Separation of Lymphoid and Myeloid Blasts in the Mixed Blast Crisis of Chronic Myelogenous Leukemia: No Evidence for Ig Gene Rearrangement in CALLA-Positive Blasts

By Kyungsae Ha, Melvin H. Freedman, Angela Hrincu, Dieter Petsche, Annette Poon, and Erwin W. Gelfand

Recent studies suggest that lymphoid blast crisis cells of chronic myelogenous leukemia (CML) expressing the common acute lymphoblastic leukemia antigen (CALLA) are B precursor cells, based on the demonstration of immunoglobulin (Ig) gene rearrangement similar to common acute lymphocytic leukemia. There is little evidence to suggest whether the cells with similar lymphoid characteristics in the mixed blast crisis of CML are also committed to B cell lineage. A patient in "mixed" blast crisis of CML was studied. On the basis of morphology, cytochemistry, and immunological studies, the blasts were classified as having either lymphoid or myeloid characteristics. A proportion of the leukemic blasts expressed CALLA, whereas others expressed My7 antigen. In order to characterize both populations of cells further, CALLA- blasts and My7- (myeloid) blasts were isolated by fluorescence-activated cell sorting. The My7- cells were highly proliferative in cell culture blast colony assays, retained the Ph1 chromosome, and were indistinguishable from acute myelogenous leukemia blasts. The CALLA- cells were also Ph1-chromosome positive, but in contrast, were poorly proliferative in vitro. Of particular note was their retention of germline configuration of Ig genes, thus distinguishing them from blasts in the lymphoid crisis of CML. We conclude that the lymphoid component in mixed blast crisis may represent a stage of differentiation prior to commitment to B lineage.

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Materials and Methods

Case report. The patient, a 15-year-old boy, was diagnosed as CML as 8 years of age based on initial clinical findings of spleno-megaly, a WBC count of 48 x 10⁹/L with all stages of granulocytic development, and the presence of the Philadelphia chromosome (Ph1) in peripheral blood cells. He received busulfan intermittently for 4.5 years with good effect, but was then switched to 6-mercaptopurine for 1.5 years because of uncontrollable leukocytosis. At the time of diagnosis of blast crisis, the WBC count was 38 x 10⁹/L with 10% blasts.

Cell morphology and cytochemistry. Blood smears were prepared from peripheral blood and from cell fractions and were stained with Wright's, periodic acid-Schiff (PAS), Sudan black B, nonspecific esterase (NSE) and acid phosphatase.

Immunological studies. Leukemic blasts were obtained from peripheral blood following Ficoll-Hypaque gradient centrifugation. Detection of E rosettes, surface immunoglobulin (sIg) and cytoplasmic immunoglobulin (cIg) were carried out as described previously. Reactivity with a panel of monoclonal antibodies was assessed by indirect immunofluorescence by fluorescent microscopy and flow cytometry with the Epics V system (Coulter Electronics, Hialeah, Fla). The monoclonal antibodies designated OKT3, OKT4, OKT6, OKT8, OKT9, OKT10, OKT11, and B-1 were obtained from Ortho Pharmaceutical Co (Raritan, N.J.), and Leu-1 was obtained from Becton Dickinson (Sunnyvale, Calif). My7 antibody was kindly provided by Dr J. D. Griffin (Sidney Farber Cancer Institute, Boston); BA-3 (CALLA) and BA-2 antibodies were provided by Dr T. LeBien (University of Minnesota, Minneapolis). Anti-Ia antibody was kindly provided by Dr M. Letarte (The Hospital for Sick Children, Toronto).

Fluorescence-activated cell sorting. After preparing the cells with monoclonal antibodies, cells were separated into CALLA- and CALLA+ and My7- and My7+ populations by Coulter Epics V cell sorter.

Blast colony assays. The cell fractions were assayed in vitro for detection of myeloblasts using a modification of the method detailed by Buick et al. The cells were depleted of T lymphocytes by a second centrifugation through Ficoll-Hypaque after rosette formation with sheep red blood cells. The remaining cells were plated (2 x 10⁶ per dish) in 0.8% methylcellulose with 20% fetal calf serum, α medium and 10% phytohemagglutinin–leukocyte-conditioned medium (PHA-LCM). Cultures were incubated for five to seven days in air with 5% CO₂, and then were counted. The blast nature of colonies was confirmed by morphology, cytochemistry, and differentiation markers.

For detection of lymphoblasts, the cell fractions were assayed according to the method of Izaguirre et al. Feeder cells were obtained from peripheral blood of healthy adults, the adherent fraction was removed, and the remainder of the cells was irradiated.

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Fig 1. (A) Peripheral blood smear illustrating a mixed population of blasts. Solid arrows indicate lymphoid blasts (scanty cytoplasm and indistinct nucleoli) and open arrows indicate myeloid blasts (Wright's original magnification x 1,200; current magnification x 1,020). (B) The CALLA + blasts after cell sorting stained with periodic acid-Schiff (PAS; original magnification x 1,200; block-positive; current magnification x 1,020). (C) The blasts of CALLA + fraction stained with PAS (original magnification x 1,200; diffuse-fine-positive; current magnification x 1,020). (D) The My7 + blasts positively stained with peroxidase (original magnification x 1,200; current magnification x 1,056).

After depletion of T cells as described above, the cells (5,000 rad). After depletion of T cells as described above, the cells (10⁶) were mixed with feeder cells (10⁶), 20% PHA-LCM, 0.8% methylcellulose, and α medium. Aliquots (0.1 mL) were dispensed into Linbro titration wells and were then covered tightly and cultured for five to seven days at 37 °C in a special chamber containing 5% to 7% O₂, 5% CO₂. Colonies were then counted and characterized as described.20

Chromosome analysis. The cells from each fraction was prepared directly for cytogenetic analysis. Metaphases were examined for the presence of the Ph₁ chromosome, and selected cells were karyotyped.

Immunoglobulin gene analysis. High mol wt DNA was extracted from the mononuclear cells in each fraction obtained after cell sorting. Genomic DNAs from thymocytes that have already been shown to have germline Ig genes were used as controls. The genomic DNAs were digested with EcoRI restriction endonuclease, which is known to permit recognition of both rearranged and germline configuration of Ig genes.23 Digested DNA was size-fractioned by agarose gel electrophoresis and transferred to nitrocellulose filters.24 The filter-bound DNA fragments were then hybridized to nick-translated [³²P]-JH probe and visualized on autoradiograms.25 In parallel to the analysis of the sorted cell populations, at least ten samples from common ALL were run showing rearrangement of one or both alleles. The J₅₆ germline clone was kindly provided by Dr P. Leder (Department of Genetics, Harvard Medical School, Boston).

RESULTS

Characterization of mixed blast crisis cells. Two different populations of blasts were observed in the peripheral blood smears (WBC count was 125 x 10⁹/L with 28% blasts). Twenty-eight percent of the blasts were PAS positive and had the morphological appearance of lymphoid blasts, whereas 30% were peroxidase positive, compatible with myeloid blasts (Fig 1).

The results of immunological marker studies on the sample before cell sorting is shown in Table 1. CALLA + cells and My7 + cells in the mononuclear cells of the blood sample were 23% and 25%, respectively. To recover adequate numbers of cells from each population, 300 x 10⁶ cells were reacted with either monoclonal antibody anti-CALLA or anti-My7, and separated using the fluorescence-activated cell sorter (FACS). Cytochemical studies and karyotypic analysis and Ig gene organization were performed on each of the four populations of cells recovered as soon in Table 2 and Fig 2. CALLA + cells were positively stained with PAS (36%), but not with peroxidase and/or Sudan black B. Among My7 + cells, 30% of the cells were stained with peroxidase. Although 30% of the My7 + cells were also stained with PAS, the pattern of the staining was different from that of CALLA + blasts (diffuse faint versus block, Fig 1). The results of karyotype analyses appeared to be of little difference between these four populations (Table 2). They all showed a clonal change manifested by the Ph₁ chromosome, and in addition, 80% to 90% of the cells examined showed clonal evolution by the presence of an additional Ph₁ chromosome.

Blast colony growth in the four cell populations was quantitated. The CALLA + and CALLA - fractions yielded

<table>
<thead>
<tr>
<th>Table 1. Characterization of Cells</th>
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<tr>
<td>Positive (%)</td>
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<tr>
<td>E Rosettes</td>
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<td>2</td>
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Numbers represent the percentage of fluorescent cells analyzed by flow cytometry except for E rosettes or clg (cytoplasmic Ig). By double labeling, almost all CALLA + cells were shown to be BA-2 +; BA-2 + cells were similarly CALLA +.
Table 2. Characterization of Cells After Sorting

<table>
<thead>
<tr>
<th>Histochemistry</th>
<th>CALLA⁺</th>
<th>CALLA⁻</th>
<th>My7⁺</th>
<th>My7⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS</td>
<td>36</td>
<td>18</td>
<td>30</td>
<td>32</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Negative</td>
<td>29</td>
<td>30</td>
<td>Negative</td>
</tr>
<tr>
<td>Sudan black B</td>
<td>Negative</td>
<td>26</td>
<td>28</td>
<td>Negative</td>
</tr>
<tr>
<td>Blast colony growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colonies per culture</td>
<td>3</td>
<td>1,100</td>
<td>925</td>
<td>800</td>
</tr>
<tr>
<td>Karyotypic analysis</td>
<td>47, XY, Ph¹, Ph¹</td>
<td>47, XY, Ph¹, Ph¹</td>
<td>47, XY, Ph¹, Ph¹</td>
<td>47, XY, Ph¹, Ph¹</td>
</tr>
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three and 1,100 colonies per culture, respectively. The My7⁺ and My7⁻ fractions yielded 925 and 800 colonies per culture, respectively. Cellular composition defined by morphology and cytochemistry of individual and of pooled colonies per culture plate was found to be myeloblastic in the CALLA⁺, My7⁺, and My7⁻ cultures. No lymphoblasts were seen in any of the studies.

When genomic DNAs from each population were digested with EcoRI and hybridized to the JH probe, all subgroups demonstrated germline configuration of Ig heavy chain genes (Fig 2).

DISCUSSION

Identification of the cellular lineage of blast crisis cells in CML has been clinically important because a high response rate to vincristine and prednisone therapy can be achieved in lymphoid blast crisis. Immunological marker studies have shown that about one third of cases express CALLA and that these are indistinguishable from common ALL cells. More recently, by means of Southern blotting techniques, it was shown that lymphoid blast crisis cells of CML underwent Ig gene rearrangements, an essential property of B-lineage cells, similar to the studies of common ALL.

In contrast to the studies of lymphoid blast crisis in CML, it is not known whether the lymphoid blasts in the mixed crisis are also B precursor cells. Although the latter are CALLA⁺, it is now known that the expansion of CALLA is not restricted to B-lineage cells. We therefore attempted to determine the cellular lineage of the lymphoid blasts in the mixed crisis of CML. Based on morphology, cytochemistry, and marker studies, it was obvious in our patient that the blasts consisted of two different populations. To study each population further, CALLA⁺ cells and My7⁺ cells were isolated using the FACS. Among the CALLA⁺ and My7⁺ fractions, no blasts stained with peroxidase (Table 2). In contrast, CALLA⁻ and My7⁻ blasts did stain with peroxidase, characteristic for myeloblasts. Some blasts in the CALLA⁺ and My7⁻ fractions were stained with PAS, but differently from CALLA⁺ blasts (diffuse block).

No blast colonies were observed in the CALLA⁺ fraction (Table 2). This is to some extent different from CALLA⁺ blasts of ALL in which blast colony growth can be seen in the same assay. Blast colony growth characteristic of myeloblasts was observed in the CALLA⁻, My7⁺ and My7⁻ populations. The My7⁻ fraction, although peroxidase negative, yielded large numbers of myeloblast colonies in vitro, suggesting heterogeneity among myeloblast progenitors, some being peroxidase positive and others peroxidase negative.

![Fig 2. Southern blot analysis and restriction enzyme map of human germline \( \mu \) gene. Upper panel: genomic DNAs from thymocytes that have already been shown to have germ line Ig genes were used as controls (C). The \( J_H \) probe contained a 3-kilobase (kb) germline EcoRI-HindIII fragment that could detect germ line configuration in an 18-kb EcoRI fragment. Leukemic cells from each fraction (CALLA⁺, CALLA⁻, My7⁺ and My7⁻) demonstrate germ line configuration of \( J_H \) region as did the cells from bone marrow (BM). Lower panel: restriction enzyme map. The wavy line corresponds approximately to the length of the switch \( \mu \) gene. The restriction endonuclease cleavage sites are shown as E (EcoRI), H (HindIII), and B (BamHI).](image-url)
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tive. It is possible that My7 expression is closely linked to peroxidase expression.

To date, almost all instances of lymphoid blast crisis cells of CML and non-T-ALL cells have shown Ig gene rearrangements.3,11,12 We therefore expected to see Ig gene rearrangements in the CALLA+ and My7+ populations. However, as shown in Fig 2, all four different populations as well as bone marrow cells demonstrated germline configuration of Ig heavy chain genes. These findings are quite distinctive because, in a parallel study from our laboratory, 32 cases of common ALL defined as CALLA+ and Ia+ showed Ig gene rearrangements.27 Thus, the lymphoid blasts in the mixed blast crisis of our patient—demonstrated by conventional morphology, cytochemistry, and immunological marker studies to be indistinguishable from non-T-ALL and lymphoid blast crisis cells of CML—seem not to be committed to B lineage and may be derived from a different stage of hematopoietic cell development. Since they do not express T-lineage antigens, it is more likely that these blasts arise from an earlier stage of hematopoietic precursor cells before undergoing Ig gene rearrangement. Bakhshi et al13 have reported on eight patients with CML who developed a blastic phase: seven patients were lymphoid blast crisis, and one was mixed lymphoid and myeloid blast crisis (CALLA+ blasts were 17% and myeloid marker blasts were 30%). Only cells from the patient with the mixed type blast crisis failed to demonstrate Ig gene rearrangements.

The techniques for studying Ig gene rearrangements are sensitive enough to detect small populations of clonal B-lineage cells among heterogenous populations of cells.3,4 More than 10% of the blasts in our patient were CALLA+ and we failed to detect Ig gene rearrangements. Furthermore, we isolated CALLA+ and My7+ blasts by fluorescence-activated cell sorting and characterized the separated populations using blast colony assays and Ig gene analyses. The results indicate that the CALLA+ blasts in the mixed blast crisis of our patient may emerge prior to commitment to B lineage. Taken together, CALLA+ blasts in mixed blast crisis may be different from those of non-T-ALL and lymphoid blast crisis cells of CML. Although mixed blast crisis of CML is rare (one in 30 blast crises seen in study by Griffin et al16), the CALLA+ blasts in mixed blast crisis may provide a unique population for studying hematopoietic stem cell differentiation.

ACKNOWLEDGMENT

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REFERENCES

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