ABSTRACT

The establishment and the biological properties of a new leukemic cell line (KBM-5) derived from a patient in the blastic phase of chronic myelogenous leukemia are described. The cells exhibited multiple copies of the Philadelphia chromosome, and a high level of p210Bcr-ABL kinase activity was detected with rabbit anti-Abl and anti-Bcr (exon 3) peptide antisera. Use of specific primers and polymerase chain reaction followed by Southern blotting revealed that KBM-5 cells carried a bcr3-ABLII splice junction. While a normal BCR message was detected, no normal ABL message was found. The cells were phenotypically myeloid with monocytic differentiation. The high cloning efficiency in semisolid media was independent of the presence of exogenous colony-stimulating factors. In vitro exposure to inducers of differentiation, such as retinoic acid, dimethyl sulfoxide, or hemin, failed to influence the growth rate of the cells and their level of differentiation. KBM-5 cells are highly resistant to the antiproliferative action of recombinant α- and γ-interferons. Although sensitive to recombinant tumor necrosis factor α, they were completely resistant to natural killer cell action. KBM-5 cells constitutively expressed mRNA for tumor necrosis factor α but not for γ-interferon, other interleukins, or hematopoietic growth factors. The KBM-5 cells that were transplanted into SCID mice manifested metastatic potential and tissue invasiveness similar to the way leukemic cells in humans do. This new KBM-5 cell line represents a helpful model for examining in vitro and in vivo modulation of the growth and properties of leukemic cells by using biological and chemotherapeutic agents.

INTRODUCTION

In CML, the long-term prognosis is poor despite recent advances in biological therapy and chemotherapy (1–3). Although a fraction of patients can be cured with allogeneic bone marrow transplantation (4), in the majority of patients, the development of a blast phase heralds a terminal stage of the disease for which there is no effective treatment (5). The cellular processes that cause such a transformation are unknown. Once developed, the blast phase of CML is highly resistant to therapy, including chemotherapy combinations and allogeneic bone marrow transplantation (4–6).

To develop new treatments, an understanding of the events of blast transformation is necessary, which is best gained through the study of in vivo and in vitro preclinical models. Studying leukemic cell lines can help us to obtain this information. By manipulating these cell lines, we can study the mechanisms that regulate the proliferation and differentiation of leukemic cells as well as evaluate the response of the cells to chemotherapeutic and biological agents. Myeloid leukemic cell lines are relatively difficult to establish, and their availability is limited. It is difficult to generalize the validity of observations made from one particular cell line, because it may represent a selected subclone of leukemic cells that are able to continue growth in vitro. Most existing lines are also poorly characterized. Thus, there is a need to establish cell lines that have defined biological properties to (a) study the leukemic process and (b) test the efficacy of various treatment approaches.

Ph+ cell lines derived from patients in the CML blastic phase may be valuable for investigating transformation events, the production of autocrine and paracrine growth factors, the significance of additional chromosomal abnormalities, and the role of cellular oncogenes in leukemic transformation. Only a limited number of Ph+ cell lines have been established (7–17). Here we describe a new leukemic cell line, KBM-5, established from the peripheral blood of a patient in the blastic phase of CML. Because these cells were found to have a metastatic potential in SCID mice, their grafting in such animals creates a new human/animal model of the disease that could be used to compare in vitro with in vivo results and that could provide unique opportunities for preclinical in vivo studies on chemotherapeutic and biological agents potentially active in the blastic phase of CML.

MATERIALS AND METHODS

Case Report of the Patient from Whom the KBM-5 Cell Line Was Derived.

A 67-year-old female with a 2-year history of Ph+cML and a recent rapid increase in peripheral WBC presented with a hemoglobin of 6.0 g/dl, a platelet count of 188 × 10^12/l, and a WBC of 74 × 10^12/l, 50% of which were blasts. The morphological and cytochemical characterization of the blasts was compatible with the myeloid blastic phase of CML. The patient was treated with 9 doses of 1-β-D-arabinofuranosylcytosine (3 g/m² for 2 h every 12 h). Although this treatment reduced circulating blast cells, on day 15 of therapy, the peripheral blast count rose again. At this time WBC were obtained for cultures from which the KBM-5 cell line was derived.

Culture of Leukemic Cells and Establishment of the Cell Line.

Mononuclear cells were separated by Hypaque-Ficoll density gradient sedimentation (1.077 g/cm³). 10 million cells were suspended in IMDM (GIBCO, New York, NY) with 25% FCS (GIBCO) and placed in 25-cm² plastic flasks (Corning Glass Works, Corning, NY). The cultures were maintained in closed boxes at 37°C in an atmosphere of 5% CO₂, 12% O₂, and 83% N₂. After a lag period of 3 weeks, the cells resumed proliferation and were split and reseeded, initially at a cell density of 5 × 10^4/ml. After the tenth passage, the cell line was maintained by biweekly passages in IMDM supplemented with 15% FCS at an initial density of 3 × 10^4/ml, but without the addition of conditioning factors. For clonogenic assays, the cells were cloned in IMDM supplemented with 15% FCS and 0.3% agar (Bacto Difco Agar) and plated in 35-mm Petri dishes at 1 × 10^4 cells/ml/dish. After incubation for 7–8 days in a fully humidified atmosphere of 5% CO₂, 12% O₂, and 83% N₂, clones of more than 50 cells were counted using an inverted microscope.
Morphology and Cytochemical Staining. Cytocentrifuge preparations of original leukemic cells and KBM-5 cells were stained with Wright-Giemsa stain and examined by light microscopy. Smears were evaluated for morphology and also cytochemistry for MPO, naphthol-ASD chloracetate esterase, esterase using a-naphthyl butyrate as a substrate, acid phosphatase, and periodic acid-Schiff stain using the standard methodology. For transmission electron microscopy, cells were fixed with 1.25% glutaraldehyde in cacodylate buffer (pH 7.6) for 30 min. Ultrathin sections were stained with uranyl acetate and lead citrate and evaluated for morphology, platelet peroxidase, and MPO (18). Terminal deoxynucleotidyl transferase was assayed using indirect immunofluorescence (19). Epstein-Barr virus nuclear antigen expression was tested using the method of Reedman and Klein (20), and the presence of EBV DNA was tested by PCR using BamW primers (21). Cytogenetic Analysis. Chromosomes were studied on the cells of the KBM-5 cell line from the original tumor, the cell line (passage 12 and higher) and primary frozen leukemic cell lines were analyzed for isoenzyme pattern. Isoenzyme analysis was conducted by starch gel electrophoresis and histochemical staining to determine the phenotype of each cell line at 13 polymorphic human enzyme loci: phosphoglucomutase 1 and 3; esterase D; adenylate kinase 1; adenosine deaminase; acid phosphatase; 6-phosphogluconate dehydrogenase; glucose-6-phosphate dehydrogenase; peptidases A, C, and D; and the mitochondrial form of malic enzyme and glyoxalase I. Methods for preparing cell extracts, gels, and histochemical stains and the conditions of electrophoresis have been described previously (22, 23).

Cytogenetic Analysis. Chromosomes were prepared using standard techniques (24). Fifteen metaphases were analyzed on Giemsa-bandied preparations of both the original leukemic cells and the KBM-5 cell line from passage 12.

Induction of Differentiation. KBM-5 cells (passages 13 and 204) were seeded at a density of 3 x 10^6 cells/ml culture medium and incubated with either 1.25% DMSO, 0.5 mg/ml hemin, or TPA. The cultures were analyzed 5–6 days later for markers indicating induction of differentiation (NBT reduction). For this assay, cells were resuspended in media and mixed with an equal volume of 0.2% NBT (Aldrich, Milwaukee, WI). The percentage of cells containing intracellular black-blue formazan granula was determined from neutral red-stained cytocentrifuge preparation prior to and after exposure to inducing agents. At least 500 cells were counted.

Immune Complex Kinase Assay for Bcr-Abl Protein. To determine if p210bcr-Abl was detectable in KBM-5 cells and, if so, to compare its level to those in cells with amplified bcr-abl (K562 cells), immune complex kinase assays were performed as described previously (25, 26) using rabbit anti-Abl and anti-Bcr (to exon 3) peptide sera.

Immunophenotyping. The presence of sheph RBC receptors was investigated using standard methods. OX RBC, sensitized with IgM-antibody-complement complex, were used as the indicator system for complement receptors (27). Only cells with three or more bound RBC were counted as positive.

The original leukemic cells and KBM-5 cells (passages 13 and 204) were analyzed for T, B, and myelomonocytic lineage surface markers with an Ortho (27). Only cells with three or more bound RBC were counted as positive.

The original leukemic cells and KBM-5 cells (passages 13 and 204) were analyzed for T, B, and myelomonocytic lineage surface markers with an Ortho Spectrum III flow cytometer (Ortho Diagnostics Systems, Westwood, MA) equipped with a 2140 data analyzer. Results were recorded as a percentage of positive cells over background (set at 2%). The following fluorochromes were conjugated to monoclonal antibodies: anti-CD3, OKT4/CD4, OKT8/CD8, OKT11/CD11 (Ortho); Leu 1/CD5, calia/CD10, Leu 12/CD19, and HLA-DR (Becton-Dickinson Monoclonal Center, Hialeah, FL). Purified monoclonal antibodies were utilized to detect the following myeloid antigens: My4/CD14, My7/CD13, (Coulter Immunology, Hialeah, FL); OKM1 (Ortho); My9/CD33, My10/CD34 (Becton Monoclonal Center, Inc., Mountain View, CA). The second step reagent was goat anti-mouse IgG (TAGO, Inc., Burlingame, CA). Non specific binding was controlled with mouse IgG control (Becton-Dickinson) or with second step reagent in the absence of a purified antibody. The cells were washed twice in DPBS and resuspended in DPBS with 30% human AB serum at a concentration of approximately 10^6 cells/ml for 1 h to block non specific binding. The cells were then stained according to standard methods recommended by commercial suppliers.

NK Cytotoxicity Test. The test was performed as previously described (28) using ^3^Cr-labeled cells of K-562 and KBM-5 lines as targets in a 4-h assay. Cytotoxicity was evaluated from results of four different target-effector cell ratios (1:5-1:40). They were expressed as specific target cell lysis after subtracting spontaneous lysis. Mononuclear WBC were obtained from the blood of normal donors by density separation on Hypaque-Ficol gradient (density ≤0.177 g/cm²; Pharmacia Fine Chemicals, Piscataway, NJ). Washed cells were either used directly or after activation by exposure to recombinant IFNγ (500 units/ml; Hoffmann LaRoche, Nutley, NJ; specific activity, 2 x 10^6 units/mg) for 2 h followed by repeated washing in DPBS.

Detection of the Chimeric BCR-ABL Transcripts in Leukemic Cells and in Tissues of SCID Mice Grafted with KBM-5 Cells. The same methods were used for tissue culture cells as for mouse tissue samples. Total RNA was extracted from 1–5 x 10^6 cells (29) and transcribed into first-strand cDNA using reverse transcriptase and random hexamer primers as described previously in detail (30, 31). The BCR-ABL transcript was amplified with Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) with antisense oligonucleotide primer ABL(-) 5'-TGAAGCTTAAAGCTGAC-3' derived from the sequence of the second exon of the c-abl oncogene along with oligonucleotide derived from Isola 1.5 (32). p210bcr-Abl protein is encoded by an 8.5-kilobase BCR-ABL mRNA, which results from coupling of either exon b2 or b3 of the major breakpoint cluster region (bcr) in the c-abl exon 2. p190bcr-Abl results from coupling of the final exon of the BCR gene with ABL exon 2 (32). RNA from K562 cells (7) and RNA from a CML patient were used as positive controls for mRNA containing bcr exon 3 ABL exon II (33) and bcr exon 2 ABL exon II (34) junctions, respectively. ALL-1, a Ph^+^ positive acute lymphoblastic cell line (kindly provided by Dr. G. Rivera at the Wistar Institute) was used as a positive control for the p190bcr-Abl transcript (el-a2 junction). The RNA from HL-60 cell line and human endometrial RNA were used as negative controls. One μg of total RNA from each sample was used for amplification reactions. The amplification method, the primers, and the probes used have been described (33).

For detection of the normal ABL cDNA, the 5' primer was a 20-mer beginning 65 bases upstream of the c-abl exon 1b-II junction; the 3' primer used was one previously described; the probe was a 24-mer spanning the splice junction with 14 bases in exon II. For detection of the normal BCR cDNA, the following primers and probe were used: BCRa, 5'-AAAAAGGCTTCTTTTCAGTGCACGCTGAAGGACGAGGTCTGCTTCGCTATTGACTGCCTCCTTGACACATCAAC-3'; BCRb, 5'-GACTCTGGTGGAAGACAGGATGCT-3'; probe, 5'-ATTGACTGCTCTTTGGTGGCAGCCGACGCGGACAGCT-3'. Amplification was carried out for 40 cycles. While using this exquisitely sensitive technique, the following steps were taken to ensure the accuracy of the PCR results: (a) the thermal cycler was kept in a separate laboratory, away from the room where cell collection, RNA extraction, and cDNA synthesis were carried out; (b) no one was allowed to bring amplified samples back into the room where RNA processing was performed; (c) at least one negative control was run for each experiment; and (d) all samples were run on at least two separate occasions. For hybridization, 10 μl of the reaction product were run on 5% Nusieve/1% Sea-Kern agarose gel, transferred overnight to GeneScreen Plus (New England Nuclear, Boston, MA) membranes, and hybridized to 5' end-labeled oligonucleotide probe recognizing the junctional BCR-ABL sequences. The filters were washed as recommended by the manufacturer and exposed to Kodak XAR film for 6–36 h. The BCR 3/ABL II and BCR 2/ABL II probes detect 200- and 125-base pair amplification products, respectively, while the probe for the p190 transcript detects a 307-base pair product. For detection of the normal ABL, a solution hybridization method, hybridization-protection assay, was used (35). This assay has the same sensitivity and specificity as Southern blotting of PCR products. The probe was an acridinium-ester-labeled 24-mer spanning the ABL exon 1b-II junction (Gene Probe, Inc., San Diego, CA).

Expression of Hematopoietic Growth Factors and Cytokines Measured by PCR. Total RNA isolation was carried out as described previously (29, 36). First-strand cDNA synthesis was performed (70°C for 45 min) with denatured total RNA using random hexamer primers (Pharmacia) and murine Moloney leukemia reverse transcriptase (200 units/μl; BRL). Cytokine and growth factor-specific primers used in the present experiments were designed and the sequence specificity verified from GeneBank data as reported elsewhere (36).
Amplification was carried out in a Thermocycler (Perkin-Elmer Cetus) for 30 cycles. The temperature profile, identical for all cytokine primers, was as follows: 1 min at 94°C for denaturation; 1 min at 58°C for annealing; 1 min at 72°C for primer extension. PCR products were separated on ethidium bromide-stained agarose gel (1.6% agarose; Pharmacia) and the size compared with molecular size standards (φX174 RF DNA/HaeIII; Gibco). The OKT3-stimulated mononuclear blood cells expressed all cytokines and served as a positive control for each set of primers to determine whether the PCR product was the correct size (36).

Amplification of Human DNA-specific Sequences (HLA-DQα) by PCR. DNA was prepared from fresh or frozen tissues (1-2 mg) or cells (1-2 X 10⁶) (37) after removal of RBC by lysis buffer. A portion (5 µl) of the DNA extract was used for amplification of DNA in the Thermocycler in a total volume of 50 µl with 1.25 units of Taq DNA polymerase under the following reaction conditions: annealing for 1 min at 55°C; extension for 1.5 min at 72°C; and denaturation for 1 min at 95°C. Previously published primers for α chain of HLA-DQα1 were used (38). Specific positive and negative controls were used for internal consistency in every PCR assay. The amplified product (usually one-fifth of the 50-µl volume) was separated by electrophoresis in 1.6% agarose gel and stained with ethidium bromide. The positive results were detected as a 225-base pair band.

Grafting of KBM-5 Cells into SCID Mice. Female C.B. 17 SCID mice were obtained from Bommice. Ltd., Denmark. They were kept in sterile conditions at the SCID animal facility of the Department of Immunology, Karolinska Institute, Stockholm, Sweden, without antibiotic prophylaxis. They were in good health with NK activity close to zero, indicating pathogen-free conditions. There were no electrophoretically detectable immunoglobulin levels in their plasma.

KBM-5 cells (1 X 10⁶) from Mycoplasma-free, exponentially growing cultures were injected into animals using s.c. (2 animals), retroorbital (2 animals), i.p. (2 animals), or i.v. (2 animals) routes. The animals were not conditioned prior to or after grafting. Animals were observed twice daily for signs of disease and killed when they became terminally ill, at which time the blood was also obtained. Postmortem examination detected the presence of macroscopic tumors and splenomegaly and samples of tissues were collected so that the histology and presence of human DNA in the tissues could be determined.

RESULTS

Establishment of the Cell Line. The search for optimal culture conditions using various supplementations of IMDM did not result in an advantageous growth in any of the conditions tested. Between weeks 3 and 4 in culture, the cells resumed growth and could be split to grow at a high density (5-7 X 10⁶/ml). After the tenth passage, the cells were diluted to an initial density of 3 X 10⁶/ml. In medium supplemented with 15% FCS, the cells grew with a doubling time between 24 and 60 h, depending on the passage, to a saturation density of 1.5-2.0 X 10⁶/ml. The cells grew in suspension as single, round, nonadherent cells. Characterization of exponentially growing, acridine orange-stained KBM-5 cells with flow cytometry revealed a cell population with a DNA index of 1.0 and a high RNA content (data not shown). Different passages of the cell line, designated KBM-5, were frozen at regular intervals. The cell line was kept free of Mycoplasma.

Morphology and Cytochemical Profile of Fresh Leukemic Cells and KBM-5 Cells. On Wright-Giemsa-stained smears, the patient’s leukemic cells were immature blasts with morphological characteristics of acute myelomonocytic leukemia according to the French-American-British classification (39). Auer rods were not observed in the original cells or KBM-5 cells. The original cells were positive for naphthol-ASD chloroacetate esterase (100%), MPO (20%), acid phosphatase (100%), and α-naphthol butyrate (30%) but negative for periodic acid-Schiff. The cytochemical profile of KBM-5 cells was similar, with the exception of a lower MPO positivity (3%), and compatible with the myelomonocytic lineage. On Wright-Giemsa-stained smears (Fig. 1A), the KBM-5 cells exhibited some anisocytosis and variation in the nucleocytoplasmic ratio. The nuclei were pleomorphic and had fine chromatin and rather small nucleoli. The relatively scarce cytoplasm was intensely basophilic and contained free eosinophilic granules and many vacuoles. The ultrastructural morphology of KBM-5 cells (passage 13) was blast-like and had an invasive nucleus and some marginate chromatin. The cytoplasm contained few mitochondria, some granules, and a moderate amount of endoplasmic reticula (Fig. 1B). The KBM-5 cells were terminal deoxynucleotidyl transferase negative, and they did not express Epstein-Barr virus nuclear antigen protein. PCR amplification of the BamW region of EBV did not reveal the presence of EBV-DNA integrated in the KBM-5 genome.

Phenotypic Characterization. The patient’s leukemic cells from which the KBM-5 cell line was established demonstrated a phenotype consistent with monocytic lineage based on My4 and OKM1 expression (40), although the absence of la reactivity was unusual (Table 1). Phenotypic changes occurred with the establishment of the cell line and persisted with a trend towards increased My7, My9, and OKT4 expression and loss of My4 and OKM1 expression. KBM-5 cells were negative for surface immunoglobulin. The cells did not form rosettes with sheep erythrocytes. Approximately 25-30% of the KBM-5 cells formed EAC rosettes, which indicated the presence of complement receptors.

Isoenzyme Analysis. Eight loci proved to be informative in that genetic variants were present in either the control cells (HL-60 and HeLa) or the KBM-5 cell line. The phenotypes (which directly reflect the genotypes, because alleles at isozyme loci are codominant) for the material studied at each of the informative loci are shown in Table 2. The coidentity of KBM-5 and the original tumor was documented. All passages of KBM-5 were the cell line and derivatives of the original tumor (KBM-5-0). The presence of the rare PGM1.7 allele clearly
Table 1 Reactivity of original leukemic cells and KBM-5 cells with monoclonal antibodies

<table>
<thead>
<tr>
<th>Monoclonal antibodies (% of positive cells)</th>
<th>HLA-DR</th>
<th>OKM1</th>
<th>MY4</th>
<th>MY7</th>
<th>MY9</th>
<th>MY10</th>
<th>OKT4</th>
<th>OKT8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original cells</td>
<td>3</td>
<td>71</td>
<td>69</td>
<td>13</td>
<td>38</td>
<td>0</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>KBM-5, pass 13</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>81</td>
<td>97</td>
<td>0</td>
<td>82</td>
<td>2</td>
</tr>
<tr>
<td>KBM-5, pass 204</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>92</td>
<td>97</td>
<td>2</td>
<td>79</td>
<td>2</td>
</tr>
</tbody>
</table>


Table 2 Phenotype of KBM-5 cells as defined by isoenzyme analysis of polymorphic human enzyme loci

<table>
<thead>
<tr>
<th>Informative loci</th>
<th>PGM1</th>
<th>ESD</th>
<th>AK1</th>
<th>ACP1</th>
<th>PGD</th>
<th>G6PD</th>
<th>GLO1</th>
<th>ADA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL60</td>
<td>1</td>
<td>1.2</td>
<td>NE*</td>
<td>a, b</td>
<td>a, c</td>
<td>b</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>HeLa</td>
<td>1</td>
<td>1</td>
<td>a, b</td>
<td>a</td>
<td>b</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>KBM-5-0, original tumor</td>
<td>1.7</td>
<td>1</td>
<td>a, b</td>
<td>a</td>
<td>a</td>
<td>b</td>
<td>2</td>
<td>1.2</td>
</tr>
<tr>
<td>KBM-5, all subsequent passages</td>
<td>1.7</td>
<td>1</td>
<td>a</td>
<td>b</td>
<td>a</td>
<td>b</td>
<td>2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* NE, not expressed.

marked the KBM-5 cells and distinguished them from HL-60, K-562, and two other new myeloid leukemia cell lines, KBM-7 and KBM-3, recently established in our laboratory (14, 41).

Cytogenetic Analysis. In each case, cytogenetic analysis of a G-trypsin-banded karyotype was performed on 50 metaphases. The analysis of KBM-5 metaphases (Fig. 2A) showed a broad range in the number of chromosomes, with 46 metaphases having between 69 and 87 chromosomes and 4 having between 107 and 170 chromosomes. The cells in the triploid range exhibited similar numerical and structural alterations: +6, +7, +8, +8, +8, -9, -9, +9q, -11, -11, -13, 14q-, +15, 17p+, +20, +22q-, +22q-, +frag, +DMs. The presence of common chromosomal changes indicates multiclonality and clonal evolution. The Ph1 chromosome t(9;22) and 17p+, +6, +8, -9, and a few minute chromosomes have also been identified in metaphases from a bone marrow sample obtained from the patient (Fig. 2B) at the initiation of the cell cultures. Minute chromosomes observed at a low frequency in the direct marrow preparation were present in multiple copies in all metaphases of the cell line. The KBM-5 cells carried multiple copies of the Philadelphia chromosome and also chromosomes 7, 8, 15, and 17 (Fig. 2A).
Table 3 Clonogenic growth of myeloid leukemia cell line KBM-5 in semisolid cultures: effects of HPCM or media conditioned by various myeloid leukemia cell lines (mean ± SD of quadruplicate cultures)

<table>
<thead>
<tr>
<th>Source of stimuli</th>
<th>≤50 cells</th>
<th>8–49 cells</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>42 ± 13</td>
<td>103 ± 5</td>
<td>146 ± 16</td>
</tr>
<tr>
<td>HPCM</td>
<td>36 ± 5</td>
<td>129 ± 10</td>
<td>165 ± 15</td>
</tr>
<tr>
<td>KBM-5, PASS 5</td>
<td>63 ± 10</td>
<td>135 ± 23</td>
<td>198 ± 29</td>
</tr>
<tr>
<td>KBM-5, PASS 9</td>
<td>47 ± 19</td>
<td>132 ± 17</td>
<td>179 ± 32</td>
</tr>
<tr>
<td>HL-60</td>
<td>28 ± 2</td>
<td>108 ± 13</td>
<td>136 ± 15</td>
</tr>
<tr>
<td>K-562</td>
<td>45 ± 3</td>
<td>107 ± 7</td>
<td>152 ± 9</td>
</tr>
</tbody>
</table>

* HPCM, human placental conditioned medium as the source of colony stimulating activity (GM-CSA). Added at 15% (v/v).

*b Media conditioned for 3–5 days by exponentially growing leukemia cells. Added at the final concentration of 15% (v/v).

No of clones/1 X 10^3 cells

Clonogenic Growth and Response to Colony-stimulating Activity. KBM-5 cells grow as compact colonies in agar- or methylcellulose-containing media. The cloning efficiency, initially 10–15%, increased after 20–30 passages to 20–30%. When cloned at low density, the cells tended to grow better in the presence of autologous conditioned media (Table 3). Neither human placental conditioned medium added in concentrations optimal to stimulate growth of granulocyte-macrophage colony-forming cells nor media conditioned by several other myeloid leukemia cell lines affected the number or size of the clones (Table 3). The cell growth and differentiation of KBM-5 cells is not affected by recombinant G-CSF, granulocyte-macrophage CSF, interleukin 3.* Additional of human recombinant stem cell factor (kindly provided by Immunix Research Laboratory, Washington, DC) at concentrations between 25 and 200 ng/ml failed to influence growth, while 400 ng/ml proved inhibitory (40% inhibition of the growth).

Response to Inducers of Differentiation. Noninduced cells only occasionally reduced NBT and the number of NBT-reducing cells increased only marginally after stimulation with TPA. When exposed to either RA or DMSO in concentrations optimal to induce differentiation of HL-60 cells, the KBM-5 cells retained the growth rate of untreated controls and morphology of undifferentiated cells. When incubated with TPA over a 5–6-day period, between 5 and 10% of the cells became loosely attached to plastic; less than 1% of the cells became adherent and assumed a morphology of macrophage-like cells with a low nucleus:cytoplasm ratio. Hemin exposure for 4 days failed to induced maturation toward erythrophagocytosis as evaluated by benzidine stain for hemoglobin. Only a small percentage of uninucleated cells phagocytized polystyrene beads, and their phagocytic activity increased less than 10% after induction with DMSO, RA, and TPA.

Sensitivity to NK-mediated Cell Lysis. Chromium-labeled KBM-5 cells were exposed to both noninduced and α-interferon-activated NK cells from normal donors (target:effector cell ratio, 1:5–1:50). K562 cells were studied as a positive control. KBM-5 cells proved to be completely resistant to any cytolytic action of NK cells in a 4-h chromium release assay (Table 4).

Immune Complex Kinase Assay for p210 Bcr-Abl. Assays were performed concomitantly with K-562 cells that were known to have amplified c-ABL (42). As expected, high levels of p210 Bcr-Abl phosphokinase activity were detected in K562 cells when rabbit anti-Ab as well as anti-Bcr (exon 3) peptide antisera were used (Fig. 3). The M, 190,000 protein in K-562 cells, detected in variable amounts by the two antisera, is thought to be a proteolytic fragment of p210 (42). In KBM-5 cells, p210 Bcr-Abl kinase activity was observed using both antisera (Fig. 3), albeit at lower levels than in K562 cells. It has been demonstrated previously (43) that the break on chromosome 22 may occur between bcr exons 3 and 4, presumably generating alternate BCR-ABL messages that either contain or lack exon 3. The significance of these molecular variations remains unclear. The detection of p210 Bcr-Abl by an anti-BCR serum, which was made against a domain within exon 3, demonstrates that KBM-5 cells, like K-562 cells, express the variant which includes BCR exon 3.

Detection of BCR-ABL Splice Junction and BCR-ABL, ABL, and ABL Message. Use of the polymerase chain reaction followed by Southern blotting revealed that KBM-5 cells carried a BCR-ABL II splice junction. There was no BCR-ABL II splice junction or c1-22 junction (the latter encodes the p190 Bcr-Abl). In addition, normal BCR message was detected by the polymerase chain reaction followed by Southern blotting. Because the primers used for the latter assay where derived from cDNA sequences distal to the junction where BCR is spliced to ABL, they would be expected to identify normal BCR message, but not the BCR-ABL message, since only the more proximal BCR sequences are found in the BCR-ABL mRNA. No normal ABL message was discerned, as determined by polymerase chain reaction followed by the hybridization protection assay.

Cytokine mRNA Expression. PCR and specifically designed primers were used to analyze the early and late passages (13 and 250, respectively). This approach detected constitutive expression of only TNFα in KBM-5 cells (with β-actin expression being consistently positive (Fig. 4). The assay failed to detect mRNA for hematopoietic growth factors, i.e., G-CSF; granulocyte-macrophage CSF, c-kit ligand; macrophage CSF; interleukins 3, 4, 6, and 10; and IFNγ. The efficacy of the PCR procedure and quality of the primers were monitored with OKT3-stimulated human monoclonal blood cells expressing mRNA for all factors/cytokines studied, with the exceptions of c-kit ligand, and G-CSF.

Growth of KBM-5 Cells in SCID Mice. Eight SCID mice were given injections of a dose of 1 X 10^7 cells, and all became terminally ill between 18 and 62 days after inoculation. The results are summarized in Table 5. Engraftment was achieved in all of the mice regardless of the mode of inoculation. In the two animals that were given s.c. injections, s.c. and regional lymph node tumors developed. In two other animals that were inoculated i.p., one developed intraabdominal tumors and ascites and another a s.c. tumor. After retroorbital inoculation, one animal developed a regional lymph node tumor and the second an abdominal tumor. The last two i.v.-injected animals developed both intraabdominal and submandibular tumors. All of the tumors were composed of blast cells. The majority of the tissues studied, e.g., blood, marrow spleen, liver, lungs, and brain tissues that were examined using primers for the α chain of HLA-DQα1 (38), demonstrated the presence of human DNA (Table 5). The presence of a specific leukemic marker, i.e., bcr/abl mRNA, was documented by amplification of bcr/abl transcript from most tissues of SCID mice studied in 3 animals after i.v. (1 animal) and i.p. (2 animals) injection of KBM-5 cells. (Fig. 5; Table 6). The histology of selected tissues revealed the presence of foreign cells the blast cell morphology of which was compatible with that of the grafted KBM-5 leukemic cells. Fig. 6 shows splenic involvement and intraabdominal KBM-5 tumor

Table 4 Sensitivity of leukemic cells to NK-mediated effect

<table>
<thead>
<tr>
<th>Cell line</th>
<th>E/T ratio</th>
<th>NK cell sensitivity (% 51Cr release) following effector cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-562</td>
<td>10:1</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>40:1</td>
<td>19.8</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>30.7</td>
</tr>
<tr>
<td>KMB-5</td>
<td>10:1</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td>40:1</td>
<td>28.5</td>
</tr>
</tbody>
</table>

* M. Beran, unpublished data.

** MWBC, mononuclear (density <10,077 g/cm^3) WBC from normal blood.

a MWBC from normal blood activated by exposing 1 X 10^6 cells/ml to 500 units recombinant IFNα for 2 h.
Fig. 3. Immune complex kinase assay in KBM-5 cell extracts. p210\textsuperscript{bcrl/abl} phosphokinase activity in KBM-5 cells was detected by immune complex kinase assay. Anti sera used were anti-abl antiserum (Lanes 1, 2, 5, 6, 9, 10) and anti-bcr\textsuperscript{3} antiserum (Lanes 3, 4, 7, 8, 11, 12). In even lanes, the reactivity is blocked by addition of cognate peptide. Lanes 1–4, KBM-5 cells; Lanes 5–8, K562 cells; Lanes 9–12, HL-60 cells (Ph\textsuperscript{-}negative leukemic cell line). Kd, molecular weight in thousands.

Fig. 4. Cytokine expression (mRNA) in KBM-5 cells as measured by PCR. PCR products are electrophoretically separated on 3% agarose gels (70 V for 60 min). Lane 1, molecular size standards; Lane 2, KBM-5, β-actin; Lane 3, H\textsubscript{2}O control; Lane 4, TNFα; Lane 5, H\textsubscript{2}O control; Lanes 6 and 7, interleukin 1 and interleukin 1, H\textsubscript{2}O control, respectively; Lane 8, IFNγ. bp, base pairs.

Fig. 5. Amplification of bcr/abl transcript from the organs of SCID mice, 4 weeks after i.p. inoculation with 1 × 10\textsuperscript{7} KBM-5 cells. Lane 1, molecular markers; Lane 2, KBM-5 cells, positive control; Lane 3, KBM-7 cells (14), positive control; Lane 4, blood; Lane 5, liver; Lane 6, negative control, normal SCID liver; Lane 7, CML, chronic phase, bone marrow. bp, base pairs.

growing adjacent to spleen (Fig. 6A) as well as involvement of the subarachnoidal space with KBM-5 cells (Fig 6B).

In vitro cultures of bone marrow and spleen cells from animals grafted with KBM-5 cells which have leukemic cells in these organs, documented by PCR/DQα, produced outgrowth of KBM-5 cells with phenotype identical to that of inoculated KBM-5 cells (CD33 97.8% and 98.8% positive KBM-5 and KBM-5/SCID, respectively; CD13 98.6% and 96.4% for KBM-5 and KBM-5/SCID, respectively).

DISCUSSION

The significance of chromosomal abnormalities other than t(9;22) evolving during the transformation of CML into the blastic phase is obscure (39, 40). Knowledge about the relationship of these chromosomal aberrations to the biology and phenotype of leukemic cells may contribute to the development of future treatments. The availability of immortalized cell lines derived from patients with blastic phase of CML might facilitate such studies. The studies with the first such established line, K-562 (derived from the pleural fluid of a patient in the lymphoid blastic phase of CML) (7), have revealed an immature phenotype of these cells compatible with transformation at the multipotent stem cell level. Another remarkable characteristic of K-562 cells was their sensitivity to lytic action of NK cells (7, 28). Questions arise, however, about the extent to which K-562 cells are representative of the blastic phase phenotype. To answer these questions, other cell lines must be obtained from patients in the blastic phase of CML.

Additional CML blastic phase-derived myeloid cell lines (8–16) as well as lymphoid cell lines (17) with various phenotypes from the CML blastic phase have been reported. A review of their principal characteristics indicates their phenotypic heterogeneity (8–17). Some lines carry multiple copies of the Ph chromosome whereas others have only one copy. Descriptions of other chromosomal markers have been reported in detail only for KBM-7 (14), TOM-1 (17), and the present KBM-5 cells. Isoenzyme analysis has proved that, like KBM-7 cells, KBM-5 cells originate from the donor-derived leukemic population. Furthermore, these cells display a chromosomal constitution similar to that in the original donor cells. Despite their blastic appearance and large nucleus:cytoplasm ratio, many cells are strongly positive for butyrate which indicates differentiation along the monocytic pathway. Unlike K-562, KBM-5 cells are completely resistant to the action of both unstimulated and α-interferon-activated NK cells. They are also completely resistant to the antiproliferative action of recombinant α-

Table 5 Growth of KBM-5 cells in SCID mice

<table>
<thead>
<tr>
<th>Route of inoculation</th>
<th>No. of animals inoculated/no. engrafted</th>
<th>Survival (days)</th>
<th>Organs analyzed and involved(a)</th>
<th>Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>s.c.</td>
<td>2/2</td>
<td>28, 30</td>
<td>Blood, liver, lungs, brain</td>
<td>s.c.: regional lymph nodes in both animals</td>
</tr>
<tr>
<td>i.p.</td>
<td>2/2</td>
<td>28, 35</td>
<td>Blood, liver, lungs, brain</td>
<td>Abdominal: ascites; s.c.</td>
</tr>
<tr>
<td>r.o.(b)</td>
<td>2/2</td>
<td>21, 56</td>
<td>Lungs, brain, liver, blood</td>
<td>Submandibular nodes: abdominal</td>
</tr>
<tr>
<td>i.v.</td>
<td>2/2</td>
<td>56, 62</td>
<td>Blood, marrow, lungs, liver, brain</td>
<td>Submandibular nodes: abdominal</td>
</tr>
</tbody>
</table>

\(a\) Human DNA sequences detected.

\(b\) r.o., retroorbital; involves i.v. and local implantation.
and γ-interferons; this resistance is accompanied by an absence of 2'–5'-oligoadenylate synthetase induction and an inability to enhance the production of this enzyme when challenged by interferons.

Because the cell lines are constantly in danger of being cross-contaminated, their origin must be carefully documented. Enzyme markers trace the origin of the new KBM-5 cell line to the patient sample, and compatible cytogenticstaces trace the origin to the original tumor. As with other leukemic cell lines, the biological factors that allow the cells to survive in vitro are not known. It is noteworthy that molecular analysis of the original frozen sample and the KBM-5 cell line revealed an approximately 8-fold amplification of c-abl, bcr, and C immunoglobulin genes in the KBM-5 cells but not in the fresh, uncultured cells from which the line was derived. These findings indicate a possible role of c-ABL and BCR amplification in the establishment of in vitro growth or the selective advantage of a minor preexisting clone in the establishment of a cell line. Interestingly, while the expression of c-ABL mRNA is consistently found in many fresh samples of blast cells from patients with CML in the blastic phase that do not display c-ABL amplification (46), no ABL transcript was detected in KBM-5 cells in the present studies. This finding makes KBM-5 cells suitable for studies of the role of c-ABL gene expression for the biological properties of cells. A recently described Ph+ lymphoid cell line, TOM-1, failed to show c-ABL gene amplification, and although the BCR was rearranged, the cell line contained BCR gene transcripts of only normal length (17).

The KBM-5 cell line has interesting biological characteristics. Although this cell line shares with K-562 cells the property of being highly resistant to the inhibitory action of recombinant IFNγ and recombinant IFNα in vitro (47), the K-562 cells are very sensitive to NK cells and resistant to recombinant TNFα, whereas the KBM-5 cells are completely resistant to NK cell action and highly sensitive to the inhibitory action of recombinant TNFα (47). Finally, KBM-5 and K-562 lines share comparable in vitro dose responses to a variety of chemotherapeutic agents, including doxorubicin and amsacrine. Thus, we believe that a series of well-defined CML blast-like phase-derived cell lines will offer new opportunities to study the biological and therapeutic significance of various phenotypic and genotypic phenomena in the blastic phase of the CML.

The observation that KBM-5 cells engraft and disseminate in tissues of SCID mice in a mode comparable to the clinical situation is particularly relevant, because it opens the possibility of studying such phenomena in vivo. Only a limited knowledge exists about the growth of chronic myelogenous leukemia-derived cells in SCID mice. The engraftment in SCID mice of cell lines derived from blast phase CML was first reported for K-562 cells (48) and also for two other PH+ myeloid and lymphoid cell lines, EM-2 and ALL-1, respectively (48). Primary cells from 2 patients in blast phase of CML were also reported to grow locally and invasively after implantation under kidney capsule and spread to bone marrow as demonstrated by flow cytometry (48). Attempt to engraft cells derived from patients with chronic phase CML were thus far unsuccessful. Transient local engraftment in the peritoneal cavity was noted in few animals after i.p. injection with cells from two of six patients. No human cells were detected in tissues of animals given injections of cells from patients with chronic phase of CML by any route (48).

We have recently studied the engraftment of acute myelogenous leukemia-derived cell lines (HL-60, KBM-3) as well as their counterparts, which were rendered resistant to antileukemic drugs. All lines engrafted consistently and were found to invade numerous tissues, including the central nervous system. After passage through SCID mice, the phenotype of both sensitive and drug-resistant cells remained unchanged. Similar results were recently reported for other cell lines derived from patients with AML, i.e., HL-60 (48, 49) and KG-1 (48). The engraftment of primary myeloid leukemic cells has been less consistent although successful engraftment of primary blasts from AML was reported from four patients (48). Only in one case was the presence of human AML cells detected after i.v. injec-

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* M. Beran et al., unpublished data.

* M. Beran and P. Pisa, submitted for publication.
tion of AML cells. After i.p. injection or kidney capsule implantation, myeloblasts from all four patients grew locally and at least in two cases spread into murine hematopoietic organs (48). In another study blast from four patients with AML failed to engraft in SCID mice (49). Because the proliferation of most myeloid and lymphoid leukemic cell lines is growth factor independent, the differences in the growth of primary leukemic cells might be related to the requirement, by primary leukemic cells, of species-specific human growth factors not present in mice. The KBM-5 cell line was established without exogenous growth factors. This, together with the lack of expression of mRNA for most hematopoietic growth factors, indicates a factor-independent proliferation of KBM-5 cells both in vitro and in vivo. Engraftment of KBFM-5 cells in mice without prior conditioning of recipients with irradiation or drugs makes this model useful for preclinical evaluation of antileukemic drugs and biological agents, currently ongoing in our laboratory.

REFERENCES
Biological Properties and Growth in SCID Mice of a New Myelogenous Leukemia Cell Line (KBM-5) Derived from Chronic Myelogenous Leukemia Cells in the Blastic Phase

Miloslav Beran, Pavel Pisa, Susan O'Brien, et al.


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