Establishment and Characterization of a Human Myeloid Cell Line From Philadelphia Chromosome-Negative Myeloblastic Leukemia Arising in a Patient With Myelodysplastic Syndrome

By Todd M. McCarty, S. Rajaraman, F.F.B. Elder, P. Gadson, and E. Brad Thompson

A new hematopoietic cell line derived from a patient with Philadelphia chromosome (Ph')-negative myeloblastic leukemia arising from a form of myelodysplastic syndrome (MDS) is described. This cell line, designated TMM, consists of immature cells with the morphological characteristics of young myeloblasts and grows in suspension culture with a doubling time of about 30 hours. By cytochemical analysis the cultured cells were positive for acid phosphatase. They were free of the Epstein-Barr virus-associated nuclear antigen as well as terminal deoxynucleotidyl transferase. Further phenotypic analysis revealed the expression of the myelomonocytic-specific antigen Leu-M1 and receptors for the Fc portion of IgG. Partial differentiation of these cells could be induced by dimethyl sulfoxide, tetradecanoyl phorbol acetate, or hypoxanthine and resulted in cells of the myeloid series expressing lysozyme and receptors for the C3b complement protein. The karyotype was 46,XY, lacked the Ph' chromosome, and displayed no abnormalities at the light microscopic level. No rearrangement of the bcr-c-abl gene complex was found. This cell line should be useful for studying an important type of the heterogeneous population constituting Ph' negative myeloblastic leukemia, arising in this instance from MDS, as well as for studying differentiation and proliferation of human pluripotent stem cells.

MATERIALS AND METHODS

Case history. The TMM cell line was derived from the peripheral blood of a 62-year-old white male originally diagnosed as having Ph'-negative CML in blast crisis. He was diagnosed as having chronic obstructive pulmonary disease in 1981 and treated with cephadine, vitamin K, theophylline, and terbutaline. In August 1984 he presented with a chronic bacular mucosa ulcer and was found to have thrombocytopenia (75,000/μL) and splenomegaly. His WBC was 17,100/μL with monocytosis (13%), and abnormal platelet morphology was observed. From that time until his death, the patient's blood cellularity was characterized by monocytosis (up to 59% on differential counts), frequently by eosinophilia (up to 24%), and by thrombocytopenia. In November 1984 he was diagnosed as having Ph'-negative CML with a bone marrow karyotype of 46,XY, and a bone marrow biopsy that showed an M:E (myeloid:erythroid) population constituting Ph'-negative myeloblastic leukemia. He was treated with hydroxyurea and Depo-Testosterone. His WBC was 326,000/μL with 76% blasts, 1% banded neutrophils.

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1% segmented neutrophils, 17% monocytes, and 5% lymphocytes. Leukopheresis was performed for three consecutive days, and although the WBC dropped to 100,000/μL, the patient continued to deteriorate and died on October 23, 1985, of bronchopneumonia.

**Cell culture.** The leukopheresed blood was collected in a heparinized bag on October 17, 1985, when the patient’s WBC was 223,000 with 69% blasts. Mononucleated cells were separated by Ficoll/sodium diatrizoate (Sigma Chemical Co, St Louis) density gradient centrifugation and suspended in RPMI 1640 medium (GIBCO, Chagrin Falls, OH) supplemented with 10% heat-inactivated fetal calf serum (KC Biological, Lenexa, KS). After a lag phase, cells with blast morphology began to grow. There was no massive loss of cells, and the cultured line seemed to develop from many cells rather than a few clones. Cells were grown as stationary-suspension cultures at 37°C in a humidified atmosphere of 95% air/5% CO2. Although the WBC dropped to 100,000/μL the patient continued to improve in clinical condition, and the cultured line seemed to develop from many cells rather than a few clones. Cells were grown as stationary-suspension cultures at 37°C in a humidified atmosphere of 95% air/5% CO2. Although the WBC dropped to 100,000/μL the patient continued to improve in clinical condition.

Morphological and cytochemical analysis. Cytoctrifuge preparations were stained with Wright-Giemsa in the University of Texas Medical Branch (UTMB) Special Hematology Laboratory for morphological analysis. The slides were examined by several members of the Department of Hematology and the experienced technicians of the Special Hematology Laboratory. For cytochemical analysis the following staining reactions were performed: peroxidase, α-naphthyl butyrate esterase (α-NBE), naphthol ASD chloroacetate esterase (CAE), acid phosphatase, alkaline phosphatase, PAS, and α-naphthyl acetate esterase (Sigma), with L cells used as a positive control.

**Epstein-Barr virus-associated nuclear antigen (EBNA) terminal deoxynucleotidyl transferase.** Testing for the presence of Epstein-Barr virus-associated nuclear antigen (EBNA) was kindly performed by Dr. Harry Connell, Jr., Department of Internal Medicine, by the method of Reedman and Klein. 28 Raji cells were used as a positive control while K-562 cells were used as a negative control. Terminal deoxynucleotidyl transferase (TdT) was assayed by indirect immunofluorescence using the BRL TdT kit. (Bethesda Research Laboratories, Bethesda, MD)

**Ultrastructural analysis.** Unstimulated TMM cells and TMM cells stimulated to differentiate with dexamethasone were fixed for electron microscopy in 1.25% glutaraldehyde in Dulbecco’s phosphate-buffered saline (PBS, Sigma) for 30 minutes at room temperature. Localization of endogenous peroxidase was carried out according to the procedure of Gershon and Karnovsky. 29 The cells were viewed by using a Philips EM 300 transmission electron microscope (Mahwah, NJ). Electron microscopy was carried out in the UTMB Cancer Center Core Facility.

**Analysis of cell marker antigens.** Cytoctrifuge preparations were stained for one hour, fixed in cold acetone for ten minutes, and stained after reaction with various antibodies by using the avidin-biotin-peroxidase complex method. The slides were incubated with monoclonal antibodies Leu-M1, Leu-14, Leu-11B, Leu-15, Leu-9, and anti-CR1 (Becton Dickinson, Sunnyvale, CA) and monospecific polyclonal antibodies to lysozyme and α-antichymotrypsin (Dako Laboratories, Santa Barbara, CA) for 60 minutes at room temperature in a humidified chamber. Sheep antivon IgG F(ab’)2, goat antivon IgM F(ab’)2, or goat antirabbit IgG F (ab’)2 (Cooper Biomedical, Westchester, PA) and streptavidin-biotin-peroxidase complex (Amersham Corp, Arlington Heights, IL) were used as the second and third reagents. The final reaction product was visualized after incubation with diaminobenzidine, 0.05% in PBS, pH 7.2, containing 0.02% H2O2. A single-step procedure using peroxidase-conjugated rabbit antihuman Ig (polyvalent) was carried out to detect surface Ig. The reactions were scored based on the intensity of the color reaction: (-) no reaction, (+) clearly positive, (+ +) strongly positive. When a positive reaction was seen, virtually all cells on the slide were positive. Controls included (a) omission of the primary antibody, (b) substitution of the primary antibody with preimmune serum of the same species, and (c) omission of primary and secondary antibodies.

**Differentiation induction.** Cells were suspended in growth medium containing various inducing agents and incubated for six days under our standard culture conditions. Dimethyl sulfoxide (DMSO) and tetradecanoyl phorbol acetate (TPA) were obtained from Sigma, St. Louis and hypoxanthine from Calbiochem-Behring Corp. (La Jolla, CA). DMSO was diluted to a concentration of 180 mmol/L in RPMI 1640, and TPA was dissolved in absolute ethanol and diluted in RPMI 1640 to a concentration of 10-8 mol/L. Hypoxanthine was dissolved in boiling serum-free RPMI 1640 medium at a concentration of 50 mol/L and immediately diluted for use to 5 mmol/L. The cells were suspended at a density of 105 cells/mL in each inducing agent and monitored throughout the six-day period. At the end of that time the cells were stained with Wright-Giemsa for morphological analysis. Cell morphology was evaluated by the experienced staff of the UTMB Special Hematology Laboratory who were unaware of the various treatments the cells had received as the slides were read. Functional analysis was determined by the expression of cell marker antigens.

**Cytogenetic analysis.** TMM cells were treated with Colcemid (Boehringer-Mannheim, Indianapolis) at a final concentration of 5 × 10-4 μg/mL for 40 minutes. The cells were then subjected to hypotonic treatment (0.075 mol/L KCl) for 15 minutes at 37°C, fixed, and air-dried to slides by following conventional cytogenetic methodology. Trypsin G-banding followed methods modified from Seabright.30

**Restriction endonuclease analysis.** DNA was extracted from cells, digested with endonuclease, subjected to electrophoresis in agar gels, and transferred from the gels to nitrocellulose filter paper as previously described.31 The filters were probed with a 0.65-kilobase (kb) clone of c-abl or a 1.2-kb clone of c-erb (both obtained from Oncogene Sciences, Inc, Mineola, NY) and labeled to a specific activity of >106 cpm/mg by a standard nick-translation technique.32 Hybridization conditions were as described previously.31

**RESULTS**

**Establishment of TMM cell line.** The patient’s leukopheresed cells were placed in culture medium at a density of 5 × 104 cells/mL and left undisturbed for 1 week. During that time about 30% of the cell population became pyknotic and nonviable. Cells that did not adhere to the growth chamber were removed and placed into fresh culture medium. Small aggregates of viable cells were evident at that time, and the first subculture was made. In the following 2 weeks about 60% of the cell population was viable, and the cell aggregates were actively proliferating. During the next 13 weeks the cells were manually diluted once a week to a density of 104 cells/mL and fed with fresh medium. After 16 weeks in culture medium the cells were 95% viable and had a doubling time of about 30 hours. Since that time they have been stable. The cells that have been in continuous culture for over 8 months continue to have a doubling time of about 30 hours and grow as a mix of single cells and free-floating aggregates.
Morphological analysis. The patient's cells originally received in our laboratory were of myeloblastic appearance, and the differential count of peripheral blood included some mature neutrophils and monocytes. The blasts had round or lobulated nuclei with basophilic cytoplasm and a few vacuoles (Fig 1A). The morphological appearance of TMM cells was consistently that of immature, undifferentiated blasts resembling the original leukemic cells. The cytoplasm was basophilic, devoid of granules, and contained a few vacuoles. The nuclei were spherical and contained fine chromatin strands that stained reddish purple. The nuclear-to-cytoplasmic ratio was high, and one to five nucleoli with sharply defined borders were discernible (Fig 1B).

Cytochemical analysis. Cytochemical analysis of the original leukapheresed leukemic cells resulted in 58% of the cells staining positive for peroxidase, 79% for α-NBE, and 100% for acid phosphatase. TMM cells were 100% positive for acid phosphatase, with a punctate pattern observed, but were negative for α-NBE. Both the original leukemic cells and TMM cells were negative for TdT. TMM cells were negative for EBNA (Table 1).

Ultrastructural analysis. Electron microscopy studies revealed that TMM cells contain the large, spherical nuclei typical of blasts. In the cytoplasm a few vacuoles together with other usual cytoplasmic structures such as mitochondria, rough endoplasmic reticulum, and microtubule organizing centers were seen (Fig 2A). No viral structures were found. The TMM cells treated by hypoxanthine (5 mmol/L) were smaller and had indented to segmented nuclei and more mature cytoplasm. Many spherical, homogenously electron-dense, peroxidase-positive cytoplasmic granules were present, similar in appearance to those of normal granulocytic cells (Fig 2B).

Cell marker analysis. Both the original cells and TMM cells were surface Ig-negative, B-cell associated antigen (Leu-14)–negative, E rosette (Leu-9)–negative, Fc receptor (Leu-11b)–positive, myelomonocytic antigen (Leu-M1)–positive, α1-antichymotrypsin–negative, C3b receptor (anti-CR1)–negative, and C3bi receptor (Leu-15)–negative. The original cells were positive for lysozyme, whereas TMM cells were negative (Table 2).

Differentiation induction. The uninduced TMM cells contained 2% mature myeloid cells. TPA and hypoxanthine each stimulated partial morphological maturation (42% and 68%, respectively) to cells with the appearance of early myeloid precursors while also inducing lysozyme and receptors for the C3b complement protein. TPA treatment did not stimulate increased adherence to the surface of the culture vessel but did slightly increase the tendency for cells to adhere to another. TPA did not induce α-naphthyl acetate esterase. Neither hypoxanthine nor TPA caused any of the cells to take on the appearance of monococyte/macrophage-like cells. DMSO also stimulated the cells to express lysozyme, but did not induce morphological maturation (Table 2).

Cytogenetic analysis. Cytogenetic analysis of 25 G-banded metaphase cells showed a range of 43 to 46 chromosomes. The modal number was 46 (22 of 25 cells), and the karyotype was 46,XY (Fig 3). The remaining three cells had karyotypes of 45,XY,−12, 44,XY,−7,−12; and 43,XY,−12,−21,−22, respectively. Scoring of 100 randomly selected metaphases showed 2% of the cells to be in the tetraploid range.

Lack of bcr-c-abl complex rearrangement. Although TMM cells lack the Ph1 chromosome, instances are known in which euploid cells show rearrangement of the c-abl gene and bcr gene. Restriction endonuclease digestion and Southern blot analysis of DNA from TMM cells and the leukemic

![Fig 1. (A) Cytospin preparation of TMM cells stained with Wright-Giemsa (original magnification × 225; current magnification ×110). (B) Cytospin preparation of original leukapheresed leukemic cells stained with Wright-Giemsa (original magnification × 225; current magnification × 110).](image-url)
Abbreviation: SmIg, surface immunoglobulin.

Fig 2. (A) Ultrastructural appearance of a typical TMM cell cultured under usual conditions (original magnification x 12,000; current magnification x 9,000). (B) Ultrastructural appearance of a typical TMM cell cultured in hypoxanthine (5 mmol/L) for six days (original magnification x 20,000; current magnification x 15,000).

Table 2. Phenotypic Analysis of Original Leukapheresed Leukemic Cells, TMM Cells, and TMM Cells Induced to Differentiate With DMSO, TPA, and Hypoxanthine

<table>
<thead>
<tr>
<th></th>
<th>Original Leukapheresed Leukemic Cells</th>
<th>Unstimulated</th>
<th>DMSO</th>
<th>TPA</th>
<th>Hypoxanthine</th>
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</thead>
<tbody>
<tr>
<td>SmIg</td>
<td>-</td>
<td>-</td>
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<tr>
<td>E rosette</td>
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<td>B cell</td>
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<td>Fcγ, receptor</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Leu-M1 antigen</td>
<td>++</td>
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<tr>
<td>CR1 (C3b/C4b receptor)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>CR3 (C3bi receptor)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>α-antichymotrypsin</td>
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<tr>
<td>Lysozyme</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Mature myeloid cells* (%)</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>42</td>
<td>68</td>
</tr>
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Abbreviation: SmIg, surface immunoglobulin.

*Includes myelocytes, metamyelocytes, and banded and segmented neutrophils.
Ph'-NEGATIVE MYELOBLASTIC LEUKEMIA

Fig 3. Giemsa-banded karyotype of the TMM line showing 46,XY.

patient's peripheral blood cells has not as yet shown any such rearrangement (Fig 4).

DISCUSSION

We herein described a novel cell line, designated TMM, derived from the peripheral blood of a 62-year-old male with a form of Ph'-negative myeloblastic disease arising from what, in retrospect, was MDS. Several features of the patient's blood and marrow point to the blast crisis arising from an MDS, ie, thrombocytopenia with abnormal megakaryocytes, monocytosis with peripheral monocytes and promonocytes >1 x 10^9/L, and abnormal erythrocytes with anemia. Therefore a current definition of the patient's disease might be MDS with chronic myelomonocytic leukemia (CMML). No sideroblasts were seen, and disgranulopoiesis was not commented on in the patient's hematologic records. The relative eosinophilia is not typical and remains unexplained.

When compared, the similarity of its cellular characteristics to the original leukemic cells, and the lack of EBNA and TdT indicate that the line we have established is of blast cell origin. Cytotoxic analysis showed positive, punctuate staining for acid phosphatase, and morphological studies revealed a majority of undifferentiated cells with large spherical nuclei and cytoplasm devoid of granules. A few (2%) promyelocytes, myelocytes, and metamyelocytes were also observed. The majority (90%) of the cells had a karyotype of 46,XY and expressed no chromosomal abnormalities at the light microscopic level. Restriction endonuclease digestion followed by Southern blotting showed no evidence for cryptic rearrangement of the bcr-c-abl loci. As shown in Table 2 the TMM cells expressed the myelomonocytic-specific antigen Leu-M1 as well as receptors for the Fc portion of IgG. Both myeloid and monocytoid differentiation involve expression of the Fc receptor, and therefore TMM cells were induced to differentiate to help determine their classification. Phorbol esters, purines and purine analogues, and polar planar compounds stimulate myeloid, erythroid, and monocytoid differentiation in a variety of leukemia cell lines, and therefore TPA, hypoxanthine, and DMSO were tested on TMM cells. Induced differentiation of this line by TPA and hypoxanthine resulted in cells morphologically resembling somewhat more mature cells of the myeloid lineage and stimulated expression of lysozyme and receptors for the C3b complement protein. DMSO stimulated the expression of lysozyme only. Ultrastructural analysis of hypoxanthine-induced cells showed a higher degree of differentiation compared with unstimulated TMM cells. The induced cells were smaller and had indented to segmented nuclei and more mature cytoplasm containing many granules that stained positive for peroxidase. Such granules, reported to contain lysozyme, myeloperoxidase, and many acid hydrolases, are important in the inflammatory, and, in associa-
leukemias have differing properties, and these have been used to advantage in many studies. In CML, although granulocytic cells usually predominate in the chronic phase, blast crisis can result in various lineages predominating in various patients, including myeloid, lymphoid, and erythroid types. K-562 cells, derived from a patient with CML in blast crisis, express erythroid characteristics, whereas MEG-01, also established from a patient with CML in blast crisis, has megakaryocytic properties. Recent evidence indicates T-cell as well as B-cell involvement in blast crisis, the latter reported in the CML cell line NALM-1. TMM, the new line reported here, was obtained from a patient whose clinical studies fit the criteria for MDS with CMML. TMM cells have characteristics not found in these previously established lines. TMM cells therefore may prove useful in studying the process of differentiation and proliferation of human pluripotent stem cells.

Within CML, variation in prognosis has been suggested to correlate with gene rearrangements associated with the Ph chromosome. Although about 10% of CML patients lack the Ph chromosome, some of these still show an associated molecular aberration involving the protooncogenes c-sis and c-abl. C-sis is usually not expressed in these cells, but a c-sis gene product and/or platelet-derived growth factor, which share homologous sequences, may play a role in myelofibrosis, which often accompanies CML. On the other hand, c-abl has been found to be rearranged to show increased expression in all cases of Ph-positive and in some Ph-negative CML. An aberrant 8-kb c-abl mRNA transcript as well as the corresponding abnormal protein has been found in many of these patients and in the Ph-positive CML cell line K-562. Morris et al have recently reported that differences in the location and expression of c-abl correlate with the survival heterogeneity found by Canellos et al and the Rosewell Park Memorial Institute study of Ph-negative CML patients. The value of in vitro models that would enable the expansion of our knowledge in this ill-defined category of myeloid leukemia has recently been noted. It appears that patients with the normal c-abl gene have a much shorter survival time. Nonrandom cytogenetic abnormalities have been reported in MDS. Most often these have been specific deletions or translocations, including 5q-, 5q-, -7, +8, 20q-, t(8:21), and +/−21. Because of this, because the TMM cell line reported here came from a patient originally diagnosed as having Ph-negative CML, and because in any case this patient had a CMML within an MDS, the cells were tested for a cryptic rearrangement of the bcr-c-abl complex. At least one report, however, has been made of chronic MDS with the Ph chromosome. On the basis of our findings to date, the bcr-c-abl complex is normal in TMM cells. Again, this new cell line may be useful to workers interested in this poorly understood area of disease.

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