B-precursor acute lymphoblastic leukemia (ALL), the most common form of leukemia in children, is presumed to result from a series of leukemogenic events that occur in a single progenitor cell and, possibly, in its monoclonal progeny. The monoclonality is convincingly demonstrated by a specific rearrangement of the Ig heavy chain (IgH) gene, which serves as a clonal marker for each individual case of the leukemia. The immunophenotypes of B-precursor ALL cells, although often aberrant, resemble stages of normal B-cell development. B-cell maturation is characterized by the sequential appearance of differentiation antigens, which are detected by known monoclonal antibodies (MoAbs).

Immunophenotypic heterogeneity is observed when the whole leukemia cell population of an individual patient is examined for differentiation antigen expression. This heterogeneity may reflect the existence of a subpopulation of disease-sustaining cells with self-renewal capability and subpopulations of effete, benign progeny that are committed to a differentiation program. The subpopulation of cells with self-renewal capability is the appropriate target of curative treatment. Demonstration of differentiation within B-precursor ALL could lead to the identification of the disease sustaining cells and is, therefore, of importance for both the understanding of the disease process and the design of more effective treatment methods.

In normal lymphopoiesis the hematopoietic progenitor cell antigen, CD34, appears only on the earliest cells of the B-lineage, while the CD20 and CD22 antigens appear at intermediate and late stages, respectively. Because these surface markers are frequently observed on B-precursor ALL cells, we sought to determine whether maturation, described by the B-cell differentiation antigens, could be detected in B-precursor ALL. To accomplish this, we compared biologic and molecular properties of isolated leukemia cell subpopulations characterized by the CD34 antigen with those characterized by the CD20 and CD22 antigens. The results of this comparison show that these differentiation antigens define a limited program of differentiation in this leukemia that preserves the features of normal B-cell development. As far as we know, this is the first demonstration, supported by measurements of growth potential, cell surface markers, and gene expression, of maturation in B-precursor ALL.

MATERIALS AND METHODS

Cells. Heparinized diagnostic samples of bone marrow cells were obtained from children at initial diagnosis. The procedures were performed, after informed consent, as part of a protocol approved by the hospital's institutional review board. Seven of the 12 patient samples screened met the criteria for the studies reported here. The cell lines used in these studies were obtained from the American Type Culture Collection (Bethesda, MD) except for the N6F neuroblastoma line, which was a gift of Dr Garrett Brodeur (Washington University, St Louis, MO).

Morphologic and cytochemical studies. Bone marrow aspirates were routinely stained with Wright-Giemsa, myeloperoxidase, α-naphthyl butyrate, periodic acid Schiff, and chloracetate esterase. The diagnosis of ALL was based strictly on the light microscopic appearance and the cytochemical stains according to the French-American-British recommendations.

Immunophenotyping. Samples with greater than 90% leukemia cells by morphology were selected for assay. Leukemia cells were isolated from bone marrow samples by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. The MoAbs used to determine immunophenotype were anti–HLA-DR, anti-Leu12 (CD19), anti-CALLA (CD10), anti-Leu16 (CD20), anti-Leu14 (CD22), and anti–HPCA-1 (CD34), all of which were obtained from the Becton-Dickinson Monoclonal Center (Mountain View, CA). Staining of the leukemia cells followed previously described procedures, and isotype identical nonreactive MoAbs were used as negative controls. Stained cells were analyzed on a dual-beam System 50H Cytofluorograf (Ortho Diagnostics Sys-
tems, Westwood, MA) modified as previously described. These studies were restricted to B-precursor ALL cell populations that consisted of greater than 90% HLA-DR⁺CD19⁺ cells and also contained a subpopulation of CD34⁺ cells.

Subpopulation isolation. For three specimens, the subpopulations were isolated by both fluorescence-activated cell sorting and magnetic bead separation as described by Spangrude et al. Because the results of the two methods were not significantly different, the remainder of the specimens were separated by the magnetic bead technique, which required less time. For this separation method, cells were incubated with the primary unconjugated MoAb at 4°C for 30 minutes, washed, and then incubated for 30 minutes with goat antimusite IgG covalently attached to 1 μm paramagnetic beads (Advanced Magnetics, Cambridge, MA). The magnetic bead-coated cells (positive fraction) were retained by a magnet while the unstained cells (negative fraction) remained in the supernatant.

The purity of the positive fraction was determined by staining with fluorescein isothiocyanate-conjugated goat antimouse IgG (Caltag, South San Francisco, CA), followed by immunofluorescence analysis. The purity of this fraction was routinely greater than 98%. Negative fractions with purity between 90% and 95% were obtained by a second incubation with MoAb and magnetic beads followed by magnetic separation. The purity of this fraction was routinely greater than 90%. The viability of the sorted cells was always greater than 95% by trypan blue exclusion. T-cell contamination was determined by staining with an anti-Leu1 (CD5), anti-leu4 (CD3), and anti-Leu9 (CD7) MoAb mixture (Becton Dickinson) followed by immunofluorescence analysis. When T-cell contamination exceeded 1%, the cells were incubated with the anti-T-cell MoAb mixture and removed by magnetic bead separation.

Southern blot analysis. High molecular weight DNA was extracted from isolated subpopulations by the method of Hanson and Kersey or Williams. The DNA was digested with the restriction enzymes BamHI, HindIII, and EcoRI (Boehringer Mannheim, Indianapolis, IN), size fractionated on 0.7% agarose gels, transferred to NYTRAN filters (Schleicher and Schuell, Keene, NH) and hybridized as described previously. The IgH gene joining region (J₅) probe was obtained from Oncor, Inc (Gaithersburg, MD).

ALL blast colony assay. The blast colony assay has been described in detail previously. In brief, 1 x 10⁵ ALL cells were suspended in α-medium, 10% fetal calf serum, 10% phytohemagglutinin-T-cell-conditioned medium, and methylcellulose at a final concentration of 0.9%. Cells were plated in the presence of 10⁵ irradiated feeder cells in 35-mm Petri dishes. Feeder cells were normal donor peripheral blood depleted of adherent cells, incubated for 2 hours at 37°C, quickly cooled to 0°C, and exposed to 70 Gy of γ irradiation. The culture dishes were placed in a humidified atmosphere of 5% CO₂ and balanced nitrogen. Colonies were counted on days 6 and 10 of incubation, and a cluster of greater than 20 cells defined as a colony.

Northern blot hybridization. Total cellular RNA from about 5 x 10⁶ cells was recovered by the acid-guanidinium-phenol-chloroform extraction method. Three micrograms of RNA (as determined by optical density at 260 nm) per lane was electrophoretically fractionated in 1.25% agarose gels containing formaldehyde. Transfer to nitrocellulose membranes (BioRad, Richmond, CA), baking, hybridization, and washes were done as described elsewhere. Membranes were exposed to x-ray film with two intensifying screens. P-labeled probes were prepared by the random-primed labeling method, purified by Sephadex G-50 (Pharmacia) chromatography, ethanol precipitated with salmon sperm carrier DNA, and resuspended in water before use in Northern blot analysis. The CD34 probe was a gift from Dr Brian Seed (Massachusetts General Hospital, Boston, MA) and the c-myb probe was obtained from the ATCC.

RESULTS

Subpopulations defined by the CD34, CD20, and CD22 surface antigens have identical IgH gene rearrangements. The characteristics of the patients and the laboratory data obtained at diagnosis are presented in Table 1. Three-color immunofluorescence studies confirmed that in all seven cases there was a distinct CD34⁺CD19⁺HLA-DR⁺CD10⁻ subpopulation that was negative for the CD20 and CD22 antigens. In two of the cases, greater than 90% of the leukemia cells were CD34⁺ with subpopulations that simultaneously bore the CD20 and CD22 antigens on their surfaces. These results are in agreement with previous studies which indicate that immunophenotypic subpopulations corresponding to the early and late stages of normal B-cell differentiation are present in B-precursor ALL.

The status of the IgH gene rearrangement, the clonal marker for the leukemia, was then examined by Southern analysis using the J₅ probe for isolated subpopulations corresponding to early and late stages of normal B-cell differentiation. The subpopulation corresponding to the early stage of development consisted of CD34⁺ cells that did not express either the CD20 or the CD22 antigens. The other subpopulations either lacked CD34 or were positive for one of the late B-cell differentiation antigens, CD20 and CD22. In all seven cases the gene rearrangement band obtained for the two isolated subpopulations were identical, indicating that both subpopulations of leukemia cells

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Sex</th>
<th>White Blood Cell Count (x 10⁶)</th>
<th>Surface Markers (%)</th>
<th>J₅ Gene Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD34</td>
<td>CD19</td>
<td>HLA-DR</td>
</tr>
<tr>
<td>1</td>
<td>15</td>
<td>M</td>
<td>24</td>
<td>60</td>
<td>97</td>
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<tr>
<td>2</td>
<td>5</td>
<td>F</td>
<td>160</td>
<td>27</td>
<td>95</td>
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<tr>
<td>3</td>
<td>2</td>
<td>M</td>
<td>63</td>
<td>63</td>
<td>93</td>
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<td>4</td>
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<td>26</td>
<td>96</td>
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<tr>
<td>5</td>
<td>7</td>
<td>F</td>
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<td>34</td>
<td>96</td>
</tr>
<tr>
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<td>7</td>
<td>12</td>
<td>M</td>
<td>74</td>
<td>97</td>
<td>98</td>
</tr>
</tbody>
</table>

Abbreviations: G, germline; R, rearranged; D, deleted.
DIFFERENTIATION IN B-PRECURSOR ALL

were members of the same clone. Typical results of this analysis are shown in Fig 1.

CD34+ leukemia cells have a growth advantage when compared with CD34- leukemia cells. To determine whether the differences in immunophenotype were accompanied by differences in biologic activity, leukemia cells from fresh bone marrow specimens of patients 1 through 4 were separated into CD34+ and CD34- subpopulations and the growth of the two groups compared using the in vitro ALL blast colony assay. The results of the ALL blast colony assay are presented in Table 2. When 10^6 cells were plated for each subpopulation, the CD34+ leukemia cells consistently formed a greater number of colonies than did the CD34- leukemia cells \((P = .0117, \text{Wilcoxon sign rank test})\). Furthermore, the CD34+ colonies remained viable for at least 10 days, whereas the CD34- colonies deteriorated after 7 days.

The number of colonies per plated cell ranged from 4.3 \times 10^{-4} to 4.4 \times 10^{-3} for the CD34+ cells and from 3.9 \times 10^{-4} to 4 \times 10^{-3} for the CD34- cells. The average overall plating efficiency, measured at 6 days, was 1.5 \times 10^{-3}, in agreement with previous results using this assay.16,17,22,23 The cells from the colonies had lymphoid morphology when Wright-Giemsa stained preparations were examined. Cytochemical studies showed that they were periodic acid Schiff positive but myeloperoxidase and nonspecific esterase negative. Marrow from healthy donors cultured under identical conditions did not develop colonies with the characteristics of the leukemia cell colonies at days 5 to 7.

CD34 mRNA is mostly in subpopulations with progenitor immunophenotype. Samples from patients 6 and 7 are typical examples of the simultaneous appearance of early and late differentiation antigens on the leukemia cell surface. Greater than 90% of the leukemia cells of these two patients were CD34+, with subpopulations that also expressed either the CD20 or CD22 antigens. Figure 2a shows the results of the immunofluorescence analysis of the cells from patient 7 labeled with the anti-CD34 and

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**Fig 1.** Identical IgH gene rearrangement patterns are observed in the isolated subpopulations. DNA was isolated from leukemia cells of the unsorted specimen (lane 1), the CD34+ subpopulation (lane 2), and the CD34- subpopulation (lane 3) and examined by Southern blot analysis. DNA was simultaneously digested with the BamHI and HindIII restriction enzymes and the blots hybridized with the J5 probe. The arrows indicate the locations of the germine bands.

**Fig 2.** Simultaneous appearance of early and late differentiation antigens on the surface of B-precursor ALL cells of patient 7. Panel (a) shows the double-labeling of single cells with antigen combinations not found in normal B cells as determined by dual immunofluorescence. In panels (b through d), the numbers correspond to RNA from the following sources: lane 1, Nalm 6 cell line (positive control for c-myb); lane 2, KG1 cell line (positive control for CD34); lane 3, normal bone marrow; lane 4, total leukemia specimen; lane 5, CD34+CD20- subpopulation; lane 6, CD34+CD20+ subpopulation; lane 7, T-ALL cell line (positive control for c-myb); lane 8, HL60 cell line (positive control for c-myb); lane 9, N6F cell line (nonhematopoietic negative control). Subpopulations were isolated by sorting using the flow cytometer. The CD34+CD20- cells contained about 10% contaminating CD34-CD20+ cells that could be responsible for the very weak band seen at 2.7 kb in lane 5. For this specimen, one allele of the IgH gene remained in the germline configuration, while the other was rearranged. An identical band pattern was observed for the CD34+CD20- and the CD34-CD20+ subpopulations.

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**Table 2. Colonies Formed by Isolated CD34+ and CD34- B-Precursor ALL Cells From Four Patients**

<table>
<thead>
<tr>
<th>Cell Surface Phenotype†</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+</td>
<td>51:55</td>
<td>40:45</td>
<td>420:451</td>
<td>49:51</td>
</tr>
<tr>
<td>CD34-</td>
<td>36:41</td>
<td>5:3</td>
<td>31:28</td>
<td>15:14</td>
</tr>
</tbody>
</table>

Results from magnetic bead separated cells are shown.

*Numbers of colonies from duplicate cultures are shown.

†Both subpopulations were shown to have identical rearrangement patterns for the IgH gene.
anti-CD20 MoAbs. We compared CD34 mRNA levels in cells from the fresh bone marrow specimen sorted according to the presence or absence of these later appearing antigens to determine whether the CD34 gene is active in both the CD34+CD20− and the CD34+CD20+ B-precursor ALL cells. Total cellular RNA was isolated from the sorted cells and analyzed. The distinct 28S and 18S bands seen in the ethidium bromide stained gel (Fig 2b, lanes 4 through 6) show that the sorting procedure did not significantly affect RNA integrity. However, the spreading of the 28S band in lane 5 illustrates the consistent finding, in these studies, that the RNA isolated from subpopulations characterized by the late-stage differentiation antigens was both less in amount and lower in integrity when compared to that from subpopulations with precursor immunophenotype. This result was unaffected by either isolation of the same subpopulation by positive rather than negative selection or differences in the total time to recover the RNA after the specimen was drawn. The results of Northern blot hybridization (Fig 2c, lanes 4 through 6) indicated that by far the greatest portion of the CD34 mRNA was present in the CD34+CD20− subpopulation (lane 6), although a very weak band was observed in the CD34+CD20+ subpopulation (lane 5). CD34 mRNA was not detected in an identical amount of RNA recovered from the mononuclear fraction of normal bone marrow cells in which there were approximately 1.5% CD34+ cells (Fig 2c, lane 1). Similar measurements were performed using the CD34+CD22− and CD34+CD22− cells from patient 6 (data not shown). In this case, the CD34+CD22− subpopulations was larger than the CD34+CD20+ subpopulation and contained the latter. CD34 mRNA was detected in the CD34+CD22− cells with the immature phenotype but not in the CD34+CD22− cells. The low levels of CD34 mRNA in the CD34+ B-precursor ALL cells that also have the CD20 and CD22 antigens on their surface indicate either a marked reduction or a cessation of the synthesis of the CD34 molecule in this subpopulation. Whether the low levels of CD34 mRNA in the cells with the more mature phenotype are due to the lack of gene transcription or to posttranscriptional regulation of the message was not determined.

The results described in the previous paragraph demonstrate that the presence of the CD34 antigen on the cell surface is not a reliable measure of ongoing intracellular synthesis. To further establish whether CD34 mRNA can be used to measure the extent of differentiation in this leukemia, we determined these mRNA levels in CD34+ and CD34− subpopulations isolated from the fresh bone marrow specimens of patients 3 through 5. The result of Northern blot hybridization of RNA from the cells of patient 3 with the CD34 probe (Fig 3) is representative of the results obtained for these three cases. CD34 mRNA was detected in the CD34+ cells (lane 3) but not in an equal amount of RNA isolated from the CD34− cells (lane 4). These data suggest that CD34 gene transcription is a useful measure of differentiation in these leukemia cell populations. Furthermore, they confirm that the CD34 message has a lifetime of sufficient duration to allow its detection in B-precursor ALL cells after they are separated into subpopulations.

c-myb mRNA is limited to subpopulations containing CD34 mRNA. Because the c-myb nuclear oncogene is expressed in primitive hematopoietic cells, including early B cells, it could also be present in the ALL cells with the B-cell progenitor immunophenotype.24,25 This possibility was examined by rehybridizing the nitrocellulose filters with a c-myb cDNA probe. Figure 2d shows that the c-myb message was detected only in the RNA isolated from CD34+ cells of patient 7 that did not express CD20. The c-myb mRNA was also found only in the cells with precursor immunophenotype from patients 3 and 6. No c-myb message was detected in the leukemia cells of patients 4 and 5 (data not shown). Thus, when the c-myb gene expression was detected it was limited to those cells that also expressed CD34 mRNA.

DISCUSSION

The results presented here are consistent with the presence of a differentiation program in untreated B-precursor ALL that is characterized by the sequential appearance of B-cell differentiation antigens in a pattern similar to normal development. The kinetics of CD34 gene transcription during normal B-cell development is unknown, but it is reasonable to assume that transcription is downregulated during the earliest stage, after which the protein is no longer present. The presence of the c-myb protein has been demonstrated in normal B-cell progenitors, but neither the c-myb protein nor the corresponding mRNA have been observed in mature resting B cells.24,25 Thus, while the in
vitrin clonogenic assay consistently demonstrated greater colony-forming capability for the leukemia cells with early B-cell immunophenotype, the mRNA measurements showed downregulation of those genes that are active in early hematopoiesis as development progresses to more mature immunophenotypes. These results are in agreement with the view that malignant cells deviate only minimally from the differentiation programs of their normal counterparts. The simultaneous appearance of the CD34 antigen and an antigen that appears much later in normal differentiation, eg, CD20 or CD22, is common in B-precursor ALL and has been termed "asynchronous expression." The presence of CD34 mRNA only in cells that lack surface expression of the later differentiation antigen indicates that "asynchronous expression" is the result of the persistence of the CD34 molecule on the cell surface rather than the simultaneous synthesis of the two antigens. This conclusion is consistent with the results of other studies that examined the behavior of the CD34 antigen in KG1 and KG1a cells. Thus, the appearance of cell surface markers on leukemia cells in a pattern that deviates from normal development is not the result of grossly deranged gene expression. A demonstrable pattern of differentiation in leukemia implies, by analogy with other developing biologic systems, the presence of a progenitor cell population with in vivo self-renewal capability. Because this group of progenitor cells is likely to sustain the disease and produce relapse when therapy fails, it is self-evident that cure requires its elimination. The identification of the immunophenotypic subpopulation that contains these cells could have immediate application to the detection of residual leukemia using either the polymerase chain reaction or the in vitro ALL blast colony assay. Restriction of the detection procedure to cells in the immunophenotypic subpopulation containing the most primitive leukemia cells would improve the reliability of interpretation. Furthermore, if the proportion of leukemia cells in this subpopulation is increased relative to all narrow cells, then the increased signal-to-background ratio would improve the chances of detecting residual leukemia. This could be especially important if the only remaining leukemia cells were precursors in a quiescent state.

The ability to define the general features of a maturation process using known reagents provides the motivation to identify and characterize the earliest leukemia progenitors. Ultimately, this identification could lead to the identification of those regulatory genes associated with leukemogenesis and allow treatment based on properties unique to these cells.

REFERENCES

Differentiation in B-precursor acute lymphoblastic leukemia cell populations with CD34-positive subpopulations

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