Granulopoietic Differentiation in AML Blasts in Culture

By J. P. Marie, C. A. Izaguirre, C. I. Civin, J. Mirro, and E. A. McCulloch

A newly described monoclonal antibody detected an antigen (My-1) that is expressed on the cell surface during normal granulopoietic differentiation. The kinetics of expression of My-1 and NASD chloroacetate esterase were studied during the formation in culture of blast colonies and normal granulopoietic colonies. My-1-positive cells increased during blast colony formation in 6 of 9 experiments, while NASD esterase-positive cells decreased. In contrast, in normal granulopoiesis in culture both markers increased coherently. We suggest that components of granulopoietic differentiation programs are expressed abnormally during blast colony formation in culture.

LAST CELLS from the peripheral blood of patients with acute myeloblastic leukemia (AML) form colonies in culture when stimulated by medium conditioned by leukocytes in the presence of phytohemagglutinin (PHA-LCM). Blast colonies consist of 20 to several hundred cells that retain characteristic blast cell morphology; however, the cells in each colony are functionally heterogeneous. A minority of the cells are capable of colony formation upon replating, while the majority are proliferatively inert under the culture conditions used to promote growth. These data have been taken to indicate that blast cell progenitors may either renew themselves or, alternatively, enter into a series of terminal divisions during which proliferative potential is lost. This latter process may be analogous to maturation occurring in normal myelopoiesis, although it is not accompanied by obvious morphological change.

The purposes of this article are (1) to use anti-My-1, together with the histochemical detection of the granulopoietic marker NASD chloroacetate esterase, to search for evidence of maturation during blast colony formation; and (2) to compare blast colony formation to normal granulopoietic colony formation with respect to the kinetics of expression of these markers.

MATERIALS AND METHODS

Blood or bone marrow was obtained from 9 patients with AML or its variants at presentation or in relapse. In one case blood samples were obtained on two occasions, separated by 1 mo. Heparinized (10 U/ml) specimens were collected as part of hematologic investigation for diagnosis or treatment.

Normal blood was obtained from volunteer donors. Marrow transplant donors provided control bone marrow; this was aspirated at the time of investigation of these individuals for their suitability as donors.

Cell Preparation

Mononuclear cell suspensions were obtained from peripheral blood or marrow by centrifugation using density 1.077 Ficoll-Hypaque. A second centrifugation after sheep erythrocytes rosette formation permitted depletion of T lymphocytes as described by Minden et al. These T-cell-depleted populations were either cultured immediately or preserved by freezing at −70°C in 10% DMSO and 10% fetal calf serum.

 Colony Assays

Blast colony formation. Blast colony formation was obtained as previously described by incubating between 10⁴ and 4 x 10⁵ T-depleted mononuclear cells in methylcellulose, growth medium, and PHA-LCM. After varying periods of incubation, cultures were examined. Colonies were counted using an inverted microscope. Cells from colonies were obtained by pooling several dishes. Progenitors of the myelopoietic lineage were picked from cultures prepared for the growth of pluripotent stem cells (CFU-GEMM) described by Fauser and Messner. In this procedure, T-depleted mononuclear preparations were plated in methylcellulose, with growth medium PHA-LCM and erythropoietin (2 U/ml). After incubation for 10–15 days, plates prepared in this manner contained not only colonies consisting of a mixture of myelopoietic elements but also erythropoietic bursts and granulopoietic colonies derived, respectively, from BFU-E and CFU-C. Thus, each class could be evaluated individually by picking individual colonies and verifying their cellular composition. Colonies of T cells, B cells, and blasts from common (non-B, non-T) acute lymphoblastic leukemia were grown as described previously.

Self-renewal of blast progenitors. Cells from blast colonies were pooled and replated in microwells as described by Buick et al.

My-1

The cell surface antigen, My-1, is present only on normal mature and developing granulocytes and a subpopulation of AML blast cells. It was defined by several monoclonal antibodies secreted by mouse lymphocyte hybridomas. Hybridomas had been prepared by polyethylene-glycol-induced fusion of P3-NS1/1-A4-1 mouse plasmacytoma cells with splenic lymphocytes from a C57BL/6J mouse that has been hyperimmunized with HEL-60 cells. Several cloned hybridomas from such fusions produced antibodies that detected the antigen designated My-1. In these studies, most experiments used monoclonal antibody 1/21/1; however, in control experiments the results obtained with this material were found to be
equivalent to those with antibody 1/12/13, the monoclonal antibody that is available for distribution. Both are IgM antibodies.

**Immunofluorescence**

My-1 was detected by indirect immunofluorescence. Cells suspended in phosphate-buffered saline (PBS), 1% bovine serum albumin, and 0.1% sodium azide (IF medium) were incubated with a 1:10 dilution of anti-My-1 (hybridoma culture supernatant) at room temperature for 45 min; cells were washed 3 times in IF medium, then incubated with 1:20 dilution of rhodamine-labeled goat IgG anti-mouse immunoglobulin (Cappel Labs) at room temperature for 30 min. After a further 3 washes in IF medium, the cells were deposited on slides by cytocentrifugation; they were fixed with cold alcohol and a cover applied using glycerol in PBS. The slides were examined using ultraviolet epi-illumination and appropriate rhodamine filters. Quantitative data was obtained from the examination of 200–400 cells.

Controls consisted of supernatants obtained from parental mouse myeloma cells used in the fusion; other controls consisted of cells exposed to the second antibody only.

**NASD Esterase**

Naphthol AS-D chloracetate esterase (NASD) stains were prepared according to the methods of Yam et al.¹⁴

**RESULTS**

Cells from the peripheral blood or bone marrow of 8 patients with AML together with primary blast colonies and secondary colonies obtained by replating were tested for the presence of My-1 and NASD esterase. A small proportion of cells were found to be positive for each in fresh preparations, but this number changed during culture. Data for 2 experiments are given in Fig. 1. In the figure, the 0 time point is the percentage of My-1 or NASD esterase positive cells in the population plated. Growth originates from a small minority of this population capable of colony formation; hence, the 0 time point is not included in the line depicting kinetics. The increase in My-1-positivity is apparent, reaching a peak on day 7. This increase was associated with cell growth, as serial observations indicated increasing colony size with time. Cell viability remained high, since when the cells were stained with anti-My-1 very few showed whole cell dull fluorescence, an indicator of cell death.

The kinetics of NASD-esterase-positive cells was different; in spite of increasing numbers of viable cells in the cultures, the percentage of NASD esterase cells declined.

Figure 2 contains photomicrographs illustrating the My-1-positive immunofluorescence reaction. The left side of the figure shows two fields under phase microscopy; on the right side the same fields are shown using u.v. illumination to demonstrate immunofluorescence. It is apparent from the phase microscopy that blast-like morphology was maintained; the immunofluorescence shows that some

![Fig. 1. Sequential analysis of My-1 antigen and NASD chloracetate esterase in blast colonies. (A) Case 1b and (B) case 3 show no correlation between both granulopoietic markers and variation from patient to patient.](image)

Two different My-1 distributions on the cell surface are illustrated in the photographs.

The data from 8 patients are presented in Table 1. The table contains percentage of blast cells positive for My-1 and NASD in fresh preparations and after 7 days in culture; data for primary (PE1) and replated (PE2) cultures are shown together with plating efficiencies of blast colonies. In 6 of the 9 experiments, My-1 expression increased during culture; in two patients no change was seen; in one instance, where My-1-positive cells were very numerous in fresh cells, their number decreased in culture. In contrast, the percentage of NASD-positive cells decreased in all but one patient and in that instance remained constant. In one patient (patient 2, Table 1) data were available for cells from two bleeds separated by a month. The patterns of change in culture were similar on both occasions.

**My-1 Antigen in Normal Hemopoietic Colonies**

My-1 is known to be present in normal granulopoietic cells. Figure 3 contains the kinetics of My-1
and NASD expression during granulopoietic colony formation from normal bone marrow precursor cells. As in Fig. 1, the 0 time point was taken as the percentage of positive cells for each marker found in the T-cell-depleted mononuclear fraction of bone marrow used to initiate the cultures and is not included in the kinetics. There was an increase in the percent of cells positive for each marker, reaching a peak on day 9 and declining rapidly thereafter. Morphological examination of the cells obtained from colonies indicated that up until day 9 they consisted of granulopoietic cells, while at later times monocytes and macrophages predominated.

In addition, erythropoietic colonies derived from BFU-E, monocyte and macrophage colonies, T-cell colonies, and colonies derived from the blast cell from CALL were all found to be negative for My-1 antigen. Colonies containing both granulocytes and erythrocytes (CFU-GEMM) were also tested; My-1 was detected only in cells with granulopoietic morphology.

**DISCUSSION**

The My-1 cell surface antigen was detected using monoclonal antibodies raised against HL-60 cells, a continuous line of leukemic promyelocytes. The data in this article confirm the specificity of the My-1 antigen for granulopoiesis; My-1 may, therefore, be assumed to be a component in granulopoietic differentiation programs.

My-1 was found in association with small proportions of blast cells from the peripheral blood or marrow of 8 AML patients and a large proportion of the blast cells from 1 AML patient. This confirms the initial description of the My-1 expression in AML, where only 1 of 9 patients was "My-1 positive" since in that study, at least 50% of the cells had to be labeled to...
be scored as "My-1 positive." My-1 is apparently present on smaller populations of blasts in most AML patients.

The major finding of this study is that the percentage of My-1-positive blast cells changed during colony formation in culture; this change was an increase in 6 of the 9 experiments. In only 1 case did the percentage positive cells decrease. In contrast, blast cell NASD chloracetate esterase percentages usually decreased during blast colony formation; an indication that the growth of blast cells was not associated with increased expression of this myelopoietic differentiation marker; indeed no correlation was found between these two markers when malignant blasts were cultured. These findings may be interpreted to mean that certain components of the granulopoietic differentiation pattern may be expressed during blast colony formation in culture even though morphological evidence of differentiation is not seen. In contrast with the finding for blast colony formation, the presence of My-1 antigen and NASD chloracetate were closely correlated during normal granulopoietic maturation in colonies. The observation is consistent with a close linkage between the two differentiation markers in normal granulopoietic programs. From this point of view, granulopoietic differentiation markers are expressed abnormally during blast colony formation.

The data in this paper support a model of cellular organization in AML that includes the suggestion that the blast population is organized functionally in a fashion similar to differentiating lineages; that is, the blast population derives from progenitors that may either renew themselves or alternatively undergo terminal divisions associated with loss of proliferative potential. The present data provide support for this model, since they show by immunologic and histochemical techniques that maturation occurs in some cells during blast cell colony formation. The finding that My-1 and NASD chloracetate esterase appear in correlated fashion during normal granulopoietic colony formation but are dissociated in the maturation of blast cells suggests to us that granulopoietic differentiation programs are only partially expressed in leukemic blasts. We have suggested that the differentiation programs of blast populations consist of abnormal assemblages of normal components. The present observations provide some evidence in favor of that view; more convincing support would require the demonstration, within the same blast cell, of markers associated with different cell lineages. Such evidence has been reported for Friend leukemia cells and human K-562 cells derived from a patient with the blastic phase of chronic myeloblastic leukemia.

---

**Table 1. My-1 Antigen and Chloracetate Esterase in Fresh Cells and Blast Colonies**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Cells Before Culture</th>
<th>Blast Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary Colonies (PE1)</td>
<td>Secondary Colonies (PE2)</td>
</tr>
<tr>
<td></td>
<td>My-1+ (%)</td>
<td>NASD-Ch Esterase (%)</td>
</tr>
<tr>
<td>1a</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>1b</td>
<td>5.4</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>2.3</td>
<td>12.7</td>
</tr>
<tr>
<td>3</td>
<td>7.5</td>
<td>34.2</td>
</tr>
<tr>
<td>4</td>
<td>6.6</td>
<td>8.3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>55.3</td>
</tr>
<tr>
<td>6</td>
<td>13.7</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>67</td>
<td>30.3</td>
</tr>
</tbody>
</table>

1a and b: patient before induction therapy and in relapse.
REFERENCES


Granulopoietic differentiation in AML blasts in culture

JP Marie, CA Izaguirre, CI Civin, J Mirro and EA McCulloch