Philadelphia Chromosome (Ph\(^1\))-Negative Chronic Myelogenous Leukemia (CML): A Clonal Disease With Origin in a Multipotent Stem Cell

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It has been shown with glucose-6-phosphate dehydrogenase (G-6-PD) mosaicism that Ph\(^1\)-positive chronic myelogenous leukemia (CML) is a clonal disease that involves multipotent hematopoietic stem cells. We now report G-6-PD studies of a 79-yr-old woman with Ph\(^1\)-negative CML. Equal amounts of B and A-type activities were found in nonhematopoietic tissues, indicating that the patient was heterozygous for G-6-PD. In contrast, only A-type G-6-PD was found in marrow cells, blood erythrocytes, leukocytes, and platelets and in granulocyte-monocyte and eosinophil colonies grown from blood mononuclear cells. Unlike most cases of Ph\(^1\)-positive CML, colony growth in this patient increased during blastic transformation and the colonies contained only immature monocytic cells. The data indicate that in this patient, Ph\(^1\)-negative CML is similar to the Ph\(^1\)-positive form of the disease in involvement of multipotent stem cells and probable clonal origin, but the two disorders differ in the rapidity with which they enter blastic transformation and in the pattern of granulocyte-monocyte colony growth at that time.

Approximately 85 percent of patients with chronic myelogenous leukemia (CML) have a very characteristic and specific chromosomal abnormality, the Philadelphia chromosome (Ph\(^1\)) rearrangement. Ph\(^1\)-negative patients have notably poorer prognoses and show other differences from Ph\(^1\)-positive cases, indicating important biologic distinctions between the two types of leukemia. However, the relationship of Ph\(^1\) to the cause, pathogenesis, and progression of the disease is unknown. It has been shown with glucose-6-phosphate dehydrogenase (G-6-PD) markers that Ph\(^1\)-positive CML is a clonal disease that involves pluripotent hematopoietic stem cells. We report similar studies in a patient with Ph\(^1\)-negative CML.

Materials and Methods

Case Report

On March 21, 1979, a 79-yr-old woman was admitted to George-town University Hospital with weakness, fatigue, and shortness of breath of 4-mo duration. Two years earlier, the hematocrit was normal and the white cell count was 3750 with 32 percent neutrophils and 64 percent lymphocytes. One month before admission, the hematocrit was 27 percent and the white cell count was 30,000. On admission, anemia and hepatosplenomegaly were noted. The white cell count was 87,100 with 12 percent neutrophils, 10 percent bands, 12 percent lymphocytes, 8 percent monocytes, 1 percent eosinophils, 1 percent basophils, 11 percent myeloblasts, 3 percent promyelocytes, 29 percent myelocytes, and 13 percent metamyelocytes. The platelet count was 125,000/mm\(^3\). The marrow showed markedly increased numbers of marrow myeloid hyperplasia with a predominance of myeloblasts (20 percent), progranulocytes, and neutrophilic and eosinophilic myelocytes. A minimal amount of fibrosis was noted on marrow biopsy. Leukocyte alkaline phosphatase score was 0 and serum vitamin B\(_12\) level was greater than 2000 pg/ml.

A diagnosis of CML was made and the patient was discharged, but 4 wk later she was readmitted with symptoms and signs consistent with a subcapsular splenic infarction. The white cell count was 128,000 with 18 percent myeloblasts. Treatment with hydroxyurea was begun. Over the next 4 wk, the white cell count steadily decreased and on May 20 it was 32,100 with 15 percent myeloblasts. Eleven weeks after initial admission, the white cell count was 103,000 with 15 percent neutrophils, 8 percent bands, 2 percent lymphocytes, 32 percent monocytes, 1 percent metamyelocytes, 1 percent basophils, 12 percent myelocytes, 1 percent progranulocytes, 13 percent myeloblasts, and 15 percent monoblasts. Marrow examination showed hypercellularity with about 70 percent blast cells and atypical monocyctic cells. Some of these cells had a morphological appearance of myeloblasts and others of monoblasts. Some blast cells stained positively with peroxidase and many were positive with a fluoride-inhibited esterase stain. Serum muramidase level was grossly elevated at 300 mg/ml (normal 4–20).

At this time, the clinical and laboratory findings were interpreted as being consistent with a myeloblastic phase of chronic myelogenous leukemia. The patient died on June 16 with septic shock. No autopsy was performed.

Methods

Specimens were sent on ice to Seattle for studies of G-6-PD and chromosomes. Cell extracts were prepared and assayed for G-6-PD as described previously. Peripheral blood or marrow mononuclear cells were separated with a Ficoll/sodium diatrizoate gradient (Isolymph, Teva, Ltd., Jerusalem) for G-6-PD studies. Blood mononuclear cells were also cultured for granulocytic colony growth as previously described with a maximum stimulatory amount of PHA-conditioned
medium. After 14 days in culture, the colonies were counted at 40x with an inverted microscope, and individual colonies were picked for G-6-PD and morphological studies. The frequency of eosinophil colonies was determined by in situ staining.

At the time the patient entered blast crisis, a cell culture was established. Mononuclear cells were placed in flasks (Falcon Plastics) in a mixture of 5 percent human type AB serum, RPMI 1640 (Microbiological Assoc., Rockville, Md.) and 20 percent fetal calf serum. After 3 days in culture, the nonadherent cells were removed and then passed every 3–4 days. At intervals, cells were taken for analysis of G-6-PD phenotype, cytochemical staining for peroxidase and nonspecific esterase, and for cytogenetic analysis. Aliquots of the cells were also tested to see whether they formed colonies in soft agar with and without added sources of colony-stimulating factor.

For chromosomal studies, marrow and peripheral blood white cells were examined after 12, 24, and 96 hr in culture without mitogen stimulation. Karyotypes were prepared with an acetone-saline-Giemsa banding technique. Karyotypes were also prepared from cells growing in the culture.

Because of the relatively low percentage of lymphocytes and the presence of numerous immature myeloid cells, attempts to make homogeneous preparations of lymphocytes directly from blood for G-6-PD assays were not successful. B-cell cultures were not obtained. For T-cell growth, lymphocytes were cultured on June 11, in the presence of human T-cell growth factor (TCGF) prepared with a modification of the method of Strausser and Rosenberg. Peripheral blood mononuclear cells prepared with a Ficoll/Hypaque gradient were cultured (10⁶ cells/ml) in RPMI 1640 medium (Gibco) with glutamine, antibiotics, 0.08 percent PHA-P (Difco), and 10 percent fetal calf serum. After 1 wk, the cells were placed in fresh medium (1.5 x 10⁶ cells/ml) containing 25 percent TCGF. The cells were diluted to this concentration every 5 days and grown until sufficient numbers of lymphocytes were obtained for G-6-PD and E-rosetting studies.

RESULTS

The ratio of type A to type B G-6-PD in skin and cultured skin fibroblasts was approximately 1:1.

Peripheral blood cells were first tested on the day of admission before any transfusions were given. Only type A-enzyme was found in red and white cells. Blood cells were tested four more times throughout the course of the disease, including preterminally during the blastic phase. Marrow was tested at that time and on two previous occasions. Only type-A enzyme was found in all leukocyte preparations and in platelets. Following transfusions, a small amount of type-B enzyme was found in erythrocytes.

Ten and 20 blood cell metaphases obtained on March 28 and April 18, respectively, were studied after 12 hr in culture. Three cells had 45 chromosomes with random chromosome loss and the other 27 cells had 46 chromosomes. Ph¹ was not found in any of these cells. Banded karyotypes prepared from 10 of these cells were normal 46,XX. Ph¹ was not found in any of 40 blood cell metaphases studied after 96 hr in culture.

Twenty-two and 19 marrow cell metaphases obtained on April 18 and May 30, respectively, were studied after 12 hr in culture. All of these cells had 46 chromosomes. Ph¹ was not found in any of them nor was it found in 22 banded karyotypes or in any of 116 metaphases (including 18 banded karyotypes) studied on the same dates after 24–72 hr in culture. No abnormalities were detected in any of the 40 marrow cell banded karyotypes.

At presentation, peripheral blood mononuclear cells formed 608 ± 40 (mean ± SEM) colonies/10⁵ cells plated. The colonies appeared normal in morphology and consisted of mature granulocytes and monocyte/macrophages. Eosinophil colonies were also present with a frequency of 23 percent ± 5 percent (mean ± SD). On restudy at the time of blastic transformation, 2534 ± 136 colonies/10⁵ cells formed. These colonies consisted entirely of immature monocytoid cells. No mature granulocytes or eosinophils were found in any of 40 separate colonies. Analysis of 123 colonies, including approximately 20 percent eosinophil colonies, from the initial specimen and 36 colonies from the blastic transformation specimen showed only G-6-PD type A.

A culture was established from peripheral blood mononuclear cells at the time of blastic transformation (Fig. 1). Cells in the culture underwent 11 passages over 2 mo with 3–5 day doubling times before becoming senescent. On Wright's stain, the cells in this culture were small, round, and granulated. Most of the cells adhered to plastic. They were peroxidase-positive and nonspecific esterase-positive, and had a 46,XX karyotype. They did not produce colony-stimulating factor. Only A-type G-6-PD was found. After 6 passages, cells from the culture were grown in agar, and 84 ± 10 colonies/10⁵ cells formed in the presence of PHA-conditioned medium (Fig. 2). The colony cells had monocyte morphology and stained positively for peroxidase and nonspecific esterase. No growth was

![Fig. 1. Cells from a short-term culture after five passages. Many cells were strongly adherent to the plastic flask and some were phagocytic (400x)](http://example.com/fig1.png)
detected in the absence of a source of colony-stimulating factor.

T-lymphocytes were also cultured from blood mononuclear cells. During the first 5 days when the cells were cultured in the absence of TCGF, cell number declined from $12 \times 10^6$ to $0.2 \times 10^6$. Thereafter, the cells were cultured in the presence of TCGF and increased in number. When the cells were harvested at day 30, 65 percent of them formed E-rosettes and less than 1 percent were peroxidase positive. In contrast to the single-enzyme type (A) found in granulocytes and erythrocytes, only 35 percent of the G-6-PD activity in these cultured lymphocytes was type A: 65 percent of the activity was type B.

**DISCUSSION**

The suggestion based on earlier studies of a few patients with chromosome markers$^{15,16}$ that Ph'-negative CML is a clonal disease is strongly supported by G-6-PD assays in our patient. As expected in this heterozygote, both B and A enzymes were found in skin and cultures of skin fibroblasts, but only a single G-6-PD type (A) was observed in the CML granulocytic cells. In over 50 subjects without leukemia who displayed both B and A-type G-6-PD in skin, both enzymes were also found in blood granulocytes.$^7$ The fact that single G-6-PD gene expression occurs in leukemic granulocytes from this patient strongly favors the postulate that the leukemia was clonal at the time of study. Similar G-6-PD studies have provided support for a clonal origin of Ph'-positive CML.$^{5,6}$ polycythemia vera,$^{17}$ agnogenic myeloid metaplasia,$^{18,19}$ and acute myelogenous leukemia.$^{20}$

Red cells and platelets from this patient also displayed only one enzyme. Thus, the leukemic clone arises in a stem cell common to the granulocyte, platelet, and erythrocyte. The finding that eosinophil colonies display only one G-6-PD type indicates that these cells also are descendants of the leukemic stem cell clone. Similar conclusions can be inferred from previously reported studies of colonies from patients with Ph'-positive CML$^8$ and have been confirmed directly (unpublished observations). In contrast, the finding of both B and A types of G-6-PD in cultured T-lymphocytes indicates that these cells did not arise from the CML clone. As in the case of Ph'-positive disease,$^{6}$ it is not known if these nonclonal T lymphocytes did not arise from the leukemic stem cell because, due to their long lifespans, they antedated the development of leukemia or because they arose from normal progenitors.

Clonal derivation of this Ph'-negative CML makes the possibility that the sole pathogenetic factor is continuous cell recruitment (i.e., the ability of a leukemic cell to continuously induce normal cells to become neoplastic, as might occur by a horizontal transfer of an infectious agent) unlikely. The fact that, at the time of diagnosis, the leukemic cells are all clonal indicates that the initiating oncogenic event is rare or that a series of steps occurs before a clone evolves into clinically evident leukemia, or both.

The initial in vitro colony growth in this patient was similar to that expected for Ph'-positive CML. Large numbers of normal-appearing colonies formed. Only clonal type-A G-6-PD was found in the colonies. Thus, as in the case of Ph'-positive CML,$^8$ no evidence for persistence of normal committed stem cells was found. However, unlike the Ph'-positive leukemia$^{21}$ an increase rather than a decrease in colony formation was noted when the disease entered the blastic phase. At that time, approximately 1 in 40 cells plated gave rise to a colony that morphologically consisted of immature monocytic cells. Furthermore, the establishment in culture of monocytoid cells that are responsive to colony-stimulating factor has not been previously reported from patients with Ph'-positive CML.

Typically, the classical course of Ph'-negative CML resembles a foreshortened course of the Ph'-positive disease. The G-6-PD results indicate that both diseases involve a multipotent hematopoietic stem cell and they appear to be clonal at the time of diagnosis. In these respects, Ph'-negative CML resembles the Ph'-positive form of the disease and the other myelo-proliferative disorders, such as agnogenic myeloid metaplasia and polycythemia vera.$^{22}$ However, despite the fact that all of these diseases involve a multipotent hematopoietic stem cell, they exhibit marked clinical and biologic differences. Thus, for example, with G-6-PD studies evidence for residual normal stem cells was found in polycythemia vera,$^{23}$ but not in Ph'-
positive⁴ or Ph'-negative chronic myelogenous leukemia. However, the Ph'-positive and Ph'-negative forms of leukemia differ in the rapidity with which they enter the blastic phase of the malignancy and at least for this patient, in the pattern of granulocyte/monocyte colony growth at that time. A likely possibility to explain these differences in the myeloproliferative disorders is that the regulatory abnormality that determines such factors as which differentiated element will be most prominent and the rapidity with which the disease evolves into a more malignant form is different in each of the diseases.

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REFERENCES

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