A Novel Leukemia Cell Line, MR-87, With Positive Philadelphia Chromosome and Negative Breakpoint Cluster Region Rearrangement Coexpressing Myeloid and Early B-Cell Markers

By Jun Okamura, Shinichi Yamada, Eiichi Ishii, Toshiro Hara, Hiroyuki Takahira, Junji Nishimura, Keiko Yumura, Keisei Kaw-Ha, Kozo Takase, Yasuhiro Enomoto, and Hideko Tasaka

We developed a Philadelphia chromosome (Ph) positive cell line, designated MR-87, from a 4-year-old boy with Ph+ acute leukemia. MR-87 cells grew in single cell suspensions with a doubling time of 120 to 144 hours. Both MR-87 and original leukemia cells were positive for myeloperoxidase (MPO) and myeloid antigen CD13. These cells exhibited the early B-cell phenotype, i.e., terminal deoxynucleotidyl transferase (+), la+, CD19+, and CD10+. Rearrangement of the immunoglobulin heavy chain was confirmed in both. Approximately 80% of MR-87 cells coexpressed CD13 and lymphoid antigens CD10 or CD19, as confirmed by a two-color analysis. Simultaneous expression of MPO and CD19 on a single MR-87 cell was demonstrated at ultrastructural level. Thus, MR-87 is a Ph+ leukemia cell line exhibiting a hybrid phenotype. The breakpoint cluster region (bcr) was not rearranged in the MR-87 cells and subsequent analysis using antisera revealed that these cells expressed a novel protein, P190\textsuperscript{+}, which was immunoprecipitated with anti-abi and anti-phosphotyrosine antibodies. The MR-87 line will be most useful for investigating the biology and pathogenesis of Ph+bcr− acute leukemia.

The Philadelphia chromosome (Ph) has been found in approximately 20% and 2% of adult patients with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), respectively.1,2 In the case of childhood leukemia, however, the occurrence of this cytogenetic abnormality seems to be less frequent. One percent to 5% of all cases of childhood ALL were reported to be Ph+,[3] and the frequency of Ph+ AML in children is not well understood.

Identification of a Ph in acute leukemia is becoming more important, since accumulating evidence suggests that the presence of this chromosome is associated with a poor prognosis.4,5

In the majority of patients with chronic myeloid leukemia (CML), the breakpoints of chromosome 22 occur within a 5.8-kilobase (kb) segment of DNA referred to as the breakpoint cluster region (bcr). In contrast, some investigators found that the breakpoints of chromosome 22 in cells from certain patients with Ph+ ALL occur outside the 5.8-kb bcr region, thereby suggesting that the genetic mechanism of activation in Ph+ bcr− ALL may differ from that in Ph+ CML.5,7

Here we report the establishment of a novel Ph+ bcr− cell line (MR-87) from a child with Ph+ acute leukemia, at diagnosis. More interestingly, immunologic studies and molecular analysis demonstrated that MR-87 as well as fresh leukemia cells had a typical phenotype of early B-cell lineage, in addition to myeloid characteristics. The MR-87 cell line should prove to be useful and important model to delineate the biology and pathogenesis of Ph+ acute leukemia.

MATERIALS AND METHODS

Case report. T.M., a 51-month-old Japanese boy, was admitted to the hospital because of cervical adenopathy and massive hepato-splenomegaly. Initial complete blood cell count (CBC) revealed WBCs 191 x 10\textsuperscript{9}/L with 92% blasts, hemoglobin 13.0 g/dL, and platelets 15 x 10\textsuperscript{9}/L. The bone marrow was filled with blasts (99%) and 5% were myeloperoxidase (MPO)-positive. The patient was tentatively diagnosed as having acute myeloid leukemia and went into a complete remission (CR) while on combination chemothera-

From the Section of Pediatrics, National Kyushu Cancer Center, Fukuoka, Japan; Departments of Pediatrics and Third Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan; Department of Pediatrics, Faculty of Medicine, Osaka University, Osaka, Japan; Department of Pediatrics, Faculty of Medicine, Tokyo Medical and Dental University, Tokyo; and Department of Pathology, Faculty of Medicine, Keio University, Tokyo.

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Address reprint requests to Jun Okamura, M.D., Section of Pediatrics, National Kyushu Cancer Center, 3-1-1 Notame, Minami-ku, Fukuoka 815, Japan.

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immunoglobulins (Clg) were identified by a direct method using FITC-labeled F(ab')2 goat antihuman λ-chains. Terminal deoxynucleotidyl transferase (TdT) (BRL Laboratories, Rockville, Md) was detected by an indirect immunofluorescence assay. TdT activity of The specificity and cluster of differentiation (CD) of each antibody were identified by a direct method using compensation procedure was used for samples of FITC-conjugated caused by the overlapping emission spectra of the fluorochromes, the conjugated and PE-conjugated MoAbs and then analyzed with a cells were then incubated with appropriate amounts of FITC-labeled F(ab')2 goat antihuman s-chains. Terminal deoxynucleotidyl transferase (TdT) (BRL Laboratories, Rockville, Md) was detected by an indirect immunofluorescence assay. TdT activity of MR-87 was quantitated.

The expression of surface antigens was analyzed using various monoclonal antibodies (MoAbs) directed to differentiation antigens. The specificity and cluster of differentiation (CD) of each antibody is shown in Table I.

A half million cells were first incubated with each MoAb, and, as the second antibody, FITC-labeled IgG F(ab')2, fragments of goat anti-mouse IgG or IgM (Cappel Laboratories, Cochranville, PA) was used. The cells were then analyzed by flow cytometry (EPICS II, Coulter Electronics Inc, Hialeah, Fla). Superoxide generation of leukemic cells was measured, as described.

Two-color immunophenotyping. The MoAbs used in dual staining were FITC-conjugated B4 (CD19) or J5 (CD10) and phycoerythrin (PE)-conjugated MY7 (CD13) or MY9 (CD33) (Coulter Immunology) as the lymphoid and myeloid markers, respectively. An aliquot of cells was first treated with 15 mg/ml human immunoglobulin to reduce nonspecific binding of the MoAbs. The cells were then incubated with appropriate amounts of FITC-conjugated and PE-conjugated MoAbs and then analyzed with a two-color flow cytometer FACS 440 (Becton Dickinson, Mountain View, Calif) equipped with a 488-nm argon ion laser and interfaced with a data processing computer. To cancel out the cross-talk signal caused by the overlapping emission spectra of the fluorochromes, the compensation procedure was used for samples of FITC-conjugated 13 or PE-conjugated 13 (Coulter Immunology) stained cells and unstained cells. We equalized the PE signal levels of the FITC-stained cells and the unstained cells by means of adjusting the subtracting gain for the FITC to PE cross-talk, and similarly in another set. The background fluorescence was determined by incubating the cells with a set of isotypic-negative control antibodies (Coulter Immunology).

Simultaneous demonstration of cell surface antigen with the immunogold method and MPO at the ultrastructure level. For the immunoreaction, approximately 1 × 10^5/mL of unfixed cells were incubated for one hour at room temperature with 200 μL of B4 antibody, then washed and incubated for one hour with 200 μL of goat antimouse Ig coupled to 15 nm gold particles (Janssen Pharmaceutica, Beerse, Belgium).

After incubation, the cells were washed and fixed in a tannic acid-formaldehyde mixture for one hour and incubated in diamobenzidine tetrahydrochloride medium to demonstrate the MPO. The cells were then washed, postfixed in osmium tetroxide (OsO4), dehydrated, and embedded.

Cytogenetic studies. Chromosome analysis was performed by the trypsin-Giemsa banding methods.

Ig gene, T-cell receptor gene, and bcr gene analysis. High-molecular-weight DNA was extracted from fresh leukemia cells (obtained from bone marrow at the time of presentation) and cultured cells. Genomic DNAs that have already been shown to have germ line Ig genes, T-cell receptor (TCR) genes (Tγ), genes (Tδ), and bcr genes were also used as controls.

These genomic DNAs were digested with BamHI, EcoRI or BglII restriction endonuclease. Digested DNA was size fractionated by agarose gel electrophoresis and transferred to nitrocellulose paper. They were then hybridized with nicktranslated [32P] DNA probes of Ig genes, Tγ genes, and bcr genes, and visualized on autoradiograms. The heavy-chain-joining gene (JH) probe used in the present study is an embryonic EcoRI-HindIII JH-containing fragment. The size of this fragment, which was provided by Dr P. Leder, Department of Genetics, Harvard Medical School, Boston, is 3 kb. The human Tγ gene probe was the 0.8-kb BglII-EcoRV fragment of the cDNA clone YT-35 that contained the constant region of the TCRγ, a gift from Dr T. Mak, Department of Medical Biophysics, Ontario Cancer Institute, Toronto. The human genomic bcr probe was the 1.2-kb BglII-HindIII fragment, obtained from Oncogene Science, Inc, New York. Using this probe, we detected rearrangement of the bcr region in about 95% of patients with Ph+ CML, and in some with Ph+ ALL.

Detection of a novel abl-related protein, P190, in cultured cells. Cells were metabolically labeled for two hours at 37°C with 300 μCi [3H] thymidine (Japan Atomic Energy Research Institute, Tokyo) per milliliter, in phosphate-free Hanks' balanced salt solution containing 5% dialyzed FCS (1 × 10^5 cells/mL). The cells were washed and solubilized in ice-cold lysis buffer (0.15 mol/L NaCl, 50 mmol/L Tris-HCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L ethylene glycol tetra-acetic acid (EGTA), 2 mmol/L MnCl2, 0.1 mmol/L ZnCl2, 50 μmol/L sodium vanadate, and 1 mmol/L phenylmethylsulfonl fluoride, pH 7.4). The clarified lysates were immunoprecipitated with either 5 μL of anti-abl sera, abl pEX-5, provided by Dr O.N. Witte,10 or 5 μL of antiphosphoty-

Table 1. Cell Surface Antigens on FrL Cells, MR-87, and MR-B Cells

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>CD</th>
<th>FrL Positive Cells (%)</th>
<th>MR-87 Positive Cells (%)</th>
<th>MR-B Positive Cells (%)</th>
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<td>7</td>
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<td>9</td>
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<td>MY7</td>
<td>13</td>
<td>5--37*</td>
<td>87</td>
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<td>MCS2</td>
<td>13</td>
<td>7--67*</td>
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<td>14</td>
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<tr>
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Abbreviations: CD, cluster of differentiation; ND, not done.
*Cells were cultured for seven days.
PH BCR CELL LINE WITH HYBRID PHENOTYPE

Fig 1. Morphology of fresh and cultured cells. (A) MG staining of FrL cells in the bone marrow (original magnification × 520; current magnification × 312). (B) Cytocentrifuged smear of leukemia cells with MPO staining. Cells had been cultured for seven days in 10% FCS RPMI (original magnification × 500; current magnification × 300). (C) Cytocentrifuged smear of MR-87 cells with MG staining (original magnification × 520; current magnification × 312).

cellosine sera, prepared as described by Pang et al. for two hours at 4°C and then incubated with 50 μL of protein A Sepharose (Pharmacia, Piscataway, NJ) for an additional two hours. The beads were washed and the absorbed materials were analyzed by 7.5% SDS-gel electrophoresis.

RESULTS

Cell culture. Cell growth became apparent 10 weeks from seeding in the flask supplemented with 20% FCS. The cell line designated as MR-87 showed a slow growth rate with a doubling time of 120 to 144 hours. The concentration of FCS was gradually reduced, and the cell line has been continuously maintained in 10% FCS in RPMI medium.

In another flask supplemented with 10% FCS, active cell growth was noted earlier than that of MR-87 (at 8 weeks). This was designated as the MR-B line. The MR-B cells formed large clumps and had a shorter doubling time of 24 to 36 hours.

Examination of Epstein-Barr virus (EBV) genome in the cells revealed that MR-87 cells were negative and MR-B cells positive for EBV. Both cell lines have been maintained in a stable condition for over 1 year and are free of mycoplasma contamination (determined by Dr J. Minowada of Hayashibara Institute, Okayama, Japan).

Morphology and cytochemistry of leukemic cells. Fresh leukemia cells were round and had a scant cytoplasm (Fig 1A). A small percentage of the cells had fine cytoplasmic granules, and special stains revealed that 5% and 7% were positive for MPO and SBB, respectively. PAS and NSE reactivity was nil. Interestingly, over 95% of these cells exhibited strong MPO reactivity seven days after initiation of the cultures (Fig 1B).

Morphology of the MR-87 cells is shown in Fig 1C. They were round and had a high N/C ratio. Oval or reniform nuclei were present in the majority of cells. One or two prominent nucleoli were present. Small proportions of MR-87 cells possessed cytoplasmic granules, and 5% of the cells were both MPO- and SBB-positive. The MR-87 cells lacked PAS and NSE reactivity.

MR-B cells, on the other hand, were large and had an abundant basophilic cytoplasm with azurophilic granules, typical characteristics of lymphoblastoid cells (not shown).

Immunologic marker studies. Both fresh leukemia (FrL) cells and MR-87 cells were negative for SmIg and Clg. Over 90% of the MR-B cells were positive for SmIg and negative for Clg. Table 1 summarizes the reactivity of various monoclonal antibodies of FrL cells, MR-87 cells, and MR-B cells. FrL and MR-87 cells were 1a+, TdT+, and MR-B cells were 1a−, TdT+.
CD19+, and CD10+, typical surface phenotype for common acute lymphoblastic leukemia (cALL). TdT activity of MR-87 cells was markedly high (79 U/10^6 cells) when quantitated (normal <0.1 unit). Interestingly, MR-87 cells were positive for CD13 (MY7, MCS2), which was reported to be a specific marker for myeloid leukemia cells. On the other hand, FrL cells expressed this antigen only when cultured for a few days in vitro. MR-B cells exhibited surface characteristics of B lymphoblasts. FrL cells and MR-87 cells did not generate superoxide when they were stimulated in vitro with wheat germ agglutinin and phorbol myristate acetate, thereby suggesting that they were not of monocytic lineage.

Two-color immunophenotyping. To confirm that a single cell possesses both lymphoid and myeloid surface characteristics, two-color analysis of the MR-87 cells was performed. The results indicated that 82% and 79% of the MR-87 cells were B4+-MY7+ and J5+-MY7+, respectively (Fig 2). On the other hand, B4+-MY9+ and J5+-MY9+ cells were only 7% and 3%, respectively.

Ultrastructural studies. MR-87 cells exhibited characteristics of immature myeloid cells. Although only 5% of the cells were reactive to MPO cytochemically, more than 90% were positive for MPO, when examined electron microscopically. MPO synthesis was clearly demonstrated in the perinuclear space, rough surfaced endoplasmic reticulum, Golgi apparatus, and granules. Reactivity to B4 antibody on each of MPO-positive MR-87 cells was also confirmed, although
interest to examine the c-abl protein expression in this cell line. We metabolically labeled the cells with $^{32}$Pi and detected MW 190,000 phosphoprotein, a novel abl-related protein that immunoprecipitated with both the anti-abl antibody and the antiphosphotyrosine antibody (Fig 6, lanes 1 and 2). Low molecular weight (mol wt) proteins reacting with anti-abl antibody might be proteolytic fragments of P190. The P190 in MR-87 cells was distinguishable from a phosphoprotein of MW 210,000, called P2190$^{bcr}$, the bcr-abl fusion protein in K562 cells (Fig 6, lanes 3 and 4).

**DISCUSSION**

From several lines of clinical evidence and from laboratory data, we concluded that the patient, T.M., had de novo Ph+ acute leukemia rather than blast crisis of Ph+ CML; the patient had no known history of a chronic phase prior to presentation. Although massive hepatosplenomegaly was evident on admission, there was no basophilia in the peripheral blood or bone marrow, a common finding in blast crisis of CML. Furthermore, the Ph+ clone completely disappeared in bone marrow when the cytogenetic analysis was repeated during CR prior to BMT. On the other hand, patients with CML in blast crisis usually retain the Ph abnormality while in clinical remission.

We obtained evidence that the MPO+ MR-87 cell line was derived from the same leukemia cell clone at diagnosis, determined using immunologic, cytogenetic, and molecular analysis. The MR-87 cells were Ph+ and exhibited a similar surface phenotype seen in the fresh cells. The DNA analysis
indicated that both cells had the same rearranged \( J_\mu \) bands, at an identical position.

Several Ph+ cell lines expressing various phenotypes have been reported; however, the majority were derived from patients in blast crisis of Ph + CML. More recently, a few Ph+ cell lines from patients with Ph+ ALL have been reported. However, to our knowledge, MR-87 is the first cell line established from a patient with Ph+ MPO+ acute leukemia at the time of diagnosis.

Results of immunophenotyping and DNA analysis of FrL cells and MR-87 cells showed that despite their MPO reactivity, both had a phenotype of 1a+, TdT+, CD19+, and CD10+, as well as a positive Ig gene rearrangement—typical characteristics of early B-cell lineage. In addition, morphology of FrL cells might be regarded as lymphoid (L1-type) rather than myeloid (Fig 1A).

Simultaneous presence of B-cell antigens and myeloid antigen CD13 or MPO on a single MR-87 cell was confirmed by a two-color analysis and an ultrastructural study. An increasing number of workers have reported similar cases of acute leukemia in which individual leukemia cells coexpress markers usually restricted to a single lineage. Expression of B-cell antigen, presence of TdT activity, and even Ig gene rearrangement in AML cells have been described. Different terms were used to refer to these leukemias, including mixed lineage, biphenotypic, hybrid, lineage infidelity, and others.

In several patients with Ph+ leukemia, the blasts carried both myeloid and lymphoid markers. The majority were patients in blast crisis of Ph + CML. The simultaneous presence of myeloblasts in Ph + ALL was also described. Ribeiro et al analyzed clinical and biologic features of 18 children with Ph + ALL. They found unique characteristics of the disease such as high leukocyte count, French-American-British L1 morphology, early CNS involvement, and common ALL immunophenotype, all of which were observed in our case. However, no single case among their series was documented to exhibit a hybrid phenotype such as additional myeloid features. More recently, Hirsh-Ginsberg et al described phenotypic and molecular heterogeneity in Ph+ acute leukemia. Using molecular probes for MPO and TdT, they found that four of five were cases of mixed lineage leukemia (AMLL), thereby suggesting a relatively high incidence for mixed lineage in Ph+ acute leukemia. However, there seems to be no report in the literature describing a case or cell line, such as MR-87, that exhibits a hybrid phenotype of blasts in Ph + MPO+ bcr− acute leukemia or AMLL.

The biologic significance of the hybrid expression on the blast is not well understood. It could be an aberrant gene expression of malignant cells or an expansion of a clone coexpressing both lineage markers, which may occur in normal multipotent progenitors.

Other investigators suggested that, as was shown in Ph+ CML, involvement of a stem cell with multipotent expression occurs in some patients with AML and childhood Ph+ ALL. This might support the existence of such a clone alluded to in the latter explanation.

Successful establishment of the B-lymphoblastoid cell line, MR-B, from the same patient was unexpected. Since the MR-B cells were Ph− and had one rearranged \( J_\mu \) band of a different size from that of the MR-87 cells, the two cell lines are clonally different. Most likely, the MR-B cells were derived from EBV-infected nonleukemic B-lymphocytes contaminated in the original cell sample. It seems likely that the cells were initially polyclonal; however, expansion and subsequent establishment of such clonal EBV+ B-lymphoblastoid cell lines during culture has been described. Different FCS concentrations (20% and 10%) used in the culture might have contributed to the establishment of two different cell lines.

In the Ph of CML, the \( c-abl \) gene on chromosome 9 is translocated to bcr on chromosome 22, an event resulting in the expression of a chimeric bcr-\( abl \) message that encodes the P210\( \text{bc}-\text{abl} \) tyrosine kinase. Several investigators have found molecular heterogeneity of chromosome 22 breakpoints in Ph+ ALL and subsequent studies demonstrated a novel \( abl \) protein, P190, in Ph+ bcr− ALL. Therefore, formation of P190\( \text{bc}-\text{abl} \), instead of P210\( \text{bc}-\text{abl} \), is considered to be a characteristic of acute rather than the chronic Ph+ leukemia. Using two antisera, we found that bcr− MR-87 cells expressed the P190\( \text{bc}-\text{abl} \) protein, which was reactive to anti-\( abl \) and anti-phosphotyrosine antibodies. Therefore, our result strongly suggests that MR-87 was of Ph+ acute leukemia cell origin. Since the production of an abnormal fusion protein, P210\( \text{bc}-\text{abl} \) or P190\( \text{bc}-\text{abl} \), was found to be associated with an enhanced protein-tyrosine kinase activity, the MR-87 cell line should prove to be a useful tool to elucidate different genetic mechanisms of activation between Ph+ acute leukemia and a chronic leukemia.

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A novel leukemia cell line, MR-87, with positive Philadelphia chromosome and negative breakpoint cluster region rearrangement coexpressing myeloid and early B-cell markers

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