Peripheral blood specimens were obtained from 22 patients with Philadelphia chromosome (Ph') positive chronic myelogenous leukemia (CML) (16 in chronic phase, 2 in an accelerated phase, and 4 in blast crisis). Studies were performed to determine the frequency of the presence of the Ph' chromosome in cells of lymphoid lineages. Rosette-positive lymphocytes (T lymphocytes) from nine patients in chronic phase and one patient in blast crisis were stimulated with T cell growth factor interleukin 2 (IL-2) and/or phytohemagglutinin (PHA). All ten patients had sufficient T lymphocyte metaphases for analysis and of a total of 461 metaphases examined, only one contained the Ph' chromosome. Nucleated cells of density less than 1.077g/mL were infected with Epstein-Barr virus (EBV). Following infection, cell lines were established from individual colonies attached to egg albumin-coated Lab-Tek slide chambers (clonal cell lines) or from suspension culture in 96-well tissue culture cluster dishes (nonclonal cell lines). Cell surface and intracellular marker analysis confirmed the B lymphocyte phenotype of all the cell lines examined. B lymphoblastoid cell lines were established from 16 of the 22 patients. All lines from 12 patients were Ph' negative. From two chronic phase patients, both Ph' positive and Ph'-negative lines were established. From one patient in an accelerated phase, only Ph'-positive lines were established. From another patient in blast crisis (of myeloblastic phenotype), only Ph'-negative lines were established initially; however, five months later, after the patient had been treated with mitoxantrone, only Ph'-negative lines were derived from this patient. Based on these results, it appears that most B cells and mature T cells in most CML patients are Ph'-negative, but that about 25% of patients have predominately Ph'-positive B cells or a mixture of Ph'-positive and Ph'-negative B cells that are capable of growing as established cell lines after transformation with EBV.

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MATERIALS AND METHODS

Patient characteristics and blood specimens. After appropriate human protection committee validation and informed consent, 25% of patients have predominately Ph'-positive B cells or a mixture of Ph'-positive and Ph'-negative B cells that are capable of growing as established cell lines after transformation with EBV.

From the Laboratories of Hematopoietic Cell Kinetics and Cancer Genetics and Cytogenetics, Memorial Sloan-Kettering Cancer Center, NY.

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Address reprint requests to Dr Bayard Clarkson, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021.
Peripheral blood specimens were obtained from 22 patients with Ph'-positive CML. The clinical and hematologic findings are summarized in Table 1. All patients had 93% to 100% Ph'-positive metaphases in their marrow on direct cytogenetic analysis, with the exception of patient 18 in blast crisis who had 45% Ph'-positive metaphases in her marrow. All but three patients (patients 9, 17, and 22) had received treatment for varying periods; treatment was usually stopped one week or more prior to performing cytogenetic studies. Sixteen of the patients were in chronic phase with <5% blasts in the marrow and blood at the time of study, from 3 weeks to 17 years after diagnosis. Two patients (nos. 21 and 26) were in an accelerated phase, with 37% and 14% blasts plus promyelocytes, respectively, in their marrow. Four patients (patients 7, 15, 18, and 23) were in blast crisis with 51%, 65%, 85%, and 70% blasts plus promyelocytes, respectively, in their marrow. Patient 7 developed low-grade fever and increased percentages of blasts (34%) and promyelocytes (17%) in the marrow, consistent with early myeloblastic transformation two years after diagnosis. After the initial blood specimen was taken on October 25, 1982, he was treated with mitoxantrone which lowered the WBC to <1,000 per cumm and resulted in marked marrow hypoplasia with reduced blasts and promyelocytes. The repeat studies were performed five months later after the marrow had regained normal cellularity and contained 3.5% blasts and 13% promyelocytes. The phenotypes of the other three blast crisis patients were: no. 15, biphenotypic, TdT+; no. 18, lymphoblastic, TdT+; and no. 23, null TdT+.

Specimens were collected in sterile heparinized syringes. Following centrifugation of the blood over Ficoll-Hypaque (sp gr 1.077 g/cm³) at 2,000 rpm for 30 minutes at 4 °C, the interphase cells were collected and washed twice with RPMI 1640 medium supplemented with 16% fetal calf serum (FCS) (Sterile Systems, Inc., Logan, Utah).

**EBV transformation of B lymphocytes.** Cell-free culture supernatant was prepared from an Epstein-Barr virus (EBV) infected marmoset line B95-8 (Institute for Medical Research, Camden, NJ). One milliliter of this supernatant and 0.1 mL of phytohemagglutinin (PHA) (Burroughs Wellcome, Research Triangle Park, NC) were added to 9 mL of mononuclear cells (~10 x 10⁶/mL) in RPMI 1640 supplemented with 30% heat-inactivated (H-I) FCS. The cells were incubated for varying periods of time with EBV at

<table>
<thead>
<tr>
<th>Pt. No.</th>
<th>Date of Specimen</th>
<th>Sex</th>
<th>Age</th>
<th>Stage</th>
<th>Rx</th>
<th>Time After Dx</th>
<th>WBCt x 10⁹/Mmol/L</th>
<th>Ph'-Positive Metaphases Established</th>
<th>B-Lymphoblastoid Cell Lines Established Following EBV Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>09/20/82</td>
<td>M</td>
<td>39</td>
<td>Chronic</td>
<td>HU</td>
<td>4 yr</td>
<td>85</td>
<td>30/30</td>
<td>58</td>
</tr>
<tr>
<td>2</td>
<td>09/30/82</td>
<td>M</td>
<td>34</td>
<td>Chronic</td>
<td>HU</td>
<td>1 yr</td>
<td>6</td>
<td>30/30</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>10/04/82</td>
<td>M</td>
<td>53</td>
<td>Chronic</td>
<td>Busulfan</td>
<td>17 yr</td>
<td>49</td>
<td>30/30</td>
<td>30/30</td>
</tr>
<tr>
<td>4</td>
<td>10/18/82</td>
<td>F</td>
<td>16</td>
<td>Chronic</td>
<td>HU</td>
<td>2 mo</td>
<td>18</td>
<td>25/25</td>
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</tr>
<tr>
<td>5</td>
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<td>61</td>
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<td>HU</td>
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<td>176</td>
<td>30/30</td>
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</tr>
<tr>
<td>6</td>
<td>10/25/82</td>
<td>M</td>
<td>29</td>
<td>BC</td>
<td></td>
<td></td>
<td>HU</td>
<td>2 yr</td>
<td>42</td>
</tr>
<tr>
<td>7</td>
<td>03/17/83</td>
<td>M</td>
<td>63</td>
<td>Chronic</td>
<td>HU</td>
<td>54 yrs</td>
<td>ND</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>8</td>
<td>11/29/82</td>
<td>M</td>
<td>63</td>
<td>Chronic</td>
<td>HU</td>
<td>2 yr</td>
<td>49</td>
<td>30/30</td>
<td>40 lines all mixed Ph'-Positive and Ph'-Negative B-Lymphoblastoid Cell Lines Established Following EBV Infection</td>
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<tr>
<td>9</td>
<td>01/03/83</td>
<td>M</td>
<td>35</td>
<td>Chronic</td>
<td>None</td>
<td>3 wk</td>
<td>125</td>
<td>30/30</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>01/20/83</td>
<td>M</td>
<td>54</td>
<td>Chronic</td>
<td>HU</td>
<td>9 yr</td>
<td>ND</td>
<td>32/32</td>
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</tr>
<tr>
<td>11</td>
<td>01/20/83</td>
<td>M</td>
<td>38</td>
<td>Chronic</td>
<td>HU</td>
<td>2 yr</td>
<td>35</td>
<td>30/30</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>01/20/83</td>
<td>M</td>
<td>38</td>
<td>Chronic</td>
<td>HU</td>
<td>1 mo</td>
<td>11</td>
<td>20/20</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
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<td>M</td>
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<td>Chronic</td>
<td>HU</td>
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<td>74</td>
<td>30/30§</td>
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<tr>
<td>14</td>
<td>05/14/84</td>
<td>F</td>
<td>48</td>
<td>Chronic</td>
<td>HU</td>
<td>4 yr</td>
<td>37</td>
<td>30/30</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>05/01/84</td>
<td>M</td>
<td>50</td>
<td>BC</td>
<td></td>
<td>VCR, Pred</td>
<td>1 wk</td>
<td>14</td>
<td>20/20§</td>
</tr>
<tr>
<td>16</td>
<td>05/24/84</td>
<td>M</td>
<td>30</td>
<td>Chronic</td>
<td>HU</td>
<td>4 yr</td>
<td>37</td>
<td>30/30</td>
<td>3</td>
</tr>
<tr>
<td>17</td>
<td>03/15/85</td>
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<td>55</td>
<td>Chronic</td>
<td>None</td>
<td>Initial</td>
<td>420</td>
<td>30/30§</td>
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<tr>
<td>18</td>
<td>06/07/84</td>
<td>F</td>
<td>31</td>
<td>BC</td>
<td></td>
<td>Adria, VCR, Pred</td>
<td>5 wk</td>
<td>4</td>
<td>48§§§</td>
</tr>
<tr>
<td>19</td>
<td>07/05/84</td>
<td>F</td>
<td>57</td>
<td>AP</td>
<td>HU</td>
<td>10 yr</td>
<td>18</td>
<td>25/26§</td>
<td>72</td>
</tr>
<tr>
<td>20</td>
<td>07/12/84</td>
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<td>41</td>
<td>Chronic</td>
<td>None</td>
<td>1 mo</td>
<td>24</td>
<td>28/30</td>
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</tr>
<tr>
<td>21</td>
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<td>M</td>
<td>52</td>
<td>BC</td>
<td></td>
<td>Relapse 10 mo post BMT</td>
<td>15 mo</td>
<td>182</td>
<td>20/20§</td>
</tr>
<tr>
<td>22</td>
<td>05/03/84</td>
<td>M</td>
<td>67</td>
<td>Chronic</td>
<td>HU</td>
<td>3 yr</td>
<td>8</td>
<td>20/20§</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>06/18/84</td>
<td>M</td>
<td>56</td>
<td>Chronic</td>
<td>HU</td>
<td>5 wk</td>
<td>24</td>
<td>29/30§</td>
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</tr>
<tr>
<td>24</td>
<td>07/30/84</td>
<td>F</td>
<td>58</td>
<td>AP</td>
<td>HU</td>
<td>8 yr</td>
<td>42</td>
<td>30/30§</td>
<td>0</td>
</tr>
</tbody>
</table>

BMT, bone marrow transplant; HU, hydroxyurea; VCR, vincristine; Adria, Adriamycin; Pred, prednisone; AP, accelerated phase; BC, blast crisis; ND, not done.

*Patients 2, 12, 19, and 20 were excluded from the study because they were Ph'-negative.
†On day of peripheral blood specimen.
‡Constitutional XXY.
§Plus additional chromosomal abnormalities.
||Myeloblastic.
†Biphenotypic TdT+.
‡Lymphoblastic TdT+.
††Null TdT+.
37 °C, from one hour to ten days prior to washing and suspension in fresh medium for further culture. There was no apparent correlation between the duration of EBV exposure and the incidence of establishment of B cell lines.

Establishment of B lymphoblastoid cell lines. To establish cell lines from individual colonies (clonal cell lines), following removal of EBV supernatant, 1 x 10^5 cells in 2 mL of medium were cultured in each chamber of sterile Lab-Tek culture chamber slides with two chambers per slide (Lab-Tek Products, Naperville, Ill) and also in 35-mm Lux Petri dishes (Flow Laboratories, McLean, Va). Culture vessels were previously coated with egg albumin to allow the cells to attach to the surface. 45 Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air (National Incubator, National Appliance Co, Portland, Ore) and every three to four days, 1 mL of medium was removed and 1 mL of new medium was added. If colonies failed to arise from cells cloned immediately following EBV infection, cloning was repeated every week (ie, cells in suspension culture were cloned again each week) generally for three weeks, but in some instances for as long as six weeks. Individual colonies attached to the egg albumin-coated culture vessels were aspirated into a finely drawn Pasteur pipette by capillary action and expressed into wells of 24-well-multiwell tissue culture plates (Falcon, Oxnard, Calif) which contained 2 mL of RPMI 1640 medium supplemented with 16% FCS. The colonies generally stayed attached to the egg albumin-coated plates for two weeks, after which time they began to detach. For this reason, colonies were aspirated at seven to ten days following cloning.

To establish nonclonal cell lines, 5 x 10^5 EBV-infected cells were dispensed into each well of a 96-well tissue culture cluster dish (Costar, Cambridge, Mass). Each well containing transformed cells was treated as a separate cell line and expanded individually. 42

T lymphocyte cell cultures. Rosette (E') lymphocytes were prepared from blood mononuclear cells using neuraminidase (Calbiochem-Behring, San Diego, Calif) treated sheep erythrocytes (SRBC) (Flow) and separated from nonrosetted cells by centrifugation over Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) at 4 °C for 15 minutes at 2,000 rpm. The rosetted lymphocytes were washed twice in medium, incubated at 37 °C for ten minutes in Tris-NH4Cl buffer to lyse the SRBC and then washed twice in medium. The resulting T lymphocyte population was incubated with 1% PHA at a concentration of 2 to 4 x 10^6 cells/10 mL of RPMI 1640 supplemented with 16% FCS. At 24 hours, an aliquot of T lymphocytes was washed free of PHA and suspended in medium containing T cell growth factor (IL-2) (kindly supplied by Dr Roland Mertelsmann) at a concentration of 1,500 U/100 mL of medium. The cells were then cultured as described above for B lymphocyte cultures. The remaining T lymphocytes were incubated with PHA for an additional two days.

Cytogenetic analysis. Chromosome preparations for cytogenetic analysis were made from both cell suspensions and from cells attached to the egg albumin-coated Lab-Tek culture chamber slides.

In brief, cells suspended in RPMI 1640 supplemented with 16% FCS were incubated for 30 minutes with Colcemid (GIBCO Laboratories, Grand Island, NY) at a concentration of 0.1 µg/mL culture medium. Chromosome preparations were then made following conventional methods, using prewarmed 0.075 mol/L of potassium chloride (KCl) as the hypotonic solution for 15 minutes and 3:1 methanol-glacial acetic acid as the fixative. Preparations were dried for 14 days in a 37 °C oven before staining with a modification of the trypsin-Giemsa banding method (G-banding) of Seabright. 47

Cells attached to egg albumin-coated chambers were incubated with Colcemid, 0.1 µg/mL culture medium, for one hour. Chromosome preparations were made using 0.051 mol/L of KCl for 30 minutes followed by gradual fixation with 3:1 methanol-glacial acetic acid. Preparations were stained with quinacrine dihydrochloride (Sigma Chemical Co, St Louis) (Q-banding method) and were examined by Zeiss fluorescence microscope.

Source of monoclonal antibodies and antisera. For flow cytometric cell surface marker analysis, anti-B1, anti-B2, anti-J2, anti-J3, anti-Mo1, anti-T11, anti-IgG, anti-IgM, anti-IgD, anti-α, and anti-λ monoclonal antibodies (Mab) were obtained from Coulter Immunology, Hialeah, Fla. Anti-BA-2 Mab was obtained from Hybritech, Inc. San Diego, Calif. Anti-IgA1 and anti-Leu-7 were purchased from Becton Dickinson Monoclonal Center, Inc, Sunnyvale, Calif. Fluorescein-conjugated F(ab')2 fragments of goat anti-serum to human total immunoglobulins (polyvalent) was purchased from Kallestad, Austin, Tex. To detect the presence of cytoplasmic IgG, IgA, and IgM, the DAKO PAP kit was obtained from Accurate Chemical and Scientific Corp, Westbury, NY.

Anti-B1 Mab identifies a human B lymphocyte-specific antigen (B1) which is present on >95% of B lymphocytes from blood and lymphoid organs. 49 Anti-B2 Mab identifies a human B lymphocyte-specific antigen which is present on a subset of B lymphocytes (midstage in B cell differentiation) isolated from blood and lymphoid organs. 30,31 Anti-J2 Mab detects a nonpolyclonal region of the human Ia-like antigen. 32 Anti-J5 Mab identifies the human common acute lymphoblastic leukemia antigen (CALLA) found on tumor cells from 80% of patients with non-T cell acute lymphoblastic leukemia (ALL), 30% to 50% of patients with CML in blast crisis and some B and T cell lymphomas. 33,34 In addition, recent studies have identified a small population of normal immature lymphoid cells in nonleukemic marrow (particularly in pediatric patients and regenerating marrows) and myeloid cells that are reactive with J5. Mab Mab recognizes an antigen present on monocytes, granulocytes, and null cells. 35 Anti-T11 Mab identifies the E rosette receptor associated antigen present on all peripheral T lymphocytes. 36 Anti-IgG, anti-IgM, and anti-IgD identify, respectively, an antigen on the γ, µ, and δ heavy chains of human immunoglobulin. Anti-IgA1 identifies a subclass of human IgA. Anti-α and anti-λ identify, respectively, an antigen on the κ and λ light chains of human immunoglobulins. Anti-BA-2 Mab identifies a human cell surface structure, p24, present on lymphoid leukemia cells and lymphohemopoietic bone marrow progenitor cells. Anti-Leu-7 (clone HNK-1) identifies most human natural killer (NK) and killer (K) cells. 37

Cell surface and cytoplasmic marker characterization. Mab reactivity was determined by indirect immunofluorescence. 10° cells were suspended in 0.2 mL of McCoy's 5A medium (modified) supplemented with Hepes buffer, 5% FCS, and an appropriate concentration of Mab. Control cells were incubated with purified mouse IgG (antiIgG) or purified mouse IgM (antiIgM) (Coulter) to monitor the degree of nonspecific staining. After incubation for 30 minutes on ice, the cells were washed twice and incubated with purified, fluorescein-conjugated goat antibody against mouse IgG1, IgG2a, IgG2b, IgG3, and IgM (GAM-FITC) (Coulter) for 30 minutes on ice. Following staining, the cells were washed twice and the percentages of fluorescent antibody-coated cells were determined using a Becton Dickinson FACS-IV.

To determine the percentage of cells positive for surface immunoglobulin, the cells were stained with FITC-F(ab')2 fragments of goat anti-serum to human total immunoglobulins for 30 minutes on ice. Control cells were incubated with purified, FITC-labeled F(ab')2 goat antibody against mouse F(ab')2 immunoglobulin (FGAM-FITC) (Coulter). Following staining, the cells were washed twice and the percentage of fluorescent antibody coated cells was determined with a FACS-IV.

To detect the presence of cytoplasmic IgG, IgA, and IgM, a
modification of Sternberger's peroxidase-antiperoxidase (PAP) technique (DAKO PAP kit system) was used. Cytocentrifuge preparations of the cells were fixed in absolute acetone and air-dried. After placing the slides in phosphate-buffered saline (PBS) (pH 7.4) for ten minutes, the slides were transferred to a 3% hydrogen peroxide solution for ten minutes, washed in PBS, and incubated with normal swine serum for 20 minutes. Following exposure to the normal swine serum, slides were treated with the primary antibody for 30 minutes, washed in PBS, exposed to link antibody for 30 minutes, and washed again before staining with the PAP reagent and substrate solution of hydrogen peroxide and aminoethylcarbazole. Mayer's hematoxylin solution was used as a counterstain.

Measurement of fluorescence of labeled cells. A FACS-IV was used to measure the distributions of fluorescence intensity of the FITC-labeled cells. Low-angle light scatter was used as a gating parameter to eliminate dead cells and debris from the fluorescence measurements. The 488-nm laser line, operating at 500 MW, was used as the incident light source.

In all cases, 10,000 cells were analyzed. Data were stored on a floppy disk and analyzed on a PDP 11/70 at the SKI Core Computer Facility.

The method used to compute the percentage of positive cells in each sample was adapted from one devised by I. Levy and T. M. Chused of the National Institutes of Health and was kindly sent to us by Dr Chused. In this method, it is assumed that any "negative" cells present in the test sample (which may have low levels of autospecific or nonspecific fluorescence) have a fluorescence distribution identical to that of control cells. After appropriate normalization of the fluorescence distribution of the control samples relative to the test sample, the former is subtracted from the latter to yield the estimated distribution (and percentage) of positive cells.

Designation of B lymphoblastoid cell lines. Cell lines were designated according to the patients' diagnosis and number, cell lineage, and karyotype. For example, SK-CML7-Bt(9;22)-1 refers to Sloan-Kettering-CML-patient 7-B t(9;22) translocation-cell line #1, and SK-CML7-BN2 identifies the normal B cell line 2 from the same patient.

RESULTS

B lymphoblastoid cell lines derived from CML patients. The frequency of Ph'-positive and Ph'-negative B lymphoblastoid cell lines established from the peripheral blood of patients with CML following EBV infection is shown in Table 1. B lymphoblastoid cell lines were established from 16 of 22 patients studied. The failure to grow colonies from six patients following EBV infection may have been due to technical factors or to a low number of target lymphoid cells in their peripheral blood as a result of expansion of the myeloid population.

From 12 of 16 patients (75%), only Ph'-negative B lymphoblastoid cell lines were established. Eight of these patients were in the chronic phase, one was in an accelerated phase, and three were in blast crisis. Due to insufficient cell numbers, the karyotype of the colonies or cells in suspension culture wells that did not continue to grow could not be determined.

From four of 16 patients (25%), Ph'-positive B lymphoblastoid cell lines were established. From three of the four patients, Ph'-negative cell lines were also established. Patient 7 was in blast crisis of myeloblastic phenotype at the time of the first study. At that time, 100% of the colonies aspirated grew into established B lymphoblastoid cell lines, and all of these lines were 100% positive for the Ph' chromosome. Five months later, after the patient had been treated with mitoxantrone, colonies were grown from his peripheral blood following EBV infection. Seventy-one percent of the colonies aspirated grew into established B lymphoblastoid cell lines; all of these lines were 100% Ph'-negative. At the time patient 8 was first studied, he was in the chronic phase of the disease; B lymphoblastoid cell lines were established from 50% of the colonies aspirated, and all of these lines had a mixed Ph'-positive and Ph'-negative karyotype. Presumably, this was due to plating too high a number of cells and, as a result, the clones did not arise from single cells. Repeat attempts to reclone the lines to obtain pure Ph'-positive and Ph'-negative lines failed, and the lines eventually died in culture. One year later, another peripheral blood specimen was obtained from patient 8 while he was still in the chronic phase. At this time, 20 cell lines were established, of which 19 were 100% Ph'-negative and 1 was 100% Ph'-positive. Patient 16 was in the chronic phase at the time of the study. All the cell lines established from this patient were nonclonal cell lines (ie, derived from 5 x 10^6 cells seeded in 96-well culture dishes). Of the lines established, 76% had a mixed Ph'-positive and Ph'-negative karyotype, 20% were pure Ph'-negative, and 4% were pure Ph'-positive. Patient 21 was in an accelerated phase with 19% myeloblasts in the marrow at the time of the study, and all of the nonclonal cell lines established were entirely Ph'-positive.

To determine the growth characteristics of the B lymphoblastoid cell lines established from CML patients, lines were defrosted and followed in culture for several months. The length of the growth period refers to the sum of the months of cell growth prior to freezing and following defrosting of the line. The growth was arbitrarily assumed to have been initiated immediately following EBV infection. Ph'-negative lines from patients 1, 5, 7, and 9 were followed for at least 12 months, and their mean doubling times in the later months were 23, 34, 14, and 23 hours, respectively. Three Ph'-negative lines from patient 6 failed to grow continuously and died in culture after approximately six months. Three Ph'-negative lines from patient 11 were followed in culture. Two of these lines have been growing continuously for nine to ten months, and their present doubling time is ~48 hours. The third line, however, died on two occasions after five months in culture. Seven Ph'-positive lines from patient 7 were studied. Six of these lines died after a growth period of five to seven months in culture. The seventh line, SK-CML7-Bt(9;22)-33 began to die after four months in culture, but by five months there was an increase in cell number; it has now been growing continuously for 11 months with a present mean doubling time of 48 hours. One Ph'-positive line from patient 8 has been growing continuously for 12 months and presently has a doubling time of ~40 hours. Two Ph'-negative lines from patient 8 have been growing continuously for eight months; their present doubling times are ~25 and ~55 hours, respectively. One Ph'-positive and one Ph'-negative line from patient 16 have been growing continuously for ten months; their present doubling times are ~49 and ~20 hours, respectively. Two Ph'-positive lines from patient 21 have been growing continuously for about seven months, and presently...
have doubling times of ~35 and ~60 hours, respectively. Therefore, in the lines studied, so far there are no consistent differences between the growth rates of the Ph\(^1\)-positive and negative lines.

Cytogenetic analysis of B lymphoblastoid cell lines. Cytogenetic analysis of all of the established cell lines was done at the time of their initiation and periodically thereafter in the case of lines selected for continuous culture. All Ph\(^1\)-negative lines at the time of establishment were karyotypically normal with the exception of the Ph\(^1\)-negative lines established from patient 7 and three Ph\(^1\)-negative lines from patient 9. All Ph\(^1\)-positive lines from patients 7, 8, and 16 at the time of establishment had no additional chromosomal abnormalities. The Ph\(^1\)-positive lines from patient 21 had an additional translocation t(2;13)(q23;q22) in 100% of the cells, and in some cells an abnormality of one of the chromosomes was observed. The same chromosomal abnormalities were seen in a bone marrow specimen studied at the same time.

Patient 7 had a constitutional XYY karyotype and metaphases from all the Ph\(^1\)-positive cell lines established from this patient had the chromosomal constitution 47,XYY,t(9;22) (q34;q11). After 11 months in culture, the chromosomal constitution of SK-CML7-Bt(9;22)-33 remained unchanged. The Ph\(^1\)-negative lines established five months later had the chromosomal constitution 47,XY,t(11q;Yq) -8 -14 +7 +14q\(^{-}\) +Mar. After ten months in culture, metaphases from the Ph\(^1\)-negative lines showed the same chromosomal constitution as at the time of establishment, and only 2% of the metaphases were tetraploid.

Metaphases from six cell lines derived from patient 9 were examined at the time of establishment of the cell lines. All were negative for the Ph\(^1\) chromosome, and most metaphases from three of the six cell lines contained a diploid number of chromosomes. The other three lines contained high percentages of tetraploid metaphases. One of the primarily diploid cell lines, after 11 months in culture, contained only tetraploid metaphases.

At the time of establishment of the lines, metaphases from representative cell lines derived from patients 1, 5, 6, and 11 contained a diploid number of chromosomes and were karyotypically normal. After six months in culture (two months prior to freezing and four months after defrosting), the lines grown continuously from patient 6 and patient 11 had died, but lines from patient 1 and patient 5 continued to grow; cytogenetic analysis still showed them to be karyotypically normal with a diploid number of chromosomes. After 11 months of continuous culture, 94% of the metaphases examined from the latter two cell lines showed a tetraploid number of chromosomes without the Philadelphia translocation.

Phenotypic markers of B lymphoblastoid cell lines. The phenotypic marker characterization of representative Ph\(^1\)-positive and Ph\(^1\)-negative B lymphoblastoid cell lines is shown in Table 2. All the lines characterized were positive for B1, I2, and sIg\(^+\) for heavy and light chains. It should be noted that SK-CML7-BN2 had a low percentage of cells expressing I2 (Ia-like antigen), this is of particular interest since this cell line also lacks expression of HLA antigens. The reasons for this are currently being investigated. All the lines (with the exception of SK-CML7-BN2) had a percentage of cells that expressed B2 and were negative for J5 (CALLA). Four of the cell lines (SK-CML-Bt(9;22)-33, SK-CML16-BN1, SK-CML9-BN3, and SK-CML11-BN6) contained both \(\kappa\) and \(\lambda\) light chain positive cells and therefore were not derived from a single cell clone. SK-CML-Bt(9;22)-33, however, after 11 months in culture, contained only \(\lambda\) light chain positive cells. Of the 19 lines characterized, 14 were

| Table 2. Phenotypic Markers of B Lymphoblastoid Cell Lines Derived From Patients with CML |

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Surface (%)</th>
<th>Cytoplasmic (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B1</td>
<td>B2</td>
</tr>
<tr>
<td>SK-CML7-Bt(9;22)-1</td>
<td>97</td>
<td>22</td>
</tr>
<tr>
<td>SK-CML7-Bt(9;22)-3</td>
<td>97</td>
<td>25</td>
</tr>
<tr>
<td>SK-CML7-Bt(9;22)-33</td>
<td>97</td>
<td>41</td>
</tr>
<tr>
<td>SK-CML7-Bt(9;22)-33*</td>
<td>98</td>
<td>33</td>
</tr>
<tr>
<td>SK-CML7-BN2</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td>SK-CML8-Bt(9;22)-3</td>
<td>83</td>
<td>94</td>
</tr>
<tr>
<td>SK-CML8-BN10</td>
<td>97</td>
<td>24</td>
</tr>
<tr>
<td>SK-CML8-BN12</td>
<td>99</td>
<td>15</td>
</tr>
<tr>
<td>SK-CML9-Bt(9;22)-1</td>
<td>98</td>
<td>70</td>
</tr>
<tr>
<td>SK-CML9-BN1</td>
<td>100</td>
<td>53</td>
</tr>
<tr>
<td>SK-CML11-BN6</td>
<td>94</td>
<td>24</td>
</tr>
<tr>
<td>SK-CML11-BN6</td>
<td>97</td>
<td>91</td>
</tr>
</tbody>
</table>

*SK-CML7-Bt(9;22)-33, after 11 months in culture.
Phagocytes. The frequency of Ph'-positive E lymphocytes from the peripheral blood of patients with CML is shown in Table 3. E+ lymphocytes from all ten patients were stimulated with PHA for three days. In addition, E+ lymphocytes from five of these patients were also stimulated with IL-2 for three to 17 days. Because of a limited supply of IL-2, it was not possible to extend the culture period. A total of 461 metaphases were examined, and only one contained the Ph' chromosome; the latter was probably a contaminating myeloid cell since it was seen in a three-day culture.

DISCUSSION

There is convincing cytogenetic and enzymatic evidence that Ph'-positive CML originates in a single pluripotential hematopoietic stem cell which serves as a precursor for the granulocytic, erythroid, megakaryocytic, and monocyte/macrophage lineages. During a preclinical period, which has been estimated to be approximately eight years in duration, the progeny of the original transformed stem cell expand to replace almost completely the normal myeloid population in the marrow. By the time clinical manifestations develop and the diagnosis is made, the greater number of marrow metaphases in almost all patients contain the Ph' marker, as is evident in the present patients as well as in previous series.

It is less clear to what extent the normal lymphoid lineages are replaced by the Ph'-positive leukemic population. CML characteristically undergoes progression to a blastic phase after an average duration of the chronic phase of about three years. In most cases, after blast transformation, the blasts have myeloid phenotypes; however, in about one-third of cases, the blasts have a lymphoid morphology similar to that seen in ALL and express TdT and CALLA.

There is now compelling evidence that the blasts in most cases of lymphoblastic transformation of CML are pre-B cells in that they have rearrangement of the immunoglobulin heavy chain genes, which is the earliest detectable step in B cell differentiation. Some of the lymphoblasts also have light chain gene rearrangements and, in a small number of cases, they may contain intracytoplasmic IgM. A few cases of lymphoblastic transformation of CML have been reported in which the blasts express properties associated with T cell differentiation or have hybrid phenotypes.

With regard to the extent of involvement of the lymphoid lineages in chronic phase CML— with a few exceptions—most investigators have found that T lymphocytes and PHA-responding cells do not contain the Ph' chromosome. The present investigation provides confirmation of the latter reports in that all but one of 461 metaphases of IL-2-stimulated and/or PHA-stimulated E rosette-positive lymphocytes obtained from the blood of 10 CML patients were Ph'-negative.

It has been shown that a small number of human mixed hemopoietic colonies derived from normal marrow contain T lymphocytes. It is possible that if multilineage colonies containing T lymphocytes could be isolated from CML marrow and grown in the presence of IL-2, the presence of the Ph' chromosome might be shown in T lymphocytes. It may be that almost all of the thymus-derived T lymphocytes were derived from normal stem cells prior to the transforming event at the onset of CML, since the thymus is populated early in human development and thus the circulating T lymphocytes would most likely be Ph'-negative. T lymphocytes, however, derived from a pluripotent stem cell stimulated to differentiate in vitro might produce T lymphocytes containing the Ph' chromosome. Fauser et al have recently shown that multilineage colonies from one CML patient did in fact contain Ph'-positive T cells.

With respect to the extent of involvement of the B cell lineage in CML, both Ph'-positive and Ph'-negative B lymphocyte metaphases have been reported, and Martin et al have found that nine of 74 EBV-transformed B lymphoblastoid cell lines established from a Ph'-positive chronic phase CML patient who was heterozygous for G6PD contained ≥93% Ph'-positive cells; only type B G6PD was detected in the Ph'-positive lines, the same enzyme found in the myeloid cells of the leukemic cell clone.

In the present series, EBV-transformed B lymphoblastoid lines were established from 16 of the 22 Ph'-positive CML
patients studied (73%). There was no clear correlation between the duration of disease and the establishment of Ph1-positive B cell lines, perhaps because of the limited number of patients studied. All lines derived from 12 of 16 patients (75%) (2 in chronic phase, 1 in an accelerated phase, and 3 in blast crisis) were entirely Ph1-negative. The duration of the disease from diagnosis at the time these 12 patients were studied varied from one week to eight years. Ph1-positive lines were established from 4 of 16 patients (25%) (2 in chronic phase (nos. 8, 16), 1 in an accelerated phase (no. 21), and 1 in blast crisis (no. 7). The duration of the disease from diagnosis at the time these four patients were studied varied from two to ten years. From the two chronic phase patients, Ph1-negative lines were also established at the same time as the Ph1-positive lines. However, only Ph1-positive lines were established initially from patients 7 and 21. Patient 7, however, was subsequently studied after treatment with mitoxantrone and, at that time, five months after the establishment of the Ph1-positive lines, only Ph1-negative lines were established.

Extensive prior cytogenetic studies on EBV-carrying B cell lines and lines established from cord blood and adult peripheral blood infected by exogenous EBV have been shown to be karyotypically normal when initially established. Although some of those lines in long-term culture developed chromosome abnormalities, in no instance has a Ph1 chromosome been reported.

The reason for the limited growth potential of some of the Ph1-negative and Ph1-positive B lymphoblastoid cell lines established in this study is unknown. A possible explanation may be that the target cells for infection with EBV are heterogeneous with respect to their growth potential. It should be noted that in the present study, not all colonies derived from the EBV-infected blood specimens were successfully grown as cell lines. Because cytogenetic analysis of these colonies was not done, it is not known whether they were derived from a Ph1-positive or Ph1-negative B lymphoblastoid cell. Martin et al. found that all Ph1-positive B lymphoblastoid cell lines derived from one patient died after three weeks to five months in culture, whereas Ph1-negative B lymphoblastoid cell lines derived from the same patient grew continuously in culture. Limited growth potential of Ph1-positive myeloid progenitors in vitro has been previously reported.

To determine if Ph1-positive B lymphocytes responsive to EBV infection are more likely to have a limited growth potential than Ph1-negative B lymphocytes responsive to EBV infection, further study is required of multiple cell lines from additional CML patients and normal subjects.

Martin et al. reported that 71% of the 63 Ph1-negative B cell lines derived from their CML patient manifested the same (type B) G6PD enzyme found in this patient's leukemic clone and that eight of these type B lines (but none of the type A lines) developed early chromosomal abnormalities after only 15 to 45 days in culture. Based on these findings, they postulated that the type B Ph1-negative B lymphoblastoid lines were genetically unstable and may have been derived from the same stem cell clone that gave rise to the Ph1-positive leukemia. Because none of our patients were heterozygous for G6PD, we were unable to confirm these observations. Further studies will be necessary to determine whether the incidence of genetic instability is generally higher in Ph1-negative B cell lines derived from CML patients than from normal subjects.

NOTE ADDED IN PROOF

After we submitted this article, B lymphoblastoid cell lines were established from an additional ten CML patients. All lines from nine patients (7 in chronic phase, 1 in an accelerated phase, and 1 in blast crisis of lymphoblastic TdT phenotype) were Ph1-negative and karyotypically normal. From one patient in chronic phase whose duration of disease from diagnosis was three years, both Ph1-positive and Ph1-negative lines were established. The Ph1-positive lines from this latter patient had no additional chromosomal abnormalities, and the Ph1-negative lines were karyotypically normal.

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Incidence of involvement of the B and T lymphocyte lineages in chronic myelogenous leukemia

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