Asynchronous Antigen Expression in B Lineage Acute Lymphoblastic Leukemia

By Craig A. Hurwitz, Michael R. Loken, Michael L. Graham, Judith E. Karp, Michael J. Borowitz, D. Jeanette Pullen, and Curt I. Civan

Cell surface phenotypes of 113 B lineage acute lymphocytic leukemia (ALL) cases, defined by the presence of HLA-DR and at least one B-cell-specific antigen (either CD19, CD20, or CD22), were compared with antigen-defined stages of normal B lymphocyte development. The cases were first evaluated for expression of HLA-DR, CD19, CD34, CD10, CD20, and CD22 by indirect one-color immunofluorescence. Pairwise comparisons of cell surface marker expression were performed for each leukemic sample: no correlations were observed for paired antigen expression on the leukemic samples using antigens expressed either early or late during normal B lymphoid development. Complete immunophenotypes of the cases were then compared with normal B-cell developmental stages. Sixteen different complete immunophenotypes were observed on the leukemias that were not found in normal marrow: at least 70% of the cases demonstrated such "asynchronous" combinations of B lymphoid-associated differentiation antigens. Several samples were subsequently studied by two-color immunofluorescence, and the presence of doubly labeled cells with "asynchronous" antigen combinations was confirmed. These results indicate that the majority of B lineage leukemias exhibit "developmental asynchrony," as compared with normal marrow B cells. The data further suggest that ALL cases do not accurately represent cells arrested at the stage where the leukemogenic event occurred. Rather, ALL appears to be a disease in which there may be maturation of leukemic blasts; but this maturation is "asynchronous" when compared with the normal developmental process.

DOMINANT concept of leukemogenesis in acute lymphoblastic leukemia (ALL) is based on the premise that the abnormal cells originate from normal counterparts at some stage of lymphopoiesis; leukemic blasts somehow lose the ability to differentiate (maintaining the phenotype of the cell of origin) but retain (or increase) their proliferative potential.14 Multiple cases, however, have been reported in which the leukemic cells appear to have no detectable counterpart in the normal lymphoid differentiation scheme.15-17 There are documented cases of myeloid antigen expression in lymphoblastic leukemias, lymphoid cell-surface marker expression in nonlymphoid leukemia, T-cell-associated marker expression in B lineage ALL, and B-cell-associated antigen expression in T-cell ALL.

One explanation of this apparent discrepancy between normal and leukemic phenotypes, termed "lineage infidelity," suggests that "aberrant" leukemic cell phenotypes reflect abnormal differentiation programs within the leukemic cell.15 It is proposed that inappropriate expression of genes in the leukemia results in expression of cell surface markers in combinations not normally observed.

An alternative view, "lineage promiscuity," argues that a leukemia cell arises from an early stem cell that normally may transiently exhibit traits of more than one lineage.18 Thus, leukemias might express phenotypes of normal progenitor cells that are extremely rare in normal bone marrow. In this model leukemia could still be thought of as a disease of maturation arrest at the stage of the cell of origin.

The qualitative expression of many B-cell-related antigens on normal marrow B cells has been characterized by several groups.19-22 Recently, our group refined these models by determining quantitative surface-marker expression of developing normal human bone marrow B cells.20 In our model normal B cells were divided into four stages based on cell-surface markers. The most immature, identifiable, committed B-cell precursor (stage I) was defined by the cell-membrane expression of CD34, CD19, HLA-DR, and high-intensity expression of CD10 ("CD10+ +"). Intranuclear terminal deoxynucleotidyl transferase (TdT) activity also consistently marked the stage I immunophenotype. Other groups have also described the early appearance of cytoplasmic CD22 in TdT+ B-cell precursors, although cell surface CD22 expression was found to occur only in the most mature stage of B-cell differentiation.23 Stage II of B lymphoid development began as CD34 was lost, and CD10 intensity decreased (from "CD10++" to "CD10+."). CD19 and HLA-DR, however, were found to be expressed throughout B-cell maturation.24 CD20 became detectable as CD10 expression was lost from the cell surface; these two concurrent events identified stage III of B lymphocyte development. Once CD10 was no longer detected, additional surface markers, such as CD22, were expressed on the cell surface, defining stage IV of B lymphoid maturation.

To more clearly define the origin of B lineage leukemias, we compared the phenotypes of 113 acute B lymphoblastic leukemias with this map of B lymphoid differentiation in normal bone marrow. In these comparisons we attempted to assess the developmental stages at which the leukemias were

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Frozen but instead observed frequent discordance in the immunophenotypes of leukemic vs normal B lymphoid cells.

MATERIALS AND METHODS

Patients and leukemic samples. Heparinized bone marrow was collected from leukemic patients before administration of chemotherapeutic agents. Acute leukemia was diagnosed in all these patients by standard clinical features, including blast cytologic and cytochemical features, according to the French-American-British (FAB) criteria. One hundred and thirteen newly diagnosed childhood and adult acute leukemia case specimens, either from The Johns Hopkins Oncology Center or from (by overnight mail) Pediatric Oncology Group institutions, were selected from all cases immunophenotyped at The Johns Hopkins Oncology Center between January 1985 and July 1987. Cases were selected for use in this study only if cells were positive for HLA-DR and at least one of the following B lymphoid specific markers: CD19, CD20, or CD22.4,21,22,23,29,31

Several cases included in this analysis were not evaluated for each of the antigens listed above. All 113 selected cases, however, were tested for a minimum of four of the six antigens under consideration.

Monoclonal antibodies. Unconjugated, fluorescein (FITC)-conjugated and/or phycoerythrin (PE)-conjugated anti-HLA-DR, anti-Leu-12 (CD19), anti-common acute lymphoblastic leukemia antigen (CALLA) (CD10), anti-Leu-16 (CD20), and anti-Leu-14 (CD22) were obtained from the Becton Dickinson Monoclonal Center (Mountain View, CA). The anti-CD22 used was derived from the clone SHC1-1.27 Rat anti-mouse IgG1-FITC, IgG1-PE, and IgG2a-FITC were the kind gifts of Dr D. Buck (Becton Dickinson Monoclonal Center). Unconjugated anti-MY10(CD34), and MOPC-21 (an IgG1 irrelevant control myeloma protein) were used as culture supernatants, as previously described.24,25

Cell preparation and immunofluorescence assays. Leukemic cells were enriched from bone marrow samples by Ficoll-Hypaque (FH) density gradient centrifugation, then immunophenotyped by one-color indirect immunofluorescence using a Spectrum III (Ortho Diagnostics, Raritan, NJ) flow cytometer, as described.29 The flow cytometer was standardized daily using glutaraldehyde-fixed chicken erythrocytes following routine procedures. The criterion for cell-surface marker positivity was set at expression by a minimum of 20% (above control background staining) of the gated cells.41 The mean fluorescence intensity (MFI) of the CD10+ cells was also determined, i.e., CD10+ samples (>20% of cells CD10+) were further classified as "CD10+" if the MFI was ≤110 and "CD10+" if the MFI was <110.

On certain specimens double-label immunofluorescence analyses were also performed using cells that had been cryopreserved at -135°C in RPMI 1640 containing 20% fetal bovine serum (FBS) and 10% dimethyl sulfoxide. Double-label studies using unconjugated antibodies (such as CD34) and directly conjugated antibodies were performed as previously described,22,25 and were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). A minimum of 12,000 events were collected for each sample using FACScan Research Software (Becton Dickinson). This software was also used to identify leukemic cells by forward and right-angle light scatter and then to correlate expression of two fluorescent labels on these cells.

Statistical analysis. Expression of paired antigens on individual specimens was compared using Pearson correlation coefficients.

RESULTS

Frequency of CD34, CD19, CD10, CD20, and CD22 expression in B lineage ALL. One-color immunofluorescence was performed on the "gated" blast population after FH density centrifugation (as described in "Methods"). A cell-surface marker was considered positive providing it was present on ≥20% of the leukemia cells. The acute leukemia cases were operationally defined for analysis in this study as "B lineage ALL" if they were HLA-DR + and positive for at least one B-cell-specific antigen (CD19, CD20, or CD22; see "Methods").

All 113 B lineage leukemias were tested for CD19. One hundred eleven (98%) were CD19+ (similar to reported values.24) One hundred nine cases of the original 113 were assayed for CD34 expression, and 73 were CD34+ (67%). From these data alone a majority of cases exhibited an "immature" B lineage phenotype (CD19+,CD34-).

One hundred six of the B lineage leukemias were studied for CD10, and 80 cases (76%) were CD10+. These CD10+ cases were divided into two groups, based on intensity of CD10 expression, in an effort to approximate the stages of normal B-cell differentiation. In normal marrow intensely labeled "CD10+" B cells were shown to be even less mature than less intensely labeled "CD10+" B cells.22,24 MFI for the entire subset of leukemic samples expressing CD10 ranged from 14 to 245, with a median of 110. No discrete interruption of continuity was noted between bright and dim CD10 MFI (Fig 1D); therefore MFI ≤ 110 was arbitrarily chosen as a cutoff so that 50% of samples expressing CD10 were "CD10+" (MFI ≤ 110), and 50% were "CD10+" (MFI ≥ 110).

Both CD20 and CD22 are expressed late in normal B lymphoid maturation.5,7,22,23,24,26,27,29,31 CD20 was assayed on 94 of these B lineage leukemia cases. Of these, 36 were CD20+ (38%). CD22 was examined on 92 patient samples, of which 60 were CD22+ (65%). These data are summarized in Table 1 and Fig 1.

Correlations between CD34, CD19, and CD22 expression on B lineage leukemias. In assessing the cell-surface antigen expression of normal B lineage cells, the progenitor cell marker, CD34, was never detected on cells expressing the
Table 1. Percentages of Cases Expressing B-Cell-Associated Antigens

<table>
<thead>
<tr>
<th>Cell-Surface Marker</th>
<th>% Cases Positive*</th>
<th>Mean % Antigen Expression†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DR</td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td>CD19</td>
<td>98</td>
<td>73</td>
</tr>
<tr>
<td>CD34</td>
<td>67</td>
<td>62</td>
</tr>
<tr>
<td>CD10</td>
<td>76</td>
<td>71</td>
</tr>
<tr>
<td>CD20</td>
<td>38</td>
<td>50</td>
</tr>
<tr>
<td>CD22</td>
<td>65</td>
<td>56</td>
</tr>
</tbody>
</table>

*Percentages were calculated as the number of positive cases (≥20% antigen-positive cells in that case) for a particular antigen divided by the total number of samples tested for that antigen.
†Values represent the sum of the % antigen expression in each antigen positive case (≥20% expression) divided by the total number of antigen positive cases.

Fig 2. Correlations between CD34, CD20, and CD22 expression. Ninety cases were tested for (A) CD34 ± CD22, (B) CD34 ± CD20, and (C) CD20 ± CD22. Percent antigen expression on patient leukemia cells is depicted on the ordinate. Cases are ordered by decreasing percent CD34 + cells in Fig 2A and B and in ascending order of CD20 + cells in Fig 2C (hatched bars), since CD34 decreases early in normal B-cell development, and CD20 increases during normal B-cell maturation. Below the hatched bars (indicating the percent CD34 + cells in Fig 2A and 2B) and CD20 + cells in Fig 2C) is the corresponding percent of the paired antigen-positive cells for that same case (clear bars). Because CD20 and CD22 are late B-cell markers, it was expected that CD34 expression would correlate inversely with CD20 and CD22 expression and that the two late markers (CD20 ± CD22) would show a positive correlation.

late antigens CD20 and CD22.22,36,44 The correlations between expression of CD34 and either CD20 or CD22, then, was expected to divide the B lineage leukemia cases into relatively mature and relatively immature groups.

Ninety of the total 113 B lineage leukemia samples were tested (one color) for both CD34 and CD22. The correlation between expression of these two cell-surface markers is shown in Fig 2A and the data summarized in Table 2. From Fig 2A there is no correlation between cellular expression of CD34 and CD22 (correlation coefficient = 0.31). In fact, using the customary 20% threshold for positivity (see “Methods”), 47 of the 90 patient samples (52%) were CD34 +,CD22 +. In 18 of these cases (20% of the 90 cases) the sum of the % CD34 + cells plus the % CD22 + cells totaled >100%. This suggested simultaneous expression of CD34 and CD22 on at least some of the leukemia cells in (at least) these cases, despite the fact that coexpression of these two antigens was not found during normal B-cell development.22,36

Ninety samples of the original 113 patients were analyzed for both CD34 and CD20. As shown in Fig 2B, there was no correlation between CD34 expression and expression of the late antigen, CD20 (correlation coefficient = −0.19). Nineteen patient samples (21%) were CD34 +,CD20 + (Table 2). In eight of these case samples (9% of the 90 cases) the sum of the % CD34 + cells plus the % CD22 + cells totaled >100%, indicating simultaneous expression of CD34 and CD20 on at least some of the leukemia cells, although coexpression of these two antigens was not observed in normal B-cell maturation.22,36,44

Both CD20 and CD22 expression were assessed on 90 B lineage ALL specimens. No correlation between CD20 and CD22 (both late B-cell antigens) expression was found (Fig

Table 2. Antigen Expression in B Lineage ALL as Determined by Single-Color Immunofluorescence

<table>
<thead>
<tr>
<th>Paired Antigen Combinations</th>
<th>CD34, CD22</th>
<th>CD34, CD20</th>
<th>CD20, CD22</th>
</tr>
</thead>
<tbody>
<tr>
<td>+, +</td>
<td>52† (105)</td>
<td>21 (9)</td>
<td>30</td>
</tr>
<tr>
<td>+, −</td>
<td>19</td>
<td>48</td>
<td>6</td>
</tr>
<tr>
<td>−, +</td>
<td>13</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>−, −</td>
<td>16</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>CD10, CD34, CD10, CD20, CD10, CD22</td>
<td>23 (12)</td>
<td>17 (12)</td>
<td>2 —</td>
</tr>
<tr>
<td>+, −, +</td>
<td>14 (14)</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>+, +, +</td>
<td>25 (12)</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>−, +, +</td>
<td>15</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>−, −, +</td>
<td>17</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>−, −, −</td>
<td>6</td>
<td>17 (17)</td>
<td>13</td>
</tr>
</tbody>
</table>

*The symbol ‘+’ represents ≥20% expression of the antigen in the order listed in the column heading.
†Percent of cases displaying this phenotype, based on number of cases assessed for the two antigens in question (using one-color immunofluorescence). Italic values are percentages of antigen combinations that are not normally found in normal B-cell maturation.
‡Cases expressing CD10 (≥20% of cells positive) were subdivided on the basis of fluorescence intensity into “CD10 + +” (MFI ≥ 110) and “CD10 + −” (MFI < 110).
§Percent of cases in which the sum of the % positive (or negative) cells for the two antigens was >100%. Therefore, both antigens in these samples must be expressed on at least some of the same cells simultaneously.
Correlation between CD10 and CD22 expression. Among normal marrow B lineage cells, CD10 was expressed early in the maturation sequence, while CD22 appeared only on the most mature cells. Ninety-two cases were tested for both CD10 and CD22 (Fig 3 and Table 2). No correlation between cellular expression of the two antigens was found (correlation coefficient = 0.21), even though a negative correlation was expected. Fifty patient samples (54%) were CD10+, CD22+. Simultaneous expression of CD10 and CD22 occurred on at least some cells in a minimum of 12% of the cases (in these cases the sum of the % positive cells for both antigens totaled >100%), although these markers were not coexpressed during normal B-cell development.

Correlation between intensity of CD10 expression and percent CD34 and CD20 expression. The most immature B lineage cells in normal marrow expressed high levels of CD10 (“CD10+”) compared with the slightly more mature “CD10+” cells.

CD10 intensity was first correlated on each specimen with the percent CD34 expression for that sample: CD10 intensity was not found to be correlated with percent CD10+ cells (data not shown). The leukemia cases expressing CD10 were then ordered according to (decreasing) CD10 MFI, and the % CD34+ cells was plotted for each case (Fig 4A). No correlation (correlation coefficient = -0.05; Table 2) was found between CD34 expression and CD10 MFI. Twenty-three patients (23%) were CD34+, “CD10+,” as are stage I normal B cells. Seventeen percent were CD34-, “CD10+,” although this phenotype was not observed in normal marrow.

In addition, 25% were CD34+, “CD10+.” In 12 of these cases (12% of the 100 CD10+ cases tested for CD10 MFI and CD34) the sum of the % CD34+ cells plus the % “CD10+” cells totaled >100%. This suggested simultaneous CD34+, “CD10+” expression on at least some of the cells, even though this phenotype was not detected on normal marrow cells.

Eighty-eight patient samples were tested for both CD10 and CD20. No relationship between the CD10 MFI and CD20 expression was demonstrated (Fig 4B; correlation coefficient = -0.13), although in normal marrow only early B cells were “CD10+,” while CD20 was expressed only on more mature B cells. Seventeen percent of the cases (Table 2) were “CD10+,” CD20+ (a minimum of 12% coexpressed both antigens). Fifteen cases (17%), however, were CD10-..CD20-., even though this phenotype was never seen among normal developing B cells. (Note: all normal CD19+ marrow B cells expressed either CD10 or CD20 or both.)

Double-label immunofluorescence studies on B lineage ALL. Expression of normal B-cell differentiation antigens was found on a large percentage of leukemic samples in combinations not seen in normal human marrow, as determined by the above paired comparisons of surface-marker expression based on single-color immunofluorescence assays. As shown in Table 2, however, not all of these cases had high enough percents of cells expressing, eg, both a late and an early antigen, to be certain that the two antigens were coexpressed on individual leukemic cells. To determine directly whether individual leukemic cells simultaneously coexpressed pairs of antigens not coexpressed on normal marrow cells, double-label immunofluorescence assays were performed on eight (cryopreserved) patient samples. Blast cell counts on the thawed samples ranged between 85% and 100%, and forward- and right-angle scatter analyses demonstrated that blast populations varied only mildly in size and granularity. Additional selective flow cytometric “gating” was performed on each sample to further eliminate nonleukemic cells from the analysis. In each case studied, combina-
Table 3. Percentages of Antigen Combinations Not Found in Normal Marrow on B Lineage ALL as Determined by Double-Label Immunofluorescence

<table>
<thead>
<tr>
<th>Patient Sample</th>
<th>CD34+ ,CD20+</th>
<th>CD34+ ,CD22+</th>
<th>CD10− ,CD20−</th>
<th>CD10+ ,CD22+</th>
<th>CD20− ,CD22+</th>
<th>CD19</th>
<th>CD20</th>
<th>CD22</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>95</td>
<td>90</td>
<td>86</td>
<td>0</td>
<td>92</td>
<td>5</td>
<td>99</td>
<td>96</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
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<td>0</td>
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<td>3</td>
<td>98</td>
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<td>16</td>
<td>84</td>
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<td>79</td>
<td>50</td>
<td>94</td>
<td>98</td>
<td>94</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>0</td>
<td>88</td>
<td>0</td>
<td>48</td>
<td>97</td>
<td>97</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>85</td>
<td>15</td>
<td>39</td>
<td>ND†</td>
<td>ND†</td>
<td>48</td>
<td>10</td>
<td>42</td>
</tr>
<tr>
<td>7</td>
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<td>10</td>
<td>5</td>
<td>75</td>
<td>35</td>
<td>95</td>
<td>55</td>
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<tr>
<td>8</td>
<td>94</td>
<td>17</td>
<td>89</td>
<td>27</td>
<td>59</td>
<td>69</td>
<td>98</td>
<td>20</td>
</tr>
</tbody>
</table>

*Antigen pairs listed are only those not normally seen in normal marrow. Values represent the percent of cells from the total leukemic population coexpressing the antigen pair in question. Totals are >100%, because more than one phenotype was observed on the same case.
†Values are percent of blasts in a 100-cell differential count after Ficoll centrifugation.
†ND, not determined.

Six of the eight cases were >95% positive for CD19, or another B lineage-specific antigen. Every (selected) case studied demonstrated simultaneous expression (on the same leukemia cells) of antigens not expressed together on normal marrow B cells (eg, CD34+,CD20+ and/or CD22+; CD10+,CD22+; CD10−,CD20−; CD20−,CD22+; Table 3). The percentages of cells expressing such antigen combinations varied from case to case, but at least one example of a phenotype not detected in normal marrow was found on individual cells in every (selected) patient sample examined, as illustrated by the case in Fig 5. Double-label immunofluorescence assays of this leukemic sample demonstrated CD34+,CD20+ cells in 89% of the entire population; CD34+,CD22+ cells in 85%; and CD10+,CD22+ cells in 92% of the leukemic population.

DISCUSSION

In the present report 113 B lineage ALL marrow samples were tested with a series of B-cell–associated and B-cell–restricted monoclonal antibodies. Each case selected for this study had to satisfy the criteria of expression of HLA-DR and at least one B-cell–specific antigen: CD19, CD22, and/or CD20. These latter three cluster designations have been shown to be restricted to B lymphocyte development. A few cases that were negative for these three antigens were (intentionally) excluded from this study by this strict definition of B lineage ALL, even though B lineage ALL might have been suspected clinically. For example, rare patients whose leukemia cells expressed only B-cell–associated antigens (but no B-cell–specific antigens) with phenotypes such as HLA-DR+,CD34+,CD10+,CD19−,CD20−,CD22−; or HLA-DR+,CD34+(or CD34−), CD19−,CD10−,CD20−,CD22− were excluded from this analysis to avoid including possible acute nonlymphocytic leukemias.

Pairwise expression of B lineage antigens was examined for each leukemic sample to compare antigen expression in B lineage leukemia with antigen expression in normal marrow B-cell differentiation. For example, the expected (negative) correlation between CD34 and CD22 expression was not observed (Fig 2A). Instead, these two antigens were coexpressed in 52% of B lineage leukemia cases (Table 2), although in normal B lymphoid maturation, CD34, the earliest B-cell–associated antigen, and CD22, a late B-cell antigen, were never expressed simultaneously on the same cells.

CD34 and CD20 expression were examined next. Again, in contrast to normal B lymphocyte differentiation (where no
cells were found to coexpress the CD34 progenitor cell antigen and the late B cell differentiation antigen, CD20\textsuperscript{22,26,36}, CD34 and CD20 expression were not (negatively) correlated (Fig 2B). Twenty-one percent of the specimens were CD34\textsuperscript{+},CD20\textsuperscript{+} (Table 2).

Expression of CD20 and CD22 (two mature B cell differentiation antigens) were similarly studied. Although cytoplasmic CD22 expression can be detected in very early (TdT+) B cell precursors,\textsuperscript{21,23} normal maturing marrow B lymphocytes express cell surface CD20 prior to surface CD22.\textsuperscript{22,26} In contrast, cell/surface expression of CD22 was found in 65% of the B lineage leukemias, while only 38% of cases expressed CD20 (Table 2). Furthermore, we expected that CD20 and cell surface CD22 expression would be related, tending to be expressed together on the most "mature cases." However, there was no correlation in the expression of the two antigens (Fig 2C).

The most mature B cell differentiation marker studied, cell surface CD22, was not coexpressed with CD10 in normal marrow.\textsuperscript{22,26} However, 53% of the B lineage ALL samples tested for CD10 and CD22 were positive for both surface markers (Fig 3 and Table 2).

The CD10 + B lineage ALL samples were then compared for CD10 MFI v percent CD34 + cells (Fig 4A). In normal marrow, intense CD10 expression ("CD10 + +") was found on a subset of CD34 + cells, but CD10 was expressed at a low intensity or was negative on more mature (CD34 -) B lymphoid cells.\textsuperscript{22,24} Unexpectedly, however, bright and dim CD10 fluorescence was randomly associated with CD34 expression in these ALL cases.

CD10 intensity was similarly compared with CD20 expression (Fig 4B). During normal B cell maturation, CD20 expression became detectable as CD10 expression declined.\textsuperscript{22} In the leukemic cases, however, CD20 was expressed on leukemic samples expressing CD10 at high as well as low intensities.

These pairwise comparisons of B cell antigen expression on B lineage ALL samples in all combinations did not map with normal B lymphocyte maturation. Instead, the leukemic expression of normal B cell differentiation markers was "asynchronous."\textsuperscript{17,47-49} Complete immunophenotypes of individual leukemic cases were also examined to estimate the frequency of this "asynchronous" surface marker expression in B lineage ALL (Fig 6). Complete immunophenotypes were obtained in 83 (73% of the total) patient samples. Twenty-two different phenotypes were observed in this population (a total of 28 surface marker combinations were theoretically possible). Of the B lineage patients defined and studied, only two were CD19 - (2%): one patient had the phenotype HLA-DR\textsuperscript{+},CD19\textsuperscript{-},CD34\textsuperscript{+},CD10\textsuperscript{-},CD20\textsuperscript{-},CD22\textsuperscript{-}; the other patient sample was HLA-DR\textsuperscript{+},CD19\textsuperscript{-},CD34\textsuperscript{-},CD10\textsuperscript{+},CD20\textsuperscript{-},CD22\textsuperscript{-}. CD20 and/or CD22 were the B lineage-specific features of the leukemias, but CD34 expression was not found on normal cell surface CD22 + cells (as seen in case 1), nor was cell surface CD22 normally expressed prior to CD20 in normal B cell development (as seen in case 2). In addition, all cell surface CD22 + (and CD20 +) normal B cells were shown to be CD19 +.\textsuperscript{22,26}

Of the 83 complete phenotypes studied, 23% fit stage I of normal B cell development to the extent that they were HLA-DR +,CD34 +,CD19 +,CD10 + +. However, 11% of the 83 specimens were HLA-DR +,CD34 +,CD19 +,CD10 + +,CD20 +,CD22 +, and 10% were HLA-DR +,CD34 +,CD19 +,CD10 + +,CD20 - ,CD22 -. These samples deviated from B cell differentiation because CD20 and cell surface CD22 were not expressed on normal CD34 +,CD10 + + "B lineage cells."\textsuperscript{22,24,36,44}

Another 27% of the leukemic specimens had subtly abnormal phenotypes (using the MFI $\geq$110 threshold for CD10 + cells) in that they were HLA-DR +,CD34 +,CD19 +,CD10 + +, even though CD10 is normally expressed at a high MFI ("CD10 + +") on CD34 + marrow B lineage

Fig 6. Schematic representation of the complete phenotypes observed in the B lineage ALL population. Eighty-three of the original 113 patient samples were tested for all six surface antigens (HLA-DR, CD34, CD19, CD10, CD20, and CD22) and are included in the analysis. Antigens are ordered as they occur in normal B lymphocyte maturation. The percent of cases expressing each surface antigen are in parentheses. Underlined values represent the percent of cases whose phenotypes were not found in normal marrow. Below this tree diagram the antigenic expression of the four stages of normal B cell development are represented, as described by Loken et al.\textsuperscript{20}
precursors. Furthermore, 8% of the samples were HLA-
DR+,CD34+,CD19+,“CD10+”,CD20+,CD22+, and another 14% were HLA-DR+,CD34+,CD19+,“CD10+”,
CD20−,CD22+, thus demonstrating additional “asynchrony” of B-cell surface antigen expression, because CD34 and the late differentiation antigens CD20 and cell surface CD22 were not expressed simultaneously on normal B-cell precursors. Another 18% of the population were HLA-DR+, CD34+,CD19+,CD10−, but in normal B lymphoid development, CD34+ cells expressed CD19 and CD10 simultaneously. Seven percent of the total cases exhibited a second deviation from normal differentiation in that they expressed early and late surface markers (2% were HLA-
DR+,CD34+,CD19+,CD10−,CD20+,CD22+, and 5% were HLA-DR+,CD34+,CD19+,CD10−,CD20−, CD22++; Fig 6).

The remaining 25% of the cases tested were HLA-DR+, CD34+,CD19+ and might be considered to represent later stages of B-cell maturation. Eight percent of these specimens were HLA-DR+,CD34−,CD19+,“CD10+”, but high-intensity CD10 expression (“CD10++”) was found only on normal B lineage precursors that were CD34+. Four percent of the cases were HLA-DR+,CD34−,CD19+,“CD10+” and appeared to correspond to this extent to stage III of normal B-cell development; but these samples were also cell-surface CD22+, even though CD10 and cell-surface CD22 were not coexpressed during normal B-cell maturation (Fig 6). In addition, one case had the phenotype HLA-DR+, CD34−,CD19+,CD10−,CD20−,CD22−, which could not be placed into a stage of B-cell development because of an absence of B lineage differentiation markers (other than CD19).

Distinction between “CD10++” and “CD10+” MFI was somewhat arbitrary. In normal marrow a definite association between CD10 MFI and B-cell differentiation was found.22,43 High CD10 MFI ("CD10++") was found on the early CD34+ cells, whereas "CD10+" expression denoted a more mature B-cell precursor (CD34−,"CD10++",CD20+). As noted from Fig 4A and 4B, however, there was no correlation between CD10 MFI and expression of CD34 or CD20 in the B lineage leukemia samples. If MFI is not considered in the analysis, then 6% of the samples that expressed both CD34 and CD10 (and were CD20−,CD22−) could be considered to follow a normal differentiation pattern. The remaining leukemia samples, however, remain unchanged in this analysis and represent 78% of the entire cases examined. In addition, 18% of these “asynchronous” phenotypes had more than one abnormality in cell-surface antigen expression. If other stage-restricted B lineage markers had been included in this analysis, a larger percentage of cases would have conceivably demonstrated “asynchronous” differentiation. For example, TdT activity is present predominantly in stage I of normal B-cell development (CD34+,CD19+,“CD10+”),22,36 whereas TdT is known to be expressed in almost all cases of B lineage ALL.30

To demonstrate directly that individual leukemic cells expressed both late and early antigens simultaneously, several (selected) cryopreserved samples were examined by double-label flow cytometry. After thawing and Ficoll centrifugation, all samples had >85% blast cells, as determined morphologically. Each of the cases analyzed contained high percentages (50% to 92%) of individual cells with simultaneous expression of both early and late B-cell antigens (Fig 5, Table 3).

Nadler et al previously deduced the stages of B lymphocyte development by evaluating the expression of HLA-DR, CD10, CD19, and CD20 (but not CD34, or CD22) in non-T-cell ALL.5 In that study the cases were divided into the following four subgroups, based on surface-marker expression: (1) HLA-DR+,CD19−,CD10−,CD20−; (2) HLA-DR+,CD19+,CD10−,CD20−; (3) HLA-
DR+,CD19+,CD10+,CD20−; and (4) HLA-
DR+,CD19+,CD10+,CD20+. Group I represented the most immature phenotype and group 4 the most mature. The percentage of cases in our study that fell into Nadler’s stages 2 through 4 did not differ significantly from his findings (Table 4). However, by the addition of CD34 and CD22 to the analysis of these cells, we found that the majority of B lineage ALL specimens had detailed phenotypes that were not found during normal B lymphocyte development. One further difference between Nadler’s model of B-cell differentiation (based on leukemia phenotypes) and our model (based on normal bone marrow) was Nadler’s conclusion that CD19 preceded CD10 expression. As previously discussed in our prior study, we found no detectable CD19+CD10− cells.

The unusual immunophenotypes in our B lineage ALL population could represent rare normal lymphoid cells that have not previously been described in bone marrow because of the inability of current technology to detect such rare cell populations. In lymphoid organs other than marrow and/or

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<th>Table 4. Phenotypes of Two B Lineage ALL Populations Defined by Ordered Expression of HLA-DR, CD19, CD10, and CD20</th>
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<td>Phenotype</td>
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<td>(I) HLA-DR+,CD19−,CD10−,CD20−</td>
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<td>(II) HLA-DR+,CD19+,CD10−,CD20−</td>
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<td>(III) HLA-DR+,CD19+,CD10+,CD20−</td>
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<td>(IV) HLA-DR+,CD19+,CD10+,CD20+</td>
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*Ninety-two of the original 113 patients were assessed for all four antigens; however, ten patients did not conform to the above phenotypes and were therefore not included in the analysis. These phenotypes included HLA-DR+,CD19+,CD10−,CD20+ (nine patients) and HLA-DR+,CD19−,CD10+,CD20+ (one patient). Six patients from Nadler’s study were excluded in the same manner.

†Nadler’s cases were defined as “B lineage” using different criteria. For example, cases with the phenotype (HLA-DR+ only) were excluded from our study.
during periods of rapid proliferation (eg, regenerating marrow, reactive lymph nodes), however, such cells may indeed exist.

With these reservations the observations in this study likely imply that B lineage ALL is not a disease paradigm of maturation arrest. Somewhat similar conclusions were also made by Ryan et al., who showed that CD20 and TdT were erratically expressed in CD10 + ALL. If cell surface phenotypes of B lineage ALL do not correlate with the developmental stages of normal B lymphocyte differentiation, separation of these leukemias according to maturational stage may be impossible. Possibly, prognosis in B lineage ALL may be related more to a deviation from normality than to previously described maturational differences.

These findings may further suggest that B lineage ALL is a disorder arising from a cell at stage I of B lymphoid development. High levels of TdT activity are found at this stage, making it likely to also be the point at which immunoglobulin gene rearrangement occurs. Concurrent alternations in genetic material plus a high proliferation rate in this early stage might make this cell particularly vulnerable to continuing "genetic misregulation," resulting in leukemias exhibiting abortive differentiation with "asynchronous" antigenic expression.

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