bcr-abl–Induced Cell Lines Can Switch From Mast Cell to Erythroid or Myeloid Differentiation In Vitro

By Andrew G. Elefanty and Suzanne Cory

**CHRONIC MYELOID** leukemia (CML) is a clonal stem cell malignancy typically distinguished by the Philadelphia chromosome (Ph)

from chromosome 22 to a shortened chromosome 22 that results from a reciprocal 9;22 translocation.

The translocation juxtaposes the N-terminal region of the bcr gene (on chromosome 22) and the body of the c-abl proto-oncogene (on chromosome 9), thereby creating a hybrid bcr-abl gene encoding a 210-Kd fusion polypeptide.

The bcr-abl gene created by a variant form of the translocation confined to Ph-positive acute lymphoblastic leukemia (ALL) involves a smaller bcr moiety and encodes a polypeptide of 190 Kd. The heightened tyrosine kinase activity of the bcr-abl proteins is believed to be the key feature inducing disease.

The chronic phase of CML, which can be regarded as a preleukemia, initially exhibits an excess of maturing myeloid elements in the blood and hematopoietic organs. After 3 to 5 years the disease accelerates and eventually culminates in a fatal blast crisis, usually an acute myeloid or lymphoid leukemia, although erythroid, megakaryoblastic, and basophilic variants occur infrequently.

The inability of chronic-phase CML cells to be maintained in culture has limited analysis of the events involved in progression to blast crisis. The existing CML cell lines, in fact, derive from patients in the terminal blast phase.

Early studies of the effect of expressing a bcr-abl cDNA in hematopoietic cells established that immortalised cell lines were relieved of their previous growth factor requirement and rendered tumorigenic.

Low-level growth factor production by one such line raised the possibility that abl oncogenes facilitate establishment of an autocrine loop.

Most attempts to mimic CML, either in vitro by infecting long-term murine bone marrow cultures with a bcr-abl retrovirus, or in vivo through bcr-abl transgenic mice, yielded only lymphoid disease. A more promising approach has been to transplant lethally irradiated mice with bone marrow cells infected with a bcr-abl retrovirus. A high incidence of hematopoietic disease was observed.

A few mice exhibited the granulocytosis expected of CML but most developed macrophage, erythroid, mast cell, or lymphoid tumors.

This report focuses on mast cell lines established from mice with erythroid and/or mast cell disease. Many were not malignant until they had been cultivated in vitro for several months. Surprisingly, several clonal bcr-abl-expressing cell lines switched in vitro from mast cell to megakaryocytic and/or erythroid character, and one became myeloid. The dramatic phenotypic shifts seem likely to involve changes occurring within progenitor cells maintaining the clone, rather than mutation of mature mast cells. The variant lines exhibited substantial spontaneous differentiation, despite being readily transplantable and therefore fully transformed. The production of hematopoietic growth factors by the mast cell lines and their phenotypic variants may implicate an autocrine loop in their evolution. These novel bcr-abl cell lines should aid in the study of genetic events in the progression from chronic to acute leukemia and facilitate analysis of hematopoietic lineage commitment.

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**MATERIALS AND METHODS**

**Establishment of Cell Lines**

Bone marrow or spleen cell suspensions were seeded at 1 to 2 × 10⁶ cells/mL in 10 mL Dulbecco's modified Eagle's medium containing 20% fetal calf serum and 0.05 mmol/L 2-mercaptoethanol in 80 cm² flasks (Nunc, Kamstrup, Denmark) and incubated at 37°C in a humidified atmosphere of 10% CO₂ in air. After 5 to 7 days, or sooner if cell proliferation was rapid, 5 to 10 mL of fresh medium was added. Thereafter, half medium changes were performed at least weekly. Cell lines that were 'slow' growing had doubling times of 2 to 7 days while 'fast' growing cell lines doubled every 1 to 2 days. Cell lines were frozen and stored in liquid nitrogen at regular intervals, especially after phenotypic 'switching,' to circumvent the problem of dedifferentiation that occurred with continuous culture of these lines.

**Analysis of DNA and RNA**

Nucleic acid isolation and fractionation was as described previously. The probes used for blot analysis included the following: IgE receptor, the 1,036-bp EcoRI fragment of the rat α subunit of the high-affinity IgE receptor (clone Edp 16); myeloperoxidase, and...
the 1.9-kb *Pst*I fragment of the murine cDNA clone mMP06; p53, 0.4-kb and 0.87-kb *Pst*I fragments of the murine cDNA clone pp53-176; and scl, the 1.9-kb *EcoRI* fragment of the murine cDNA, corresponding to nucleotides 406 to 2335. The origin of other probes is described elsewhere. Probes were labeled with [α-32P]dATP using a random hexamer priming kit (Bresatec Limited, Adelaide, South Australia).

**Hematopoietic Assays**

Conditioned medium collected from dense cell cultures was concentrated 10-fold in an ultrafiltration cell (model 8050; Amicon, Danvers, MA) and filtered before use. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) were assayed using FDC-P1 cells (responsive to GM-CSF and IL-3) and 32D clone 23 cells (responsive to IL-3) as described previously. Erythropoietin (Epo) was assayed on fetal liver cells using day-2 concentrated 10-fold in an ultrafiltration cell (model 8050; Amicon, Danvers, MA) and filtered before use. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) were assayed using FDC-P1 cells (responsive to GM-CSF and IL-3) and 32D clone 23 cells (responsive to IL-3) as described previously. Recombinant murine IL-3, GM-CSF, granulocyte-CSF (G-CSF), and leukemia inhibitory factor (LIF) were generously provided by Dr D. Metcalf, and recombinant human Epo by Dr A.W. Burgess.

**Detection of Cell Surface Antigens**

Cells were stained with the monoclonal antibodies (MoAbs) M1/70 (α-Mac-1), Rb6-8C5 (8CS'/αGr-1), F4/80, and TER-119; each had been directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (Southern Biotechnology Associates, Birmingham, AL), or biotinylated and revealed by an FITC- or PE-streptavidin second stage. All staining was performed in the presence of excess unlabeled anti-Fcγ receptor antibody to reduce nonspecific binding of antibodies, and cells were analyzed on a FACScan (Becton Dickinson, Sunnyvale, CA).

**Cytochemical Stains**

May-Grünwald-Giemsa, Luxol Fast Blue, Astra Blue, and staining for α-naphthyl acetate esterase, myeloperoxidase, and acetylcholinesterase were performed on cytospin preparations or fixed agar colonies according to standard protocols. Detection of the pseudoperoxidase activity of hemoglobin in agar colonies or cell suspensions was with benzidine HCl or 2,7-diaminofluorene. 

**RESULTS**

Erythroid and mixed erythroid/mast cell hyperplasia was a frequent cause of morbidity in mice we reconstituted with *bcr-abl* virus-infected bone marrow cells (Elefanty and Cory, manuscript submitted). To further characterize this syndrome, we sought to establish cell lines by culturing the bone marrow and spleen cells of 19 mice in the absence of added growth factors. Twelve of the 15 lines that were derived (Table 1) were mast cell in origin; three grew rapidly from the outset but the majority grew very slowly, doubling every 2 to 7 days. The proviral insertion pattern shown by Southern blot analysis established that all but one of the cell lines were clonal and, in 9 of 10 cases, the pattern obtained from the cell line was identical to that obtained from the tissue of origin. Because six of the seven mast cell lines in this analysis derived from tissue infiltrated predominantly by erythroid cells, both populations probably descended from a common infected precursor.

Six mast cell lines were maintained continuously in vitro for several months. Remarkably, they proved to be phenotypically unstable (Table 2), most evolving towards erythroid/megakaryocytic differentiation. After cloning in agar, the phenotypic variants from all six lines were shown by proviral insertion analysis to derive from the same infected precursor as the initial mast cell line (this report and unpublished results). The history of one erythroid/megakaryocytic line (96.4) will be described in detail, as will that of an exceptional line (34.6) that became myeloid. The evolution of the *bcr-abl* lines towards a fully malignant phenotype will also be considered.

**The 96.4 Line: A Switch From Mast Cell to Erythroid and Megakaryocytic Differentiation**

Clonal phenotypic variants. Figure 1A summarizes the evolution of the 96.4 mast cell line, which was obtained from the spleen of a BALB/c mouse with erythroid, mast cell, and macrophage disease (Elefanty and Cory, manuscript submitted). The slow-growing line was independent of the exogenous IL-3 required by normal mast cells, but may have been provided with growth factors by the stromal component present during the first few weeks of culture. After about 3 months, a rapidly growing subline of mast cells (designated 96.43Mst) arose in one of several cultures grown in parallel. The cells in another flask of the slow-growing line (designated 96.42Mst) underwent a dramatic change after another 2 months. Within 1 week, the culture became pleomorphic (compare Fig 1B with 1C and D) and cytospin preparations (Fig 1E and F) showed that the mast cells had been replaced by maturing erythroid cells and megakaryocytes that stained with acetylcholinesterase (not shown). Most cells (70%) in this subline (96.42Ery) contained hemoglobin, as judged by staining with benzidine or diaminofluorene and over 50% reacted strongly with the receptor for mast cell growth factor (MGF), while β globin and Epo receptor expression was found only in sublines containing erythroid cells (lanes 3 and 4). Two genes encoding transcription factors implicated in erythroid differentiation, GATA-1 and *scl*/*tal*, were expressed in the mast, erythroid, and erythroid/
Table 1. Derivation of Cell Lines From Primary bcr-abl Tumors

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Strain</th>
<th>Latency (wk)*</th>
<th>Pathology†</th>
<th>Transplantation‡</th>
<th>Cell Line§</th>
<th>Clonal Relationship</th>
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<tr>
<td>33.3</td>
<td>C57Bl/6</td>
<td>46</td>
<td>Ery, Mast</td>
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<td>Erythroid</td>
<td>Erythroid/mast</td>
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<td>BALB/c</td>
<td>22</td>
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<td>ND</td>
<td>Erythroid</td>
<td>Erythroid/mast</td>
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<tr>
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<td>30</td>
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<td>+†</td>
<td>Erythroid</td>
<td>Erythroid/mast</td>
</tr>
<tr>
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<td>Mast</td>
<td>+</td>
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<td>45.14</td>
<td>DBA/2</td>
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<td>+‡</td>
<td>Mast</td>
<td>Mast</td>
</tr>
<tr>
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<td>BALB/c</td>
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<td>Ery</td>
<td>++</td>
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<td>Mast</td>
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<tr>
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<td>C57Bl/6</td>
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<td>–</td>
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<td>ND</td>
</tr>
<tr>
<td>33.1</td>
<td>C57Bl/6</td>
<td>7</td>
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<td>–</td>
<td>Mast</td>
<td>ND</td>
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<tr>
<td>33.2</td>
<td>C57Bl/6</td>
<td>7</td>
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<td>–</td>
<td>Mast</td>
<td>ND</td>
</tr>
<tr>
<td>34.6</td>
<td>C57Bl/6</td>
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<td>–</td>
<td>Mast</td>
<td>ND</td>
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<tr>
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<td>T†</td>
<td>–</td>
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<tr>
<td>45.11</td>
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<td>3</td>
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<td>–†</td>
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<td>I</td>
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<td>BALB/c</td>
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<td>ND</td>
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<td>I</td>
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Abbreviation: ND, not done.

*Time elapsed (in weeks) between transplantation of irradiated mice with bcr-abl virus-infected bone marrow cells and autopsy.
†Histopathology of primary bcr-abl mice that exhibited erythroid (Ery) and mast cell (Mst) disease (Elefanty and Cory, manuscript submitted). Two of these mice also had a lymphoid (T) tumor component and all had a variable amount of macrophage tumor that, at least in some cases, derived from the same infected progenitor as the erythroid/mast cell tumor. 
‡Transplantability of the primary tumor was usually assessed by injecting 10^6 spleen cells intraperitoneally into 2 to 6 sublethally irradiated (4.5 Gy) or nonirradiated (two daggers) recipients, except for those (two asterisks) that were tested as cell lines within 25 days of initiating in vitro culture. Recipients were monitored for tumors for 12 months; +, transplantable; –, not transplantable.
§Cell type in primary line derived by culturing spleen or bone marrow cells of sick mice.
||Retroviral integration pattern of cell line determined by Southern blotting (see Fig 2A) to be identical (I) or different (D) to that of tissue of origin or, for mouse 34.8, of tumor deposits elsewhere in the primary mouse. ND, not done due to insufficient material; these four animals had oligoclonal tumors at other sites.
†A clonal T-lymphoid thymoma was present that derived from the same progenitor (mouse 33.2) or a different progenitor (mouse 34.8) to the macrophage or mast cell disease.
#Four of six recipients developed tumors following a long latency (6 months).

Table 2. Phenotypic Shifts of bcr-abl Mast Cell Lines

<table>
<thead>
<tr>
<th>Cell Line*</th>
<th>Culture Period (mo)†</th>
<th>Secondary Lineage‡</th>
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<tr>
<td>96.4Mst</td>
<td>5</td>
<td>Erythroid/megakaryocytic</td>
</tr>
<tr>
<td>34.6Mst</td>
<td>3</td>
<td>Erythroid/mast cell</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Erythroid</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Myeloid</td>
</tr>
<tr>
<td>34.4Mst</td>
<td>1</td>
<td>Erythroid/megakaryocytic</td>
</tr>
<tr>
<td>45.14Mst</td>
<td>1.5</td>
<td>Erythroid/megakaryocytic</td>
</tr>
<tr>
<td>52.5Mst</td>
<td>6</td>
<td>Erythroid/megakaryocytic</td>
</tr>
<tr>
<td>99.4Mst</td>
<td>6</td>
<td>Erythroid/mast cell</td>
</tr>
</tbody>
</table>

*Six other mast cell lines did not demonstrate a phenotypic shift in vitro, but were only cultured for 1 to 4 months.
†Total period in culture (in months) before the observed shift.
‡In all cases, agar cloned cell lines of the secondary lineage harbored identical proviral integrations to the primary cell line (Figs 2 and 5, Elefanty et al., and data not shown).
§These lines evolved from the erythroid/mast cell line.
|Although mast cells dominated the primary cultures from these mice, some erythroblasts persisted from the outset.
|Previously described, but the megakaryocytic component was not initially appreciated.
#Rare clones obtained after infection of the 52.5Mst line with a retrovirus expressing a mutated p53 cDNA; whether p53 played any role is obscure.

megakaryocytic variants, consistent with previous observations.20-23

Factor production. Four of five 96.4 mast cell sublines expressed GM-CSF RNA (eg, Fig 2B, lane 2) and, although no IL-3 (or MGF) RNA was detectable, more sensitive bioassays (see Materials and Methods) showed that the three clones tested secreted low levels of IL-3 (1 to 5 U/mL) as well as GM-CSF (5 to 900 U/mL). All three erythroid/megakaryocytic and both erythroid subclones tested secreted GM-CSF (5 to 100 U/mL). A low level of Epo bioactivity (0.05 U/mL) was detected in the single erythroid clone tested, but not in an erythroid/megakaryocytic clone.

Differentiation capacity of the 96.4 variants. The range of differentiation stages detectable in the 96.4EMeg and 96.4Ery lines was impressive. Both produced macroscopic colonies of erythroblasts in the spleen of lethally irradiated syngeneic mice 12 to 14 days after injection. Spleen colony formation, a property displayed by some early stem cells (CFU-S), was not obtained with the other bcr-abl erythroid cell line (33.3Ery) tested. When cultured in semi-solid agar, the 96.42 erythroid lines yielded diverse colony types (Table 3). The EMeg line produced large compact colonies containing blast cells (some colonies were white, others red due to a large erythroid component), as well as small single-
lineage or mixed colonies of maturing erythroid cells and megakaryocytes (Fig 3). In contrast, the Ery subline yielded fewer megakaryocytic colonies and many more overtly hemoglobinized colonies (Table 3). Only the white compact colonies retained a high proliferative potential upon replating (data not shown). Addition to the agar cultures of IL-3 or Epo (Table 3) or GM-CSF (not shown) affected neither the plating efficiency nor the distribution, size, or morphology of colonies.

The 96.4 line did not readily switch its differentiation pattern. Despite the phenotypic shift just described, the 96.4 primary mast cell line was not intrinsically unstable in vitro. After being maintained for 8 months in liquid culture, 96.42Mst cells could be cloned in agar, but only on one occasion over the next 5 months were erythroid colonies detected amongst the mast cell colonies. These were established as EMeg lines that displayed the same proviral insert as all other 96.4 sublines (Fig 2A, lane 5), but the liquid culture from which they derived was not overtaken by erythroid cells, perhaps because the mast cells themselves now grew more rapidly or because the second EMeg variant was not as competitive as the earlier one.

Because the demethylating agent 5-azacytidine has been shown to induce a lymphoid to macrophage switch in an Abelson-transformed cell line,44 we attempted to induce a mast to erythroid/megakaryocytic switch by incubating three 96.42 Mst clones with up to 6 µmol/L 5-azacytidine for 1 to 5 days. As only one treated culture acquired a small number (~12%) of benzidine-staining cells, we infer that the genetic change(s) required to permit erythroid/megakaryocytic differentiation of the 96.42Mst progenitor cell is not readily induced by demethylation.

The 34.6 Line: A Switch From Mast Cell to Erythroid and Myeloid Differentiation

The 34.6 mast cell line (34.6Mst) was derived from the near-normal bone marrow of a C57BL/6 mouse with a modest bcr-abl–induced erythroid and macrophage tumor burden.45 As outlined in Fig 4A, prolonged culture of this line produced both an erythroid and a myeloid variant. Once again, Southern blotting established that the subcloned phenotypic variants and the primary mast cell line derived from a single infected precursor (Fig 5A).

Evolution of the erythroid variant. After 3 to 4 months in culture, the cells became smaller, generally exhibiting a morphology intermediate between that of an erythroid and
bcr-abl MAST CELLS SWITCH DIFFERENTIATION

Table 3. Colony Formation by 96.4 Erythroid Lines in Agar

<table>
<thead>
<tr>
<th>Cell Line*</th>
<th>Stimulus</th>
<th>White Compact</th>
<th>Red</th>
<th>Megakaryocytic, Mixed b</th>
<th>Erythroid Diffuse</th>
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</thead>
<tbody>
<tr>
<td>96.42EMeg</td>
<td>Saline</td>
<td>2</td>
<td>9</td>
<td>70</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>IL-3</td>
<td>4</td>
<td>9</td>
<td>64</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Epo</td>
<td>2</td>
<td>13</td>
<td>71</td>
<td>13</td>
</tr>
<tr>
<td>96.42Ery</td>
<td>Saline</td>
<td>7</td>
<td>34</td>
<td>13</td>
<td>46</td>
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<tr>
<td></td>
<td>IL-3</td>
<td>5</td>
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<td>23</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Epo</td>
<td>6</td>
<td>17</td>
<td>27</td>
<td>50</td>
</tr>
</tbody>
</table>

*The 96.42EMeg cell line was assayed after 6 months and the 96.42Ery line after 8 months in culture. IL-3 was used at 1,000 U/mL and Epo at 1 U/mL.

†Morphology after 7 days of culture; 150 to 500 colonies from 4 to 9 plates were scored per stimulus. The plating efficiency of 96.42EMeg ranged from 0.7% to 0.9% and of 96.42Ery from 0.4% to 1.3% for the different stimuli, and was linear over cell concentrations of 10^6 to 10^7/μL. Agar plates and individual colonies were stained with benzidine or for acetylcholinesterase to confirm erythroid and megakaryocytic elements, respectively. White compact colonies were composed of blast cells with small numbers of erythroblasts and megakaryocytes, while red compact colonies were dominated by erythroblasts with few blasts and megakaryocytes.

‡Colonies containing megakaryocytes, either alone or mixed with erythroid cells.

| Colonies with a diffuse morphology (Fig 3F) that contained erythroid and megakaryocytic cells. 

Fig 3. Colony morphology in cultures of 96.42EMeg grown in agar for 7 days. (A) White compact, (B) red compact, (C) erythroid, (D) megakaryocytic, (E) mixed erythroid/megakaryocytic, and (F) diffuse colonies (original magnification x50) (see text and Table 3).

a mast cell (34.6EMst) (compare Fig 4B with C), although occasional cells resembled myelocytes and monocytes. RNA analysis showed messenger RNAs (mRNAs) for both β globin and the mast cell-specific IgE receptor α chain, as well as c-kit (Fig 5B, lane 1). At this point the cell line was cloned in agar and transplanted (see below). With time, 34.6EMst subclones assumed further erythroid character (compare Fig 4D with C), with increased expression of β globin and a loss of expression of the IgE receptor and c-kit (compare lanes 1 and 2 in Fig 5B). The clone subsequently analyzed in detail (34.6Ery.3) formed agar colonies contain-
levels of TER-119. Curiously, most cells expressed very high levels of F4/80, an antigen not seen in the 96.42EMeg line and previously thought to be macrophage specific, although other features suggestive of macrophage differentiation were not present. Attempts to differentiate 34.6Ery cells with 1.5% DMSO in liquid culture showed marked toxicity, but the surviving cells acquired some cytoplasmic granules suggestive of reversion to a mast cell phenotype.

A shift to myeloid differentiation. After 2 months in vitro, the mast cell culture generated from the spleen of a mouse transplanted with 34.6EMst cells was monopolized by maturing myeloid cells that grew in tight clumps (Fig 4F), unlike the erythroid/mast cell or its erythroid derivative (Fig 4E). The line was cloned in agar and designated 34.6Myl. All 12 clones analyzed were dominated by granulocytic cells (Fig 4G) that stained strongly for myeloperoxidase (Fig 4H). Depending on the clone, 5% to 80% of the cells were eosinophils and their precursors, because Luxol Fast Blue showed small numbers of emerald green cytoplasmic granules. Less than 5% of the cells were phagocytic adherent macrophages but ~30% expressed α-naphthyl acetate esterase. RNA analysis showed myeloperoxidase transcripts in 34.6Myl but no detectable mRNA for β globin or the IgE receptor (Fig 5B, lane 3).

34.6Myl cells formed granulocyte-macrophage colonies in agar in the absence of added growth factors. Neither plating efficiency nor colony morphology was affected by the inclusion of IL-3, GM-CSF, G-CSF, M-CSF, Epo, or LIF in the assays (not shown). However, analysis of conditioned medium showed endogenous production of

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**Fig 4. Differentiation of 34.6 cell lines.** (A) In vitro history (see text). (B through D) Cytocentrifuge preparations stained with May-Grünwald-Giemsa (MGG) of (B) 34.6Mst (2 months), (C) 34.6EMst (3.5 months), and (D) 34.6Ery (12 months) (original magnification ×500). Appearance in culture of (E) 34.6Ery (12 months) and (F) 34.6Myl (14 months) (original magnification ×125). Cytocentrifuge preparations of 34.6Myl stained (G) with MGG, (H) for myeloperoxidase, (I) with MGG after 2 days of induction with 1.5% DMSO, and (J) with MGG after 24 hours of treatment with 16 nmol/L tetradecanoyl phorbol acetate and overnight incubation with latex beads (0.8 μm; Difco, Detroit, MI) (original magnifications: G, H, I, ×500; J, ×800).
Evolution of the Transformed Phenotype

In contrast, minimal levels of GM-CSF were detected in the culture (Fig 4G). DMSO increased Mac-1 expression and most cells acquired F4/80, while a subpopulation (5% to 15%) expressed Mac-1 and lacked Sfpi-1 expression (Fig 6, lanes 3, 4, and 16). Levels were also low in the myeloid derivative of 34.6 (Fig 6B) and in three of the four mast cell lines examined (Fig 6, lanes 1, 2, and 10). Sequence analysis will be required to fully assess the role of p53, because oncogenic changes affecting p53 function may therefore have played a role in their neoplastic progression. Other bcr-abl erythroid lines, however, lacked Sfpi-1 expression (Fig 6, lanes 3, 4, and 16).

Differentiation Capacity of the Cell Lines In Vivo

Histologic analysis of animals transplanted with the established cell lines permitted assessment of their differentiation capacity in vivo (Table 4). Mice injected with 96.42EMeg cells developed erythroleukemia with greatly elevated numbers of circulating erythroblasts (Fig 1G). Although both 96.42Mst and 96.43Mst cells produced disseminated mast cell tumors, all four animals examined also contained areas of erythroid tumor in the spleen. This finding may indicate that the cells had a wider differentiation potential in vivo than in vitro, or that the frequency of 'lineage switch' mutations was relatively high in vivo. Within the 34.6 series, the 34.6Myl.1 clone produced disseminated myelomonocytic tumors, while all six recipients of 34.6EMst cells succumbed to various combinations of erythroid, mast cell, and macrophage tumors. Unexpectedly, the tumors arising after injection of the relatively undifferentiated erythroid clone 34.6Ery.3 were mast cells, implying that its differentiation potential had also been modified by environmental signals or by mutation.

DISCUSSION

The bcr-abl–expressing mast cell lines characterized here were obtained from mice that had developed erythroid and/or mast cell hyperplasia after lethal irradiation and
Table 4. Clonogenicity and Transplantability of bcr-abl Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Culture Period (wk)*</th>
<th>Clonogenicity (%)†</th>
<th>Transplanted Tumors‡</th>
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<tr>
<td>96.4 lines</td>
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</tr>
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<td>96.4Mst [s]</td>
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<td>0/4</td>
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<td>96.42Mst [s]</td>
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<tr>
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<td>33</td>
<td>15</td>
<td>37 Mst(Ery)</td>
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<td>2/3</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>11 Ery#</td>
</tr>
<tr>
<td>34.6 lines</td>
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<tr>
<td>34.6EMst</td>
<td>15</td>
<td>10</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>15</td>
<td>8 Mst(Ery)</td>
</tr>
<tr>
<td>34.6Ery.3</td>
<td>56</td>
<td>45</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>66</td>
<td></td>
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</tr>
<tr>
<td>34.6Myl.1</td>
<td></td>
<td></td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>50</td>
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</tr>
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</tr>
<tr>
<td>33.1Mst [s]</td>
<td>8</td>
<td>ND</td>
<td>0/3</td>
</tr>
<tr>
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<td>5</td>
<td>&lt;0.01</td>
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<tr>
<td></td>
<td>8.26</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>99.4Mst [f]</td>
<td>4</td>
<td></td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5</td>
<td>3 Mst</td>
</tr>
<tr>
<td>33.3Ery</td>
<td>2</td>
<td>1</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 Ery(Mst)</td>
</tr>
<tr>
<td>Abbreviation: ND, not done.</td>
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</table>

*Time of continuous culture (in weeks) before transplantation or plating in agar; [s] and [f] indicate slow and fast growing mast cell lines.
†Up to 10⁵ cells (5 × 10⁴ for 96.42Mst cells) plated in triplicate 1 mL agar cultures were scored for colony formation at day 7 and 14, and the cloning efficiency expressed as a percentage of the cells plated. No colonies were observed for 96.42Mst before 33 weeks of culture or for 52.5Mst before 42 weeks of culture. Equivalent results were obtained for mast cell lines cultured with or without IL-3 (1,000 U/mL).
‡Cells (10⁶) were transplanted intraperitoneally into sublethally (4.5 Gy) irradiated recipients, or nonirradiated mice (boldface), and monitored for up to 12 months. Additional animals were inoculated intravenously (IV) where indicated.
§Number of mice developing tumors versus total number injected.
∥Mean tumor latency (in weeks) posttransplantation.
*Histopathology of secondary tumors: Mst, mast cell; Ery, erythroblast; Mac, macrophage; Myl, myeloid. Minor tumor components are indicated in parentheses (see text).
#One of two recipients also had mast cell tumor around the common bile duct.
**Previously described.¹⁹

Fig 6. Northern blot analysis of cell lines harboring bcr-abl provirus. Polyadenylated RNA samples (2 to 4 µg) were sequentially hybridized with the indicated probes (see Materials and Methods). Samples for (A) are as for Fig 2B. In (B), (5) 34.6EMst (3 months), (6) 34.6Ery.3 (13 months), and (7 through 9) 34.6Myl.1 incubated with 1.5% DMSO for 1, 3, or 5 days (16 months). In (C), (10) 52.5Mst (3 months), (11) 99.4Mst (5 weeks), (12) 45.14EMeg.2 (14 months), (13) 33.3Ery (1 week), (14) 33.3Ery.2 (11 months), (15) 34.4EMeg (7 months), and (16) 55.3Ery (2 weeks).
reconstitution with *bcr-abl* virus-infected bone marrow (Elefanty and Cory, manuscript submitted). The virus-infected cell responsible for the syndrome appears to have been a progenitor cell with capacity for both mast cell and erythroid differentiation, as judged by clonal analysis of affected tissues and derived cell lines (Elefanty et al and this report). The *bcr-abl* mast cell lines grew in the absence of exogenous IL-3, unlike normal mast cells, but initially may have required factors produced by associated stromal cells, eg, the recently identified MGF or *Steel* factor. They produced significant levels of GM-CSF and IL-3. When first established, most lines were neither transplantable nor clonogenic in soft agar, but transplantable cells emerged after prolonged culture, presumably due to additional (undefined) oncogenic mutations. Analogous factor-independent mast cell lines produced by Abelson virus infection of multilineage or blast cell colonies or 18-day fetal liver cells also secreted multiple growth factors and initially required stromal cell support, but became fully malignant much more rapidly than the *bcr-abl* lines (2 to 3 months after virus infection vs 6 to 20 months). This finding probably reflects differences in the transforming potency of v-abl and p210 *bcr-abl* proteins.

The striking feature of the *bcr-abl* cell lines was their propensity to spontaneously change hematopoietic lineage on prolonged culture, a property never previously documented for mast cell lines. Several assumed an erythroid or erythroid/megakaryocytic character, but one became myeloid (Table 2). In each case, the variant line contained the same proviral insert as the parental mast cell line (Fig 2A and 5A and data not shown).

Several of the variant sublines exhibited a marked capacity to continuously differentiate, despite being highly malignant. The 96.4EMeg line and its subclones, for example, yielded mature erythrocytes and megakaryocytes in the absence of added growth factors (Fig 1). Because normal erythroid differentiation requires Epo and/or IL-3, and Epo was detected in the conditioned medium of one *bcr-abl* erythroid clone, an autocrine loop may have figured in their evolution. Intriguingly, spontaneous hemoglobinization is a feature of some erythroid progenitors in the chronic phase of CML. A surprisingly high proportion (5 of 13) of the cell lines established from CML blast crisis patients (eg, K562) exhibit some erythroid and/or megakaryocytic character, but induction by chemical agents is usually required to promote differentiation. Equivalent lines have not been isolated with Abelson virus; differentiating erythroid colonies isolated from infected fetal liver cells could not be established in culture.

The differentiation capacity of the *bcr-abl* myeloid line (34.6Myl) strongly resembled that of the well-studied promyelocytic line HL-60 derived from a patient with acute myeloid leukemia. Both differentiate spontaneously along neutrophil, eosinophil, and macrophage lines, the degree of differentiation being enhanced by a range of chemical inducers. 34.6Myl cells secrete GM-CSF and IL-3 and an ‘autocrine activity’ has been observed in HL-60-conditioned medium. None of the existing CML lines shows such a degree of myeloid differentiation and no equivalent lines have been isolated with Abelson virus. However, one clone of the IL-3–dependent line 32D, which was initially characterized as a mast cell line, has the capacity to terminally differentiate into granulocytes if IL-3 is replaced by G-CSF.

The nature of the cells involved in the ‘lineage switches’ is an intriguing, although unresolved, issue. Figure 7 depicts our current working hypothesis. It seems unlikely that mature mast cells are capable of the phenotypic shifts observed, so we presume that the affected cells were precursors present at low concentration in the cultures. These cells may have been committed, unipotential mast cell progenitors or oligopotent cells unable to express their full potential under the growth conditions provided. (The observation that certain lines exhibited a wider differentiation potential in vivo than in vitro may favor the latter hypothesis.) In either case, phenotypic switching in vitro presumably resulted from somatic mutation(s) that either provided necessary growth factor(s) or induced key transcriptional programming gene(s). Our failure to mimic such mutation by treatment with 5-azacytidine suggests that the mechanism of the lineage switch is more complex than activation of a single gene by demethylation.

For 96.42, the switch from mast to erythroid/megakaryocytic differentiation was accompanied by transformation, raising the possibility that the same gene(s) orchestrated both events. Many of the genes encoding nuclear oncoproteins (which might cooperate with cytoplasmic *bcr-abl*) are known to be transcription factors and might well influence the differentiation program. However, transformation and phenotypic change were not always linked. The myeloid switch in the 34.6 line, for example, occurred in a cell that was already tumorigenic and clonable in agar.

The evolution of the *bcr-abl*–expressing cell lines pro-
vides clues to the function of bcr-ab1 and its role in CML. Expression of bcr-ab1 by a hematopoietic progenitor and its progeny provides only a modest proliferative advantage and relieves certain growth factor requirements but probably not all. Whether growth factor production, particularly of IL-3, is crucial to the action of bcr-ab1 (and v-ab1) or is merely an epiphenomenon remains an important unresolved issue. The unexpected phenotypic plasticity of the cell lines suggests that bcr-ab1 expression may facilitate and/or complement the (postulated) somatic mutations in lineage-determining genes. Finally, our observation that even fully transformed lines can retain substantial capacity for maturation suggests that the chronic phase of CML may accumulate mutations that increase malignancy without significantly curtailing differentiation. Thus, more events may be needed to progress to blast crisis than hitherto suspected. The lines characterized here may facilitate identification not only of genes able to cooperate with bcr-ab1 in leukemogenesis but also those instrumental in determining lineage commitment during normal hematopoiesis.

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bcr-abl-Induced cell lines can switch from mast cell to erythroid or myeloid differentiation in vitro

AG Elefanty and S Cory