Relationship Between T200 Antigen Expression and Stages of B Cell Differentiation in Resurgent Hyperplasia of Bone Marrow

By Charles W. Caldwell and William P. Patterson

Using monoclonal antibodies (MoAbs) and dual-parameter flow cytometric techniques, bone marrow mononuclear cells (MMC) from patients with resurgent hyperplasia were analyzed for their coexpression of HLe-1 (T200) and antigens normally associated with particular stages of B cell differentiation. The marrow from those with resurgent hyperplasia contained increased numbers of B cell precursors in multiple stages of differentiation compared to controls, thus providing a useful model system for studies of B cell differentiation. These studies indicate that the quantitative expression of T200 is differentiation-related on normal and malignant B cells and B cell precursors.

PREVIOUS STUDIES have shown that lymphoblasts of patients with acute lymphoblastic leukemia (ALL) as well as cells of patients with other lymphoid malignancies exhibit decreased expression of HLe-1, an epitope of the T200 antigen. During these studies it was noted that in some marrow specimens following chemotherapy and in marrow specimens of patients with nonmalignant reactive processes, dim fluorescence peaks were present in addition to the normally bright peaks after staining with anti-HLe-1 (T200) monoclonal antibody (MoAb). In many of the cases of ALL in which these peaks were seen, the patients entered complete remission after therapy. These observations raised the possibility that these dim peaks of T200 were associated with resurgence of normal, early B cells in bone marrow recovering from the bone marrow, it is not surprising that multiple subpopulations might be demonstrable in this tissue. This paper represents an attempt to evaluate this possibility by analysis of bone marrow mononuclear cells (MMC) for their coexpression of HLe-1 (T200) and antigens normally associated with particular stages of B cell differentiation.

MATERIALS AND METHODS

Study population. Bone marrow was obtained from children (ages 3 to 15) undergoing diagnostic evaluations for various suspected or documented disease processes. These included two patients (each on two separate occasions) with cyclic neutropenia, one following chemotherapy for a T cell ALL, two with immune thrombocytopenia, three with neuroblastoma, one with Ewing's sarcoma, and one with hepatitis B. Tumor was not found in any of the bone marrow specimens at the time of study. Bone marrow aspirates used as controls were obtained from children (ages 4 to 12) undergoing routine clinical monitoring for previously diagnosed and treated ALL (n = 10). All these children were receiving maintenance chemotherapy, and all were considered to be in remission of their leukemia at the time of examination and on follow-up 6 months later. Peripheral blood was collected from healthy individuals aged 2 to 20 years (n = 50). Specimens were collected after informed consent in accordance with guidelines established by the Institutional Review Board of the University of Missouri School of Medicine.

Preparation of specimens for analysis. Peripheral blood and bone marrow were aseptically collected by standard techniques. The peripheral blood lymphocytes (PBL) and MMC were enriched by density gradient centrifugation as previously described. PBL and MMC were adjusted to 1 x 10^7 cells/mL in RPMI 1640 medium and analyzed within two hours of receipt. Bone marrow smears from aspirated cells were visually examined after staining with May-Grünewald Giemsa. The marrow was also examined for the presence or absence of malignancy in fixed tissue preparations of clotted marrow aspirate or needle-biopsy core sections.

MoAbs and immunostaining. The specificities and sources of MoAbs used in this study are listed in Table I. Several of these MoAbs were available as both fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labeled reagents. The anti-immunoglobulin reagents and anti-T200 MoAbs were unconjugated and therefore used in indirect methods of staining. The MoAb J-5 ( Coulter Electronics, Hialeah, FL) was labeled with RD-1, which is similar to PE in its excitation and emission spectra. A panel of antitymoid MoAbs was also used in some cases to determine the level of myeloid contamination of the light scatter peaks (data not shown). Aliquots (50 μL) of the cell suspensions (1 x 10^7 cells/mL) and 50 μL of FITC-labeled MoAbs (at saturating concentrations) were incubated at 4°C for 30 minutes and the cells analyzed with the flow cytometer. A monoclonal FITC-labeled isotypic negative control was analyzed in parallel with each specimen. Previous work has shown the reproducibility and stability of this "no-wash" staining procedure.

For dual-parameter staining, aliquots of 50 μL of cell suspension were added to 50 μL of FITC-labeled MoAb and 50 μL of PE (or RD-1)-labeled MoAb. After incubation at 4°C for 30 minutes, the cell suspension was washed twice with cold phosphate-buffered saline (PBS)/azide, resuspended in 0.5 mL of cold buffer, and submitted to flow cytometric analysis.

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The anti-T200 MoAbs were used in an indirect method of immunostaining. Aliquots (50 μL) of cells were first incubated with saturating concentrations of each unlabeled MoAb, washed twice in PBS, and aliquots (100 μL) of FITC-labeled sheep antimouse immunoglobulin (Fab' fragments) were added at saturating concentrations. After a 30-minute incubation at 4°C, the cells were again washed twice in PBS and submitted to flow cytometric analysis.

**Flow cytometric analysis.** An EPICS V flow cytometer (Coulter Electronics) was operated at approximately 500 mW in the 488-nm line. The FITC signal was filtered through a 515-nm long-pass filter. Standardization of forward-angle light scatter (FALS) and log integral green fluorescence (LIGFL) signals were performed as described.11,13

For demonstration of antigen expression, cells were analyzed by passing the fluorescence signal through a 560-nm dichroic mirror to split the beam for red fluorescence (collected through a 570-nm long-pass filter) and green fluorescence (collected through a 525 ± 10-nm band-pass filter). The green and red fluorescence subtraction modules were set to eliminate spectral crossover of FITC- and PE-MoAb positive cells into the photomultiplier tube (PMT).

Single (and dual) parameter histograms were analyzed for percentages of positive cells using computer-assisted programs (INTEGRATE, IMMUNO, STATS; Coulter). Fluorescence intensity was determined from single- or dual-parameter histograms with computer assistance (STATS; Coulter).

**Immunoaalkaline phosphatase staining for cytoplasmic Mu heavy chains.** The percentage of cells expressing cytoplasmic IgM heavy chains (cIgM) was determined by immunostaining with MoAb against human IgM with the cells in suspension (surface IgM) and after fixation on slides (cIgM). Monoclonal anti-IgM (Coulter) was used with a commercial immunoaalkaline phosphatase kit (Vectorstain, Burlingame, CA).

**Immunoadsorption of cell populations.** In some experiments, certain cell populations were removed from the specimen by "panning" prior to analysis for HLe-1 and other markers. The panning procedure was performed as described.13

## RESULTS

**Bone marrow morphological changes.** Morphological evaluation of resurgent bone marrow specimens revealed various patterns, some with obvious mononuclear hyperplasia and others with nonspecific changes. Although the percentage of blasts was not increased in all specimens, there was usually an increase in lymphocytes, some mature and others less mature, having diffuse nuclear chromatin, scant cytoplasm, and indistinct nucleoli. Many of these lymphocytes are actually B cell precursors.14

**Single MoAb positivity.** The overall pattern of immunostaining in resurgent marrows suggests a "left shift" toward immature B cells and is similar to the pattern in fetal bone marrow,4 while control marrows are similar to adult patterns.5 There is a difference in the percentages of B cell precursor when resurgent marrows are compared with controls (Table 2). Control pediatric marrow contains mainly T lymphocytes (OKT11) with low numbers (<10%) of B cells and B cell precursors. The percentage of HLA-DR positivity is increased over the percentage of positivity with pan-B cell markers (Leu-12 and Leu-16) and is related to contamination.

### Table 1. Monoclonal Antibodies Used in This Study

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DR</td>
<td>Nonpolymorphic HLA-DR epitopes. Present on all early B cells and lost at plasma-cell stage.</td>
<td>B</td>
</tr>
<tr>
<td>Leu-12</td>
<td>Appears earlier than Leu-16 on pre-B cells and is lost early in plasma-cell differentiation (CD 19)</td>
<td>B</td>
</tr>
<tr>
<td>CALLA</td>
<td>Common acute lymphoblastic leukemia antigen, present on early pre-B cells, lost on sIg* cells (CD 10)</td>
<td>B</td>
</tr>
<tr>
<td>J-5</td>
<td>Anti-CALLA (CD 10)</td>
<td>C</td>
</tr>
<tr>
<td>Leu-16</td>
<td>Appears on early pre-B cells and is lost at plasma-cell stage (CD 20)</td>
<td>B</td>
</tr>
<tr>
<td>B2</td>
<td>CR 3, mature B cells (CD 21)</td>
<td>C</td>
</tr>
<tr>
<td>IgM</td>
<td>Mu heavy chains</td>
<td>C</td>
</tr>
<tr>
<td>IgG</td>
<td>Gamma heavy chains</td>
<td>C</td>
</tr>
<tr>
<td>Lambda</td>
<td>Lambda light chains</td>
<td>C</td>
</tr>
<tr>
<td>OKT11</td>
<td>E-rosette receptor on T cells (CD 2)</td>
<td>O</td>
</tr>
<tr>
<td>IL 2R</td>
<td>Interleukin 2 receptor (CD 25)</td>
<td>B</td>
</tr>
</tbody>
</table>

#### Anti-T200 Antibodies

<table>
<thead>
<tr>
<th>Anti-T200 Antibodies</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLe-1</td>
<td>B and T cells, granulocytes, monocytes (CD 45) (all T200 bands)</td>
</tr>
<tr>
<td>T29/33</td>
<td>Strong expression on B cells (gp 220) and T cells (gp 200)</td>
</tr>
<tr>
<td>F8-11-13</td>
<td>Strong staining of B cells, weak staining of a small subpopulation of thymocytes and T cells (gp 215)</td>
</tr>
<tr>
<td>F10-89-4</td>
<td>B cells stain weaker than T cells (gp 190-215)</td>
</tr>
<tr>
<td>G1-14.2</td>
<td>B and T cells, all four major gp bands (190-220)</td>
</tr>
<tr>
<td>G25-1</td>
<td>Similar to G14 in pattern of reactivity, but marks different epitope</td>
</tr>
</tbody>
</table>

**Abbreviations:** CD, Clusters of differentiation, as defined by the Second International Workshop on Human Leukocyte Differentiation Antigens.27-28 C, Coulter Immunology, Hialeah, FL; O, Ortho Diagnostic, Raritan, NJ; B, Becton-Dickinson, Mountain View, CA; M, Boehringer-Mannheim, Indianapolis.

* Biotinylated HLe-1 was a generous gift of Becton-Dickinson, Mountain View, CA.
† These were generous gifts of Dr R. Dalchau, Sussex, UK.24-25
‡ These were generous gifts of Dr J. Ledbetter, Seattle.26

### Table 2. Percentages of MoAb-Defined Subsets in Control and Resurgent Bone Marrow

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Control Marrow (n = 10)</th>
<th>Resurgent Marrow (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DR</td>
<td>*23.7 ± 6.7</td>
<td>67.7 ± 9.7</td>
</tr>
<tr>
<td>Leu-12 (CD 19)</td>
<td>8.1 ± 3.7</td>
<td>45.8 ± 9.1</td>
</tr>
<tr>
<td>CALLA (CD 10)</td>
<td>4.0 ± 2.6</td>
<td>48.3 ± 14.3</td>
</tr>
<tr>
<td>Leu-16 (CD 20)</td>
<td>6.9 ± 3.9</td>
<td>27.5 ± 14.5</td>
</tr>
<tr>
<td>cIgM†</td>
<td>4.9 ± 4.1</td>
<td>10.4 ± 9.1</td>
</tr>
<tr>
<td>B2 (CD 21)</td>
<td>6.3 ± 3.4</td>
<td>7.3 ± 2.4</td>
</tr>
<tr>
<td>sIg</td>
<td>5.1 ± 3.9</td>
<td>5.9 ± 4.1</td>
</tr>
<tr>
<td>OKT11 (CD 2)</td>
<td>73.1 ± 17.0</td>
<td>44.4 ± 15.6</td>
</tr>
<tr>
<td>IL 2R (CD 25)</td>
<td>3.0 ± 2.4</td>
<td>6.4 ± 5.1</td>
</tr>
</tbody>
</table>

*Numbers represent the mean percentage (± SD) of MoAb-positive cells from within the lymphoid light scatter gates.
†Percentages of cells positive for cIgM were determined from immunoaalkaline phosphatase (IAP) staining. Values represent the percentage of mononuclear cells after correction for myeloid contamination as determined using an antimonyloid cocktail in the IAP staining procedure.
tion with a few erythroid and myeloid cells (data not shown) and possibly early Leu 12- B cell precursors.8,10 Most of the B cells in control pediatric marrow are relatively mature, as demonstrated by the similarity in percentage of positivity with B2, Leu-16, and slg. A small percentage (<5%) of cells are positive for common acute lymphoblastic leukemia antigen (CALLA) and clgM, indicating small numbers of early B cells. Additionally, a few (3%) of the cells are positive for the IL 2R, suggesting at least a low level of T or B cell activation. These values are similar to those reported in normal adult marrow.5,9

In resurgent marrow, lower percentages of T cells and higher percentages of B cell precursors are present (Table 2). The HLA-DR positivity still exceeds the percentage of Leu 12+ cells. Compared to control marrow, marked increases are seen in clgM +, HLA-DR +, Leu-12+, CALLA +, and Leu-16+ cells. These MoAbs are present on B cell precursors. Percentages of B2+ and slg+ cells remain similar to controls, thus the majority of B cells present in resurgent marrow are at immature stages of development. A mild increase is also seen in IL 2R positivity, consistent with a modest degree of cellular activation. Dual marking of these cells demonstrated both IL 2R-positive T cells and B cells (data not shown).

Fluorescence intensity of single MoAb. Differences in the FI of cells from peripheral blood, control pediatric marrow, and resurgent marrow are apparent (Table 3 and Fig 1). In peripheral blood, which contains only mature B cells, a single peak of FI was present with each MoAb. In control marrow, single peaks were also found, with the exception of HLe-1. A few (three of ten) of the specimens from children with durable remissions of ALL and recovering from maintenance chemotherapy had small secondary peaks (<5% of cells) of dim HLe-1 FI that represented low numbers of B cell precursors. In all three of these cases, the FI of the secondary peak was significantly different than that of their ALL blasts at diagnosis. This type of HLe-1 staining has been found in the course of monitoring children during maintenance chemotherapy (data not shown). Nonmalignant marrow specimens from children and adults evaluated for iron deficiency anemia contain only one HLe-1 peak of FI (data not shown).

Resurgent specimens contained more than one peak of FI with all MoAbs except Leu-12, thus suggesting the presence of multiple subpopulations of cells. Although Leu-12 produced only one peak, it was dimmer than that observed in control marrow and peripheral blood. In the case of HLA-DR, the major peak was bright and corresponded to that seen in control marrow and blood. The dim HLA-DR peak consisted of T cells and immature B cells by dual marker studies (data not shown). The peaks of FI with CALLA were of a similar pattern in all resurgent specimens and differed from controls in that neither of the two peaks corresponded to the CALLA FI of control specimens. This is probably due to the low number of both cell types (dim and bright) that blend into a single histogram of average intermediate FI in control specimens. The major peak of CALLA in resurgent marrow was the dimmest, and the bright peak (not present in normal marrows) was a lower percentage of cells. Staining with Leu-16 also produced two peaks, a bright peak similar to that in control marrow and blood and a dim peak not seen in these specimens. The MoAb HLe-1 usually produced three distinctive FI peaks (and occasionally four) in the resurgent specimens. The brightest of these was similar to that of mature peripheral blood B and T lymphocytes, while the dimmer peaks were not seen in blood. An intermediate peak corresponded in FI to the small peak seen in some control marrow specimens, but the dimmest peak was not present in controls. Therefore not only are the number of FI peaks different from normal but also the quantitative FI of the peaks.

Relationship of HLe-1 to B cell-associated MoAbs. Figure 2 illustrates the relationship of HLe-1 (T200) FI to that of other B cell-associated (or T cell) MoAbs on subpopulations of cells from a representative resurgent specimen. In each histogram, regions identified as 1 to 4 are associated with increasing FI of HLe-1-stained cells. Multiple peaks of increasing HLe-1 FI (y-axis) are present from cells within the lymphoid region by FALS (x-axis). Cells from this specimen, identified by FALS (x-axis) and stained with HLe-1 (y-axis), demonstrate a wide range of FI levels, with varying numbers of cells in each of the four regions. The highest FI peaks are present in the lymphoid region, suggesting that a subset of B cells (or T cells) is stained with HLe-1. This subset shows a wide range of FI levels, indicating a mixture of cells with different degrees of maturity.

### Table 3. Fluorescence Intensity of Single MoAb-Stained Bone Marrow Cells

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Control Marrow (n = 10)</th>
<th>Resurgent Marrow (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak 1</td>
<td>Peak 2</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>100 ± 10†</td>
<td>93 ± 11</td>
</tr>
<tr>
<td>Leu-12</td>
<td>56 ± 7</td>
<td>47 ± 9</td>
</tr>
<tr>
<td>CALLA</td>
<td>NA</td>
<td>53 ± 9</td>
</tr>
<tr>
<td>Leu-16</td>
<td>82 ± 19</td>
<td>80 ± 12</td>
</tr>
<tr>
<td>HLe-1</td>
<td>126 ± 4</td>
<td>129 ± 4</td>
</tr>
</tbody>
</table>

*Peaks 1, 2, and 3 represent separate but sometimes overlapping subsets of cells within each histogram. In all cases peak 1 is the main peak in each histogram, followed in decreasing order by peaks 2 and 3.
† The mean channel of fluorescence intensity ± the SD is expressed on a linear scale (256 channels) after logarithmic amplification and thus represents the geometric mean of fluorescence intensity.
‡ Numbers in parentheses represent the mean fluorescence intensity expressed as linear arbitrary fluorescence units (see Materials and Methods).
§ Three of the ten control specimens contain this intermediate FI peak of HLe-1. In all three this peak was 5% of the total MoAb-positive cells.
Fig 1. Comparison of MoAb-defined histograms of T200 and B cell-associated antigens between control (N) and resurgent (R) bone marrow. Directly labeled MoAbs and mononuclear cells from representative marrow aspirates were used to construct fluorescence histograms of 10,000 cells (HLe-1 contains 20,000). The resurgent and normal histograms with each MoAb are illustrated at the same scale factor. Fluorescence intensity is the logarithmically amplified signals plotted on a linear scale.

Fig 2. Dual-parameter histograms of a representative resurgent bone marrow immunostained with HLe-1–biotin-avidin PE (y-axis) and each of the indicated FITC-labeled MoAbs or forward-angle light scatter (x-axis). All histograms contain 20,000 cells. Fluorescence is illustrated after 3 decade logarithmic amplification. Cells negative for either parameter cluster on the baseline at the gain settings employed (horizontal and vertical light lines). Regions 1 to 4, delineated by heavy lines in each histogram, are shown to identify similar Fl with HLe-1.

specimen were also simultaneously immunostained with HLe-1 (y-axis) and each of the indicated FITC-labeled MoAbs (x-axis).

Simultaneous staining with HLA-DR produced three dual-stained peaks as well as a small population of HLA-DR+/HLe-1− cells and cells that were HLA-DR+/HLe-1+. Dual staining with a cocktail of antmyeloid MoAbs (My4, MY7, MY9; Coulten Immunology) and HLA-DR produced 4% dual-stained cells; therefore only a small part of these were myeloid precursors (data not shown). The HLA-DR+/HLe-1+ cells were found to be T cells on dual-staining with OKT11 (not shown). Of the three peaks of dual-positive cells, those in region 1-2 were simultaneously dim for both, while region 3 cells were bright for HLA-DR and spanned the region of dim–intermediate HLe-1 Fl. Region 4 contained mainly “activated” T cells expressing low levels of HLA-DR and a few mature B cells with bright HLA-DR. This histogram demonstrates that as HLe-1 Fl increases, HLA-DR Fl increases on B cells.

Three dual-stained peaks of Leu-12 and HLe-1 are present. Of these the Leu-12 Fl is very dim on cells in region 2, increases to a maximum on the major HLe-1 peak (region 3), and then decreases slightly in Leu-12 Fl on HLe-1 bright cells (region 4). Leu-12 first appears on cells that are somewhat brighter for HLe-1 than does HLA-DR, suggest-
Simultaneous staining with CALLA and HLe-1 produced two peaks of dual positivity. The brighter CALLA FI was associated with intermediate HLe-1 FI (region 3), while dimmer CALLA FI was associated with brighter HLe-1 FI (region 4). Therefore, as previously reported, CALLA FI decreases with maturity.\(^\text{1,16}\)

Leu-16 was dimly positive on intermediate HLe-1 FI cells (region 3) and at its maximum FI on HLe-1 bright cells. This is opposite the pattern seen with CALLA. Simultaneous staining for kappa and lambda light chains and HLe-1 demonstrated all the dual positivity to be associated with bright HLe-1 (not shown). As illustrated, OKT11\(^+\) cells are contained mainly in the brightest HLe-1 peak (region 4), with fewer (and dimmer) OKT11\(^+\) cells in the intermediate HLe-1 peak (region 3). Although every specimen did not exhibit the same percentages of precursor cells, all resurgent marrow demonstrated the same relationships of FI between MoAbs. A small number of erythroid precursors were also found in the HLe-1 dim peak (data not shown). The sequential initial appearance (from HLe-1 dim to HLe-1 bright) of HLA-DR, Leu-12, CALLA, and Leu-16 suggests a direct relationship between the level of HLe-1 FI and increasing stages of B cell differentiation.

\textit{HLe-1 FI and clgM after selective cell depletion.} Figure 3 illustrates the HLe-1 FI histograms of MMC before and after selective removal of specific cellular subsets by immunoadsorption ("panning"). In addition to HLe-1 FI, percentages of clgM\(^+\) cells were calculated on each sample undergoing MoAb-defined subset removal. The top histogram (NONE) represents the HLe-1 FI peaks from a sample of resurgent marrow in which no subsets were removed. Three discreet peaks of HLe-1 FI are present, and on this basis the histogram was divided into four regions. Selective depletion of cells by various MoAbs or combinations of MoAbs produced losses of HLe-1\(^+\) cells from particular regions of the histogram. The percentage of HLe-1\(^+\) cells remaining in each region after panning are indicated for each histogram.

In the histogram labeled "Mature Panel," OKT11\(^+\), Leu-12\(^+\), Leu-3\(^+\), and slg\(^+\) cells were removed. This reduced the percentage of HLe-1\(^+\) cells with the highest FI to 31\% and increased the relative numbers of less bright HLe-1\(^+\) cells as well as clgM\(^+\) cells. This demonstrates that losses of the more mature cells lead to a loss of cells with the brightest HLe-1 FI.

By using immunoadsorption directed at less mature cells (Leu-12, B1[Leu-16], and CALLA), decreased percentages were seen in the regions of dimmer HLe-1 FI. In addition, the percentages of clgM\(^+\) cells were decreased by 43\% to 68\%. The histograms labeled Leu-12, B1, and CALLA show an increase in percentages of the brightest HLe-1\(^+\) cells as compared to the "Mature Panel" and the "None" histogram, with a decrease in numbers of dimmer cells in the histograms in which less mature cells were removed.

\textit{Relationships of FI in dual-stained populations.} Based on the above observation that resurgent marrow contained multiple subpopulations of B cell precursors, additional dual-staining was done to determine the relationships of the FI of these MoAb-stained cell populations to each other. In this way it may be possible to define the sequence of quantitative antigen expression as a function of cell differentiation. Data from a representative resurgent marrow specimen (cyclic neutropenia) are presented in Fig 4. All the resurgent specimens produced similar patterns but different percentages of cells in each peak.

HLe-1-stained cells \(v\) FALS (cell size) demonstrates five peaks of differing FI. Peaks 1 to 4 are cells from within the "lymphoid" area, while peak 5 contains myeloid cells. Cells from peak 5 were not included in the remaining histograms. Peak 1 is contaminating RBC; peak 2 contains a few erythroid precursors but mainly Leu 12\(^+\) B cells; and peaks 3 and 4 contain lymphoid cells.

Four peaks of Leu 12\(^+\) cells are discernable after dual staining with HLe-1. Peak 1 contains few cells and is negative for HLe-1 but dimly positive for Leu-12. About half
the resurgent specimens contained a few of these cells, thus it is not entirely clear which antigen is expressed first. Peak 2 is only slightly brighter than peak 1 in terms of Leu-12 Fl, while the HLe-1 Fl is clearly brighter. A few HLe-1+/Leu-12- cells (erythroid precursors) are also found within this dim HLe-1 peak (data not shown). The third peak shows the brightest Leu-12 Fl and intermediate HLe-1 Fl. Panning experiments (Fig 3) indicate these are enriched in clgM+ and clgM- pre-B cells. A small number of T cells (OKT11+) are also in peak 3. Peak 4 contains mature sIg B cells that are brightly positive for Leu-16 but of intermediate Fl for Leu-12.

Dual staining with Leu-16 and Leu-12, both overlapping markers of B cell subsets, shows that of the four populations present, most are Leu-12+/Leu-16- (peak 1), and a small number are Leu-12-/Leu-16+ (peak 4), suggestive of plasmacytoid B cells. Peak 2 contains a small (2%) population of Leu-12-bright, Leu-16-dim cells, and peak 3 contains cells that are brightly positive for Leu-16 but of intermediate Fl for Leu-12.

Overall the cross-correlations of Fl with these combinations of MoAbs support reported schemes of B cell differentiation and relate these to HLe-1 Fl (Figs 2 and 4).

**Additional Anti-T200 MoAb.** Figure 5 shows the pattern of Fl in a reactive marrow using a panel of anti-T200 MoAb that identifies various epitopes of the antigen. This panel was used to determine if different epitopes of the T200 antigen are expressed at different times during cell differentiation or if all are expressed in a similar pattern. In general, the MoAb T29/33, F10-89-4, G1-14.2, and G25-1 all show multiple peaks of Fl similar to those observed with HLe-1. However, the histograms of some of these MoAbs show additional subpopulations or shifts in the distribution of cells.
within the histograms. It is possible that not all these epitopes are quantitatively expressed on the same cells at the same time during differentiation. In general, though, all these MoAbs appear to define immature B cells as decreased FL. F8-11-13-stained cells show a rather broad spectrum of FL. In part this is because many of the T cells are negative for this MoAb, which is restricted in its specificity (Table 1). While the brightest fluorescent peak with each MoAb remains in a similar position (~channel 120), the intermediate and dim cells shift in their relative FL with different MoAbs. Overall it appears that quantitatively the T200 epitopes identified by the FL of these MoAbs are all expressed in a differentiation-related manner, dim on immature cells and brighter on more mature cells, but not all are necessarily expressed quantitatively at the same time during B cell differentiation.

**DISCUSSION**

Bone marrow B cell precursors have been shown to be increased in several disease states associated with reactive marrow hyperplasia. These cells conform immunophenotypically to the subpopulations of precursor B cells in bone marrow previously described.\(^\text{9,10,15,16}\) thus providing an excellent model of in vitro B cell differentiation. In vitro studies by Nadler et al\(^\text{9}\) revealed at least four subsets of B cell precursors in normal marrow. Similarly, Hokland et al\(^\text{16}\) demonstrated an orderly expression of B cell antigens in fetal bone marrow stimulated to differentiate in vitro. This study confirms the in vitro work by Hokland et al\(^\text{16}\) and demonstrates the progressive increase in quantitative T200 expression that occurs as B cell precursors differentiate.

The lymphoid subsets defined in our pediatric control population are quite similar to those reported in adults by others.\(^\text{3,5}\) Control pediatric marrow contained predominantly mature T cells and a low number of B cells, a mixture of mature slg\(^*\) cells, and a few immature precursors. Specimens of resurgent marrow contained predominantly immature B cell precursors at multiple stages of differentiation and a lower percentage of T cells than controls. This study is similar to that reported in fetal bone marrow.\(^\text{8}\) Ryan et al\(^\text{15}\) reported that the percentage and fluorescence intensity of CALLA\(^*\) cells from children with immune thrombocytopenia, neutropenia, or nonhematologic malignancies not involving the marrow were no different than in children with ALL in remission. The present study shows clearly there is a difference in the percentage of B cell precursors between those with reactive processes and those on maintenance chemotherapy. The number of precursors in marrows from patients on chemotherapy depends on when the sample was obtained relative to receiving medication (data not shown).

In the absence of more than one HLe-1 peak, the percentage of CALLA\(^*\) cells was much lower. Thus it may be that the cases reported by Ryan’s group\(^\text{15}\) were actually reactive or resurgent. Similar to their findings, the present study shows that CALLA-bright cells are dim for HLe-1 and that CALLA-dim cells were brighter for HLe-1 but still dimmer than mature B cells.

Based on the comparative FL with each MoAb in resurgent marrows, a hypothetical scheme of B cell differentiation is proposed that incorporates not only the positivity with each MoAb but also the FL (Fig 6). The quantitative expression of each cell-surface antigen appears to be differentiation-related, as suggested by others.\(^\text{10,15}\) The FL of HLA-DR is dimmer on immature cells and increases with maturation. The next antigen to appear is Leu-12. This antigen is dim on immature cells, increases in intensity during the pre-B stages, and loses some intensity on mature slg\(^*\) cells before disappearing completely on plasma cells. The CALLA (J5) antigen is first expressed after Leu-12 and is brightest on earlier precursors. A second population of more mature CALLA\(^*\) cells quantitatively express less of the CALLA antigen before it disappears on mature B cells. Leu-16 first becomes positive on CALLA\(^*\) cells and becomes brighter with further differentiation before it is lost at the plasma cell stage of differentiation.

HLe-1 first appears on B cell precursors at about the same time as Leu-12. The FL is very low at this point but progressively increases to a maximum on mature peripheral blood B cells. Based on the present study it appears feasible to use HLe-1 FL as a scale of B cell differentiation.

Previous studies from this laboratory have documented quantitative alterations in expression of HLe-1 on malignant lymphoid cells.\(^\text{1,3}\) The dimmest cells are those of ALL of non-T–non-B type, followed by B cell ALL, chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL), and normal peripheral blood B cells.\(^\text{3}\) All cases of multiple myeloma have been negative for HLe-1 (data not shown). Thus quantitative expression of T200 correlates with the presumed level of cell-differentiation of these leukemic cells and normal mature B cells. Our studies also suggest that the quantitative expression of HLe-1 may relate to therapeutic outcome in some children with ALL.\(^\text{2}\)

In summary, evaluation of the FL as a reflection of total surface-antigen content is a useful parameter for studies of cell differentiation. In particular, the quantity of T200 antigen provides information about the degree of B cell differentiation. If this antigen is quantitatively conserved on
malignant B cells, it will provide a very useful marker of their differentiation state and provide a common framework on which to investigate relationships between normal and malignant B cells. Using this model it is feasible to evaluate malignant B cells to determine if they are really at a “frozen” stage of normal differentiation. The functional role of the T200 antigen on human B cells is not known, although the MoAbs have been reported to block cytolytic activity of natural killer cells and may inhibit B cell differentiation in mice. Since the antigen increases in quantity during human B cell differentiation, it is tempting to speculate on its functional role during this process.

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Relationship between T200 antigen expression and stages of B cell differentiation in resurgent hyperplasia of bone marrow

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