       | (0)   | 41    | (0)   | 2     | (20)  | 7     | (12)  |
| 10                           | 256±46               | 31                             | (52)   | 60    | (99)  | 65    | (75)  | 15       | (39)  | 17    | (34)  | 16    | (0)   | 13    | (16)  |
| 11                           | 922±6                | 65                             | (46)   | 97    | (96)  | 89    | (50)  | 69       | (0)   | 80    | (44)  | 43    | (0)   | 66    | (0)   |
| 12                           | 1,200±92             | 4                              | (57)   | 98    | (99)  | 74    | (53)  | 52       | (40)  | 39    | (12)  | 9     | (19)  | 6     | (32)  |
| 13                           | 64±10                | 72                             | (94)   | 50    | (69)  | 87    | (56)  | 93       | (0)   | 90    | (0)   | 83    | (34)  | 94    | (0)   |
| 14                           | 136±6                | 37                             | (71)   | 51    | (80)  | 99    | (96)  | 75       | (12)  | 8     | (0)   | 23    | (0)   | 16    | (11)  |
| 15                           | 622±38               | 94                             | (99)   | 25    | (84)  | 78    | (85)  | 83       | (59)  | 79    | (4)   | 63    | (12)  | 75    | (11)  |
| 16                           | 1,508±132            | 79                             | (98)   | 71    | (99)  | 90    | (98)  | 71       | (81)  | 71    | (7)   | 56    | (0)   | 64    | (0)   |
| 17                           | 64±10                | 93                             | (94)   | 66    | (91)  | 87    | (84)  | 80       | (75)  | 74    | (0)   | 54    | (5)   | 78    | (6)   |
| 18                           | 132±16               | 87                             | (94)   | 69    | (92)  | 99    | (96)  | 78       | (96)  | 89    | (61)  | 8     | (0)   | 9     | (0)   |
| 19                           | 44±8                 | 59                             | (68)   | 66    | (100) | 85    | (77)  | 79       | (55)  | 82    | (22)  | 31    | (0)   | 65    | (0)   |
| 20                           | 175±5                | 79                             | (87)   | 75    | (21)  | 84    | (93)  | 88       | (91)  | 30    | (0)   | 14    | (0)   | 18    | (0)   |
| <i>P</i> value <sup>  </sup> |                      | 0.02                           |        | 0.001 |       | 0.06  |       | 0.06     |       | 0.001 |       | 0.02  |       | 0.26  |       |

\* L-CFC/ $10^5$  cells (sham treated with control antibody and complement) (mean±SD of quadruplicate cultures).

† Percent of total AML cells positive by indirect immunofluorescence (10,000 cells analyzed).

‡ Percent decrease in L-CFC number following L-CFC lysis by antibody and complement (mean of quadruplicate cultures, data shown is one of two representative experiments).

§ *P* values based on a two-sided sign test comparing “total” versus “L-CFC” for each antigen.



**Figure 2.** Effects of leukemic cell concentration on L-CFC cloning efficiency. Cells from four cases (A, B, C, and D represent cases 8, 16, 9, and 5, respectively) were cultured in agar at cell concentrations from  $1.5 \times 10^3$  cells/ml culture to  $50 \times 10^3$  cells/culture, and L-CFC enumerated on day 7.

and MY9, were detected on the majority of L-CFC from almost all cases studied. PM-81 and AML-2-23 antigens were expressed on the L-CFC from some cases, but not from others (despite being expressed on the majority of leukemic cells from 94 and 84% of cases, respectively). Further, although several cases were identified in which L-CFC were PM-81 positive and AML-2-23 negative, the converse was not observed. The data in Table III thus suggest that several "stages" of L-CFC could be distinguished by surface antigen phenotype: a stage at which only Ia and MY9 were expressed, a stage at which PM-81 antigen was acquired in addition to Ia and MY9, and a stage at which AML-2-23 was acquired in addition to Ia, MY9, and PM-81. In an effort to investigate these apparent stages in more detail, groups of patients with similar L-CFC phenotypes were identified (arbitrarily defining an antigen as "positive" if there was 50–100% lysis of L-CFC, and "negative" if there was 0–49% lysis). It should be noted that the majority of positive results had greater than 75% lysis, and the majority

of negative results less than 25%. Using this definition, the L-CFC of the 20 cases analyzed here (Table III) were divided into three phenotypic groups. Patients 2, 3, 4, 5, and 7 (group I) expressed Ia and MY9 on L-CFC, but lacked PM-81, AML-2-23, Mo1, Mo2, and MY3. Two additional patients were included in this group. Patient 1 expressed Ia on 95% on L-CFC but MY9 was low (31%). Patient 6 expressed MY9 on 79% but Ia expression was low (42%). However, PM-81, AML-2-23, Mo1, Mo2, and MY3 were not detected on the L-CFC of either case. Cases 8–14 (group II) expressed Ia, MY9, and PM-81 on L-CFC, but lacked AML-2-23, Mo1, Mo2, and MY3. One additional patient was included in this group. Patient 11 expressed MY9 on 96% of L-CFC, and PM-81 on 50% but Ia was low (46%). Patients 15–19 (group III) expressed Ia, MY9, PM-81, and AML-2-23 on L-CFC. One additional patient (20) expressed Ia on 87%, PM-81 on 93%, AML-2-23 on 91% of L-CFC, but MY9 was low (21%).

The three arbitrarily-defined L-CFC groups were further analyzed to determine if there were any biologically or clinically significant correlations between L-CFC phenotype, surface antigens expressed by the total cell population, and FAB classification (Table IV). Within each group, the leukemic cell populations have acquired surface antigens that are not expressed on the L-CFC as noted above. For example, a majority of cells in the total cell population of cases in group I expressed PM-81, AML-2-23, and Mo1 in addition to Ia and MY9 antigens present on the L-CFC. Group II patients tend to acquire AML-2-23, Mo1, and Mo2 on the total cell population, while group III patients expressed Mo1 in 100% of the cases, Mo2 in 67%, and MY3 in 67%. The FAB subsets were disproportionately distributed among the L-CFC groups, with all of the M1 patients in group I, and 91% of the M4 patients in groups II and III. These results suggest that the surface antigens and thus the level of differentiation attained by the majority leukemic cell population (21) in many cases of AML are at least partially related to the stage of differentiation of the L-CFC.

These results also make it possible to relate the L-CFC to a counterpart normal myeloid cell. The relationship between the phenotypes of L-CFC and normal myeloid progenitor cells is illustrated schematically in Fig. 3.

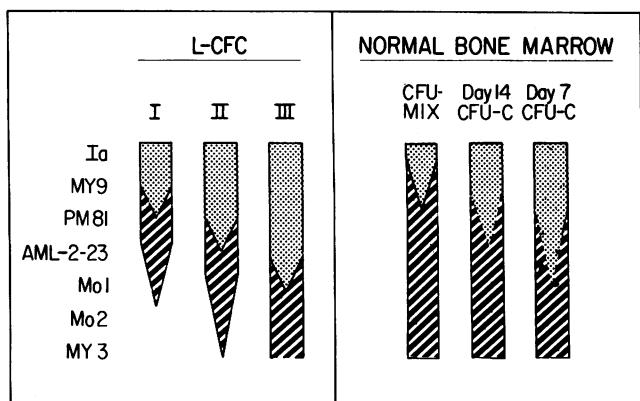
**Table IV. Phenotypic Groups of L-CFC**

| L-CFC* group                       | Percent of cases expressing antigen on greater than 20% of total cell population |      |       |          |      |      |      | Distribution of cases by FAB classification |    |        |    |
|------------------------------------|--|------|-------|----------|------|------|------|---|----|--------|----|
|                                    | Ia   | MY9  | PM-81 | AML-2-23 | Mo1  | Mo2  | MY3  | M1  | M2 | M4     | M5 |
| n                                  |  |      |       |          |      |      |      | %   | %  | %      | %  |
| I (Ia+, MY9+)                      | 7  | 86   | 86    | 83       | 50   | 86   | 0    | 29  | 4  | 1      | 1  |
| II (Ia+, MY9+, PM-81+)             | 7  | 86   | 100   | 100      | 86   | 71   | 43   | 29  | 0  | 0      | 6  |
| III (Ia+, MY9+, PM-81+, AML-2-23+) | 6  | 100  | 100   | 100      | 100  | 100  | 67   | 67  | 0  | 1      | 4  |
| P value†                           |  | 1.0‡ | 1.0   | 0.63     | 0.15 | 0.48 | 0.07 | 0.33  |    | 0.013§ |    |

\* L-CFC groups are identified by surface antigen phenotype of the L-CFC (each antigen expressed on 50–100% of L-CFC in each case).

† P values based on Fisher's Exact Test (22) to determine if the probability of expression of each antigen is independent of the L-CFC group.

‡ P value based on Fisher's Exact Test for a 4 × 3 table = 0.013.



**Figure 3.** Comparison of surface antigen phenotypes of L-CFC and normal bone marrow progenitor cells. The surface antigens expressed by the colony forming cells are shown (dotted pattern) along with the surface antigens acquired during the process of differentiation of the leukemic or normal progenitor cells (striped pattern).

## Discussion

In this study, we have explored the relationships between clonogenic AML cells and the total leukemic cell population in detail using surface antigen analysis. The results show that L-CFC are a distinct subpopulation of cells in all morphologic types of AML and that the majority of cells in each patient with AML are more differentiated than are the L-CFC of the same patient. In order to further investigate L-CFC heterogeneity, we defined three stages of L-CFC by differentiation associated phenotypes. L-CFC from 7 of 20 cases studied expressed only Ia and MY9; a second group of seven cases expressed Ia, MY9, and PM-81, and a third group of six cases expressed Ia, MY9, PM-81, and AML-2-23 (Table III). Interestingly, these antigens are acquired in normal granulocyte-monocytic differentiation in the same sequence (Fig. 1 and Table II). Thus, L-CFC from one-third of the cases studied here expressed surface antigens characteristic of multipotent progenitor cells, one-third expressed antigens of an "early" CFU-GM, and one-third expressed antigens of a "late" CFU-GM. The correlations between L-CFC and normal granulocytic-monocytic progenitor cells demonstrate that AML is a heterogeneous disorder not only at the level of the major population of "terminal" leukemic cells (21), but also at the level of the leukemic clonogenic cell, in agreement with the results of Lange et al. (23) and our previous studies in acute myelomonocytic leukemia (7).

Previous attempts to define the cell of origin of AML have also suggested that the leukemic stem cells may be derived from very early progenitor cells. Fialkow (1) determined the clonality of AML cells by glucose-6-phosphate dehydrogenase (G6PD) isoenzymes in four patients. In two pediatric cases, erythroid cells were not found to be part of the malignant clone, while in two adult cases, the disease appeared to involve stem cells multipotent for myeloid cells, erythrocytes, and platelets. Other groups have attempted to trace the AML clone using marker chromosomes. Blackstock (24) et al. and Jensen et al. (25) reported the presence of a marker chromosome in erythroid lineage cells in cases of AML, and Reid et al. (26) reported that a marker translocation  $t(1q-; 11q+)$  could be

found in normal appearing CFU-GM from a child with AML, suggesting that an early stem might be involved in the pathogenesis of some cases of AML. The results presented here support the observation of Fialkow (1) that some cases of AML may originate in a multipotent progenitor cell, while others arise in a later cell already committed to the myeloid lineage. The possibility exists, however, that pluripotent stem cells are involved in the leukemic process in most cases of AML, but fail to proliferate in the assay system used here or are present in very small numbers. Several observations tend to support the concept that the L-CFC assayed in this study are representative of the major clonogenic cell that can be cultured. First, L-CFC with the phenotype of a multipotent cell were observed in a distinct subset of cases, demonstrating that such cells can be identified with this culture system. Second, the clonogenic cells in each case of AML tended to be a relatively uniform subset. Multiple levels of L-CFC in the same patient thus were generally not observed, even though L-CFC were strikingly different in different patients. Finally, the majority of leukemic colonies in individual cases tended to be of uniform size (generally 20–40 cells) and to follow similar kinetics in culture, suggesting that the L-CFC are a reasonably homogeneous population with respect to proliferative potential, unlike normal bone marrow progenitor cells. However, it is not possible to be certain that any culture system will promote growth of the earliest possible leukemic progenitor cell, or that the *in vitro* clonogenic cells are representative of *in vivo* stem cells. Similar studies need to be done with the other L-CFC culture systems (3, 27, 28). It may be possible to determine if the different assay systems detect the same L-CFC cell by using the surface antigen phenotype methods described here. Data from other laboratories suggest that agar and methyl cellulose L-CFC culture systems may be similar (15). In our own pilot studies comparing agar and methylcellulose cultures, no reproducible differences in colony number or size were observed. Agar was selected because of the ease with which whole cultures could be stained cytochemically for myeloid specific esterase activities. We chose to avoid conditions that would support the growth of T cell colonies (phytohemagglutinin or phytohemagglutinin-stimulated leukocyte conditioned medium) (28). Every effort was also made here to avoid "contamination" of leukemic cells with residual normal CFU-GM by testing only patients with near total replacement of marrow by leukemic cells, and by testing peripheral blood when possible (where the number of possible residual normal myeloid progenitor cells is several orders of magnitude lower than the number of L-CFC).

The differentiation stage of the L-CFC did not correlate with age in this study. Pediatric cases were equally distributed among the groups of both multipotent and more differentiated L-CFC, and the two oldest patients (patients 19 and 20) had L-CFC in the most differentiated group (III). The AML patients studied here, however, were *de novo* cases and did not include patients with a prior history of a myelodysplastic syndrome or a myeloproliferative disease. In these stem cell disorders, the L-CFC might be anticipated to be more uniformly related to a multipotent cell. However, it is also possible that the final leukemic transforming event in these patients also takes place in a later progenitor cell. It would be of interest to determine self-renewal potential of the different stages of L-CFC. Wouters et al. (6) have presented evidence that a

hierarchy of AML blasts exists by demonstrating that cells with high proliferative potential also have high self-renewal capacity and can be distinguished from cells with lower proliferative potential and lower self-renewal capacity.

There is ample evidence that AML cells and AML cell lines can be induced to differentiate in vitro (8, 29-32). If the L-CFC assayed in vitro do indeed function as leukemic stem cells in vivo, then our results suggest that partial and often abnormal degrees of differentiation are an on-going process in vivo. It is interesting, however, that different stages of L-CFC appear to have different abilities to differentiate, thus resulting in different morphological and immunological phenotypes. The most immature L-CFC (group I) tended to be associated with FAB classification M1, while the more differentiated L-CFC (groups II and III) tended to be associated with M4 and M5 (Table IV). The expression of surface antigens on the total cell population was also related to the degree of differentiation of the L-CFC. Thus, although Ia, MY9, and PM-81 antigens were expressed on the leukemic cells of all patients, the percentage of cases expressing AML-2-23, Mo1, Mo2, and MY3, and the percentage of cells in each individual case expressing these antigens, tended to increase from group I to group II to group III. The analogies between leukemic and normal differentiation are summarized in Fig. 3.

The results presented here have several implications for the therapy of AML. The ability to immunologically purge leukemic progenitor cells from a remission bone marrow could allow autologous bone marrow transplantation despite the lack of leukemia-specific markers. This approach has been used successfully in the treatment of acute lymphoblastic leukemia (anti-CALLA) (33) and aggressive histology lymphomas (anti-B1) (Nadler, L. M., T. Takvorian, L. Botnick, R. C. Bast, R. Finberg, S. Hellman, G. P. Canellos, and S. F. Schlossman, submitted for publication). The heterogeneity of AML cells, especially at the level of the clonal cells, may make application of this technique more difficult in AML. Our results do demonstrate that antigens can be identified, such as AML-2-23 and PM-81, that are not expressed on the multipotent normal progenitor cells needed for marrow recovery, but are expressed on clonal cells from at least a subset of AML patients. Anti-MY9 could also have clinical utility since it reacts with only a fraction of normal multipotent progenitor cells. Clinical trials with antibodies [AML-2-23 and PM-81 (34)] are underway, and may ultimately allow an assessment of the biological significance of L-CFC.

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