Evidence for a Multistep Pathogenesis of Chronic Myelogenous Leukemia

By Philip J. Fialkow, Paul J. Martin, Vesna Najfeld, Grace K. Penfold, Robert J. Jacobson, and John A. Hansen

To study the relationship of the Philadelphia chromosome (Ph') to the pathogenesis of chronic myelogenous leukemia, multiple B-lymphoid cell lines were established from a patient with Ph'-positive leukemia who was heterozygous for the X-chromosome-linked enzyme glucose-6-phosphate dehydrogenase. Both A and B types of enzyme were found in a 1:1 proportion in normal tissues, but 45 of 63 (71%) Ph'-negative B-lymphoid cell lines derived from this patient showed only the single glucose-6-phosphate dehydrogenase (type B) found in the Ph'-positive leukemic clone. Furthermore, 8 of 33 analyzable lines with B-type enzyme had chromosomal aberrations compared to 0 of 14 lines with A-type glucose-6-phosphate dehydrogenase. These results provide evidence for the suggestion that some cells of the abnormal clone do not express the Ph' abnormality. Thus, acquisition of Ph' may not be a sufficient cause for the disease. It is possible that at least two steps are involved in the pathogenesis of Ph'-positive chronic myelogenous leukemia, one causing abnormal proliferation of a clone of pluripotent hematopoietic stem cells and the other inducing Ph' in descendants of these progenitors.

MULTISTEP CAUSATIONS have been suggested in the development of neoplastic cells, but there is little reported direct experimental evidence to support these postulates in human malignancies. In this article we describe studies of the pathogenesis of chronic myelogenous leukemia in which the Philadelphia chromosome rearrangement (Ph') and the X-chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) were used as markers of cells that originated from the leukemic clone.

Since only one of the two X chromosomes is active in XX somatic cells, women who are heterozygous for the usual G6PD gene (GdA) and a variant gene such as GdA have two cell populations—one producing B-type G6PD, and the other A-type enzyme. Nonhematopoietic tissues of GdA/GdA heterozygotes with chronic myelogenous leukemia show both B and A enzymes, while marrow cells and blood granulocytes, erythrocytes, platelets, monocytes, and some lymphocytes manifest only one enzyme type. These data indicate that chronic myelogenous leukemia involves pluripotent hematopoietic stem cells and that the leukemic cell population extant at the time of study has arisen from a single cell (i.e., it is clonal).

Our previous studies with G6PD suggested that some blood lymphocytes that arose from the chronic myelogenous leukemia clone were Ph'-negative and prompted the hypothesis that at least two steps are involved in leukemogenesis, one causing growth of a clone of pluripotent hematopoietic stem cells and the other inducing Ph' in descendants of these progenitors. In the present studies this question was more directly investigated by evaluating Ph' and other chromosomal abnormalities in multiple B-lymphoid cell lines established in vitro by exposing blood cells of a patient with chronic myelogenous leukemia to Epstein-Barr virus (EBV). Peripheral blood B cells infected with that virus are transformed and thus become permanently established as cell lines capable of unlimited growth in vitro. Analysis of these cell lines for G6PD, cytoplasmic immunoglobulin (cIg) and for cytogenetic abnormalities provides evidence for the existence of clonally derived, genetically unstable cells that lack Ph'. These data suggest that Ph' is not a sufficient cause for the leukemia.

MATERIALS AND METHODS

Subject
The patient whose cells form the basis of this report has been described previously (subject 6 in ref. 2 and M.K. in ref. 3). The diagnosis of chronic myelogenous leukemia was first made in 1975 when she was 60-yr-old and had a white cell count of 253,000/cu mm. Ph' was found in 28 of 30 marrow cells. Blood for the present study was collected in heparin on 8 different dates between April 1979 and January 1980. The patient remained in chronic phase throughout this period with less than 2% circulating blast cells. The absolute blood lymphocyte count ranged between 700 and 8100/cu mm. When last studied in January 1980, the patient's white cell

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count was 31,000 with 1% blast cells, 18% other immature myelogenous cells, 64% polymorphonuclears, and 13% lymphocytes.

Cell Lines
Leukocytes were isolated from the patient's blood by sedimentation in dextran. The leukocyte fraction was incubated in RPMI 1640 supplemented with 12% fetal calf serum for 4 days at 37°C and 5% CO₂ in 95% humidified air. The cells were then centrifuged over Ficoll-Hypaque (specific gravity 1.077). This procedure significantly reduced the number of myeloid cells and enhanced the yield of cell lines.

The interface cells were washed and resuspended (2-4 x 10⁶ cells/ml medium), diluted with an equal volume of cell-free culture supernate from the EBV-producer cell lines MCUV (kindly provided by Dr. George Miller, Yale University), and dispensed into wells of 96-well microculture trays (2-4 x 10⁴ cells in 0.2 ml/well). Cultures were fed twice weekly by aspirating half of the medium from each well and replacing it with an equal volume of fresh medium. When sustained growth was observed, generally within 10-21 days, transformed cells from each individual well were passed separately to successively larger tissue culture containers. Once established, the cell lines were cryopreserved in RPMI medium with 12% fetal calf serum and 7% dimethyl sulfoxide.

Cell Characterization
All tests on the cell lines were done between 15 and 45 days after EBV transformation had occurred. Cytoplasmic Ig was determined with rhodamine-conjugated F(ab')₂ fragments specific for μ, γ, α, κ, and λ.ILA antigens were stained with rhodamine-conjugated F(ab')₂ fragments specific for the human p29.34 la-like membrane biomolecular complex. These reagents were generously provided by Dr. Shu Man Fu (Rockefeller University). EBV nuclear antigen (EBNA) was detected as described. For immunofluorescence studies, cells were examined with a Leitz Ortholux Two microscope with a 200 W mercury lamp and a Ploemopak two filter system. A minimum of 200 cells was counted.

G6PD Analyses
Cell lysates made by freezing and thawing were subjected to starch gel electrophoresis, and the relative activity of the enzyme bands was estimated visually. The sensitivity of this technique is such that a minor enzyme activity component of 5% can be detected.

Cytogenetic Analysis
Blood cells from the sample obtained on September 18, 1979 were studied after 24 or 72 hr in culture with and without phytohemagglutinin. Mitoses in the cell lines were examined between 15 and 45 days after they emerged from the primary cultures. The preparations were exposed to 1 μg/ml of colchicine for 3 hr before fixation and staining. Ph' was scored in randomly examined, conventionally stained well spread metaphases in which all G-group chromosomes could be identified. With three exceptions, 30 or more conventionally stained metaphases were examined from each preparation. In addition, depending on the availability of metaphase spreads, between 5 and 30 karyotypes were examined with a modified Giemsa banding technique. Scoring for Ph' was conducted without prior knowledge of the G6PD phenotype.

RESULTS
Establishment of Cell Lines
More than 850 microculture wells were plated with cells from the 8 specimens of peripheral blood, and cell lines with the typical growth characteristics and morphology of B-lymphoblastoid lines grew in all 8 experiments. Sufficient numbers of cells for analysis of chromosomes and G6PD were obtained from 74 cell lines.

Characterization of Cell Lines
Cytoplasmic Ig was found in 25%-95% of cells in each line. With 5 exceptions, each of the 42 tested lines expressed clg of a single light and heavy chain class. Two of these exceptions were lines in which cells expressing μ and cells expressing γ were found. In 3 other lines, cells expressing κ and cells expressing λ were observed. Each of these 5 lines expressed a single G6PD type. There was considerable diversity in the classes of Ig from line to line. Twenty-three percent of the B-lymphoid cell lines expressed μ, 43% γ, 30% α, 52% κ, and 55% λ Ig chains. Each of 30 lines tested for la antigen and 8 lines tested for EBNA was positive.

G6PD and Ph' in Blood Cells
As on previous occasions, myeloid cells, erythrocytes, and platelets from the patient consistently displayed only one G6PD (type B), although skin and cultured skin fibroblasts showed both B and A types of enzyme in a 1:1 ratio.

Ph' was found in each of 50 banded karyotypes of white blood cells cultured for 24 hr without mitogen. In addition to the two chromosomes usually involved in the Ph' translocation (numbers 22 and 9), the rearrangement in all of these cells also involved chromosome 7, giving the karyotype 46,XX,t(7;9)(q11 or 21;q34;q11). These abnormalities had been present in white blood cells since at least 1976, shortly after the patient's initial presentation.

Ph' and t(7;9)(q22;q22) were found in 7 of 27 karyotypes prepared from cells cultured for 72 hr with phytohemagglutinin [one cell also had t(11;12) (p15;q14)]. The other 20 cells were normal 46,XX.

G6PD and Ph' in Lymphoid Cell Lines
A cell line was considered to be Ph'-positive if more than 95% of the metaphases had Ph'. Negative lines had less than 5% Ph'-positive mitoses. Of the 74 lines evaluated, 9 were Ph'-positive and are described elsewhere. The remaining 65 lines were Ph'-negative. Eighteen of these 65 Ph'-negative cell lines manifested only type-A G6PD, and 45 displayed only B enzyme (Table 1). Two of the cell lines showed both A- and B-type enzymes. Since these two lines were not homogeneous, they were excluded from further study. The difference between the observed ratio of type-A to type-B lines (18:45) and the expected ratio (1:1) was statistically significant (p < 0.001, χ²).
### Table 1. G6PD Phenotypes in Ph́-Negative Lymphoblastoid Cell Lines*

<table>
<thead>
<tr>
<th>Date Blood Sample Obtained</th>
<th>G6PD Phenotypes (No.)</th>
<th>Ph́-Negative Cell Lines</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>4/3/79</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5/29/79</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>6/26/79</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>8/21/79</td>
<td>5</td>
<td>0</td>
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<td>7</td>
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<td>10</td>
<td>5</td>
</tr>
<tr>
<td>1/15/80</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>65</td>
<td>18</td>
</tr>
</tbody>
</table>

*The ratio of A:B-type G6PD in the normal tissues from this patient was 1:1. Only B-type G6PD was found in the CML clone.

### Table 2. Chromosomal Abnormalities and Cytoplasmic Immunoglobulin Light Chain Classes in 8 Ph́-Negative Lymphoid Cell Lines*

<table>
<thead>
<tr>
<th>Date</th>
<th>Line</th>
<th>No. of Banded Metaphases Studied</th>
<th>Abnormal Cells</th>
<th>Karyotype</th>
<th>Percent of Cells</th>
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<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>6/26/79</td>
<td>MK 243.2.4</td>
<td>20</td>
<td>14</td>
<td>47, XX, +19</td>
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<tr>
<td>8/21/79</td>
<td>MK 273.2.6</td>
<td>16</td>
<td>3</td>
<td>46, XX, 13q+</td>
<td></td>
</tr>
<tr>
<td>9/18/79</td>
<td>MK II.3.2</td>
<td>14</td>
<td>1</td>
<td>46, XX, -20, +mar 1†</td>
<td>0 61</td>
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<td></td>
<td>1</td>
<td>46, XX, -19, +mar 2†</td>
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<tr>
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<td></td>
<td></td>
<td>1</td>
<td>48, XX, -10, -10, +12q-, +mar 1†, +mar 2†, +mar 3, +mar 4</td>
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<td>1</td>
<td>46, XX, -5, +mar 3§</td>
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<td></td>
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<td>1</td>
<td>46, XX, -4qicb, +mar 3§</td>
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<td></td>
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<td>1</td>
<td>47, XX, +2, +13, -18, -19, +mar 1†</td>
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<td></td>
<td></td>
<td>1</td>
<td>45, XX, 4p- (qicb), -11</td>
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<td>1</td>
<td>46, XX, -18, +mar 1†</td>
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<td>44, X, -X, -D, -17, +mar 1†</td>
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<td></td>
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<td></td>
<td></td>
<td>1</td>
<td>43, X, -X, 4qicb, 6q-, -11, -14</td>
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<td></td>
<td>MK 12.26</td>
<td>17</td>
<td>1</td>
<td>46, XX, 6q+</td>
<td>86 0</td>
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<td></td>
<td>MK 31.2.7</td>
<td>13</td>
<td>5</td>
<td>Hyperploid, +mar 1</td>
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</tr>
</tbody>
</table>

*Each of these cell lines manifested only B-type glucose-6-phosphate dehydrogenase. The chromosome classification and nomenclature was done according to the International System for Human Cytogenetic Nomenclature.21
†Mar 1 is a G-group-like chromosome and was similar in II.3.2 and II.3.19 and similar to mar 1 in 12.26.
‡Mar 2 is a C-group-like chromosome and was similar in II.3.2 and II.3.19.
§Mar 3 in both cells of 12.26 was similar, but differed from mar 3 in other cell lines.

#### DISCUSSION

In a previous study of blood cells from patients with chronic myelogenous leukemia, mitogen-stimulated lymphocytes grown in vitro for 92-120 hr showed only the G6PD type present in the abnormal hematopoietic cell clone, indicating that those cells were derived from this clone. Nonetheless, Ph́ was not generally found in the mitoses of these preparations. The findings thus suggested that not all cells arising from the abnormal clone are necessarily Ph́-positive. An alternative explanation, however, was the possibility that the mitogen-stimulated mitoses that lacked Ph́ originated from a small population of normal cells. If this population contributed less than 5% of the preparation's total cell mass, it would be difficult to detect by conventional cytogenetic methods. The findings of this study suggest that the use of mitogen-stimulated lymphocytes may be more sensitive than the use of mitoses to detect the presence of Ph́ cells in the abnormal clone.
Fig. 1. Karyotype of a cell showing 47,XX,+12 from line MK II.3.9 (1700 x).

G6PD activity, it might be detected. In order to examine this question further, we established from a single patient a large number of B-lymphoid cell lines that were homogeneous with respect to G6PD and cytoplasmic Ig. We then studied the chromosomes and G6PD of the cell lines to determine whether there was an abnormal clone of Ph'-negative cells in this patient.

EBV is a polyclonal activator of B lymphocytes. Previous studies of non-neoplastic tonsil-derived B-lymphoid cell lines using G6PD and Ig markers have shown that EBV-transformed cell lines are initially polyclonal, but that with passing time in culture, they become homogeneous, presumably by random selection and overgrowth of a single clone. The length of time required for this process probably depends on the number of cells used to initiate the culture. Only 2 of the 65 Ph'-negative B-lymphoid cell lines displayed an A/B G6PD phenotype. All other lines were either A or B. Furthermore, with few exceptions, clg of a single light and heavy chain class was identified in the cells of each line. These findings suggest that at the time of observation, most of the cell lines were homogeneous for the markers studied. The relatively rapid evolution

Fig. 2. Partial karyotypes of two cells showing 10q+ from line MK 12.18. The origin of the extra chromosome segment could not be determined (1700 x).
of these cell lines to a homogeneous state probably resulted from the small number of B cells seeded in each microwell, which is also reflected in the low transformation efficiency (<10% of wells produced transformed cell lines). With conventional methods, it is not possible to determine simultaneously the Ig class, G6PD type, and chromosomal composition of a single B cell. However, with these homogeneous B-cell lines, we were able to examine the progeny of one or a few cells for all three markers, and thus infer the phenotypic expression of the progenitor(s) for each line.

The G6PD phenotype of 18 of the 65 Ph' negative lymphoid cell lines was A. Since the leukemic clone in this patient showed B-type enzyme, it is concluded that these 18 lymphoid cell lines originated from normal progenitors. Either these progenitors were long-lived and antedated the development of chronic myelogenous leukemia or they arose during the course of the disease from pluripotent stem cells not involved by the leukemia. Similar possibilities were advanced to explain the occurrence of populations of T lymphocytes that did not arise from the leukemic-stem-cell clone in this and in other patients with chronic myelogenous leukemia.

In nonleukemic subjects, the proportions of A:B-type G6PD activities in mesenchymal tissues, including muscle, skin, and blood lymphocytes, granulocytes and erythrocytes, are highly correlated. Since the ratio of A:B enzyme in skin from this patient is 1:1, it is assumed that the frequency of normal B-cell progenitors that express A-type G6PD should be equal to the frequency of progenitors that express B enzyme. However, 71% of the 63 lines that showed a single enzyme type manifested only B-type G6PD, a figure that is significantly different from the expected 50% (p < 0.001, x2); the excess of lines that expressed B-type enzyme strongly suggests that some of these lines were derived from a cell population that was abnormal, yet was also Ph' negative.

It could be argued that the discrepancy between the observed and expected frequencies of lymphoid cell lines showing only B-type G6PD resulted from a selective advantage in culture for progeny of abnormal precursors. On the contrary, it is likely that B-lymphoid cells that arose from normal progenitors had a growth advantage in vitro, causing an overrepresentation of lymphoid cell lines of G6PD type A. This suggestion follows from several observations. First, approximately 30% of the B-lymphoid cell lines manifested A-type G6PD, even though type-A enzyme was not detected in short-term cultured B lymphocytes from this patient. Second, as reported elsewhere, it has thus far not been possible to maintain in vitro the Ph' positive B-lymphoid cell lines from this patient for long periods of time, whereas Ph' negative cell lines have been maintained in culture under identical conditions without difficulty. These observations indicate that B-lymphoid cells arising from progenitors of a chronic myelogenous leukemia clone may be less capable of adaptation to culture conditions than progenitors arising from normal stem cells. Recently, we have demonstrated that normal cells from patients with chronic lymphocytic leukemia may have an in vitro growth advantage over leukemic B-lymphoid cells.

The conclusion that some Ph'-negative B-lymphoid lines were derived from abnormal progenitors is supported by the results of detailed chromosome studies of the lines. B-lymphoid cell lines established from the blood of normal donors generally are chromosomally normal during the first year of growth in vitro. In contrast, chromosomal abnormalities were detected in 8 of the Ph'-negative B-lymphoid lines from our patient after only 15–45 days of growth in culture. Moreover, the aberrations were not found at random, but were detected only in cells from lines that expressed the same G6PD type as the chronic myelogenous leukemia stem cells.

In two lymphoid cell lines with chromosomal abnormalities, all of the metaphases had the same aberration (II.3.9 and 12.18) (Table 2), suggesting that each line was clonal, a conclusion also supported by the clg studies. The other five tested lines also had nonspecific Ig types, but were composed of mixtures of normal and chromosomally abnormal cells. Either these lines arose from more than one cell or each of them arose from a single cell and the chromosomal abnormalities occurred in vitro. If these aberrations did occur in vitro, the similarity of the marker chromosomes found in three lines (II.3.2, II.3.19 and 12.26) would suggest that the chromosomal abnormalities arose nonrandomly as specific changes characteristic of an abnormal cell population in this patient. Another possibility is that these chromosomal aberrations could confer a selective advantage in culture.

The cause of the chromosomal abnormalities is not clear and could conceivably be related to patient therapy or culture conditions. Furthermore, it is possible that EBV infection could have been the factor that elicited the chromosomal alterations in the genetically unstable cells. In any event, regardless of the immediate cause of the chromosomal abnormalities, it is clear that some B-lymphoid cells were unusually susceptible to the development of chromosomal changes. That such changes were found only in cell lines with B-type G6PD indicates that the chromosomal abnormalities occurred specifically in progeny of the abnormal stem cells. Chromosomal instability is frequently observed in cells derived from a malignant or premalignant clone.

Both the G6PD and the chromosomal findings in the cell lines indicate that there exists in this patient...
an abnormal population of B-lymphoid cells that is Ph'-negative and has the same G6PD phenotype as the leukemic clone. One explanation for this observation is that this patient has, in addition to chronic myelogenous leukemia, another clonal hematologic disorder, a postulate for which there is no supporting clinical or laboratory evidence. Another possibility is that the process that caused the Ph'-positive leukemia could have also affected nonleukemic progenitors for B-lymphoid cell lines. Were this the case, chromosomal abnormalities should have appeared in B-lymphoid lines of G6PD type A as well as of type B. However, chromosomal abnormalities were found only in cell lines that manifested B-type G6PD, the same enzyme type that was observed in the leukemic clone. A more likely explanation is that the genetically unstable B-lymphoid cells that are Ph'-negative arose from the same clone of stem cells that gave rise to the Ph'-positive leukemia.

Several possibilities could explain the existence in chronic myelogenous leukemia of clonally derived cells that are Ph'-negative. For example, formation of Ph' may be but one of several steps in leukemogenesis, or the abnormality could occur secondarily in cells of an already leukemic clone as the disease progresses. Compatible with both of these possibilities are the reports of occasional patients with typical chronic myelogenous leukemia, whose marrow cells were Ph'-negative at presentation, but later become Ph'-positive. Several facts, however, support the postulate that Ph' is involved in the pathogenesis of chronic myelogenous leukemia: Ph' is generally detected in 90%-100% of dividing marrow cells in patients with the leukemia; it is very specific and characteristic of the disease; and it has been found in some cases years before overt leukemia has developed. Thus, the likely explanation is that at least two steps are involved in the evolution of this leukemia—one causing proliferation of pluripotent hematopoietic stem cells and the other inducing Ph' in one or more descendants of this progenitor clone. The data presented here were obtained from detailed study of a single patient with chronic myelogenous leukemia. The demonstration of cells that lacked Ph', yet were apparently derived from the abnormal hematopoietic stem-cell clone, indicates that formation of Ph' is not the sole event in the pathogenesis of this patient's leukemia.

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REFERENCES

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