Flow Cytometric Analysis of Human Bone Marrow. II. Normal B Lymphocyte Development

By Michael R. Loken, Virendra O. Shah, Karen L. Dattilio, and Curt I. Civin

A panel of B lymphoid-reactive monoclonal antibodies was used to analyze the phenotypic changes that accompany B lymphocyte development in normal human bone marrow. The B lymphoid cells were identified using light scattering and the expression of CD19 on a flow cytometer. Quantitative three-color immunofluorescence was then used to correlate other cell surface antigens on these cells identified as B lymphoid in normal marrow. CD10 and CD20 identified almost exclusive populations and provided a convenient means of discriminating between the less and more mature B lineage cells. The CD10− cells could be further subdivided using CD34. The population of CD19+, CD10−, CD34− cells comprised only 0.6% of marrow cells, but these contained the majority of terminal deoxynucleotidyl transferase (TdT+) cells. In the assessment of class II antigens, HLA-DR was expressed on all B lineage cells whereas HLA-DP preceded HLA-DQ in appearance during the developmental process. Among the later antigens expressed on B lineage cells, cell surface IgM, CD20, and HLA-DQ were expressed at essentially the same time. Cell surface CD10 was lost at the time when CD21 and CD22 were acquired on the cell surface. These data illustrate that multiparameter flow cytometry can be used to define a continuous progression of stages of B lymphocyte development based on cell surface antigen expression even though these cells represent a minor fraction of normal marrow cells.

The bone marrow is the site of production and early maturation of B lymphocytes in humans. A correlation between maturation stage and cell surface antigen expression of B lymphocytes has been made, primarily on the basis of cell surface phenotypes of acute lymphoblastic leukemias (ALL) and lymphomas. Counterparts of many of the malignant cells have been identified in normal bone marrow.

Previous studies of B lymphocyte surface antigens have identified CD19 (Leu-12, B4) as a marker for all B lymphocytes. CD19 is expressed very early in B cell development and is retained until the latest stages of cell activation and antibody secretion. This marker is expressed on most non-T ALL (>95%), but is not found on T cell ALL or on myeloid lineage leukemias. Thus, CD19 defines most cells of the B lineage with exceptional lineage specificity.

Another B cell antigen, CD20 (Leu-16, B1), is expressed later during B lymphocyte development. As compared with CD19, CD20 is expressed on fewer normal B lymphocytes in the marrow and on a lower percentage of leukemias. CD10 (common ALL antigen, CALLA, J5) has been found on most cases of non-T ALL but is expressed on only a subset of immature B lymphocytes. Mature peripheral blood B lymphocytes express both CD19 and CD20, but do not express CD10. The progenitor cell-associated antigen, CD34 (My10) is also observed on some B lineage ALL cases, but is not expressed on normal peripheral blood B cells.

Mature B lymphocytes in peripheral blood express class II antigens including HLA-DR, HLA-DP, and HLA-DQ, as well as several other B lymphocyte specific antigens: CD21 (CR2), CD22 (Leu-14), cell surface IgM (sIgM), and IgD (sIgD).

In this article, we correlate the expression of multiple cell surface markers on normal bone marrow B lineage cells. By using multiparameter quantitative analysis on a flow cytometer, we examined all low-buoyant-density cells obtained from the bone marrow sample without preenrichment. The entire B lymphoid lineage was identifiable using CD19 in combination with light-scattering characteristics. Pairs of other cell surface antigens were then identified and correlated on the CD19+ cells by using three-color immunofluorescence. From these data in combination with cell sorting and immunostaining for terminal deoxynucleotidyl transferase (TdT), we could define the sequence of appearance during B lymphocyte development of these antigens: HLA-DR, HLA-DP, HLA-DQ, CD10, CD19, CD20, CD21, CD22, CD34, sIgM, sIgD, and nuclear TdT.

Materials and Methods

Monoclonal antibodies. Fluorescein-conjugated (FITC) and/or phycoerythrin (PE)-conjugated CD3(Leu-4), CD10(CALLA), CD11b(Leu-15), CD14(Leu-M3), CD16(Leu-11), CD19(Leu-12), CD20(Leu-16), CD21(CR3), CD22(Leu-14), anti-HLA-DR, anti-HLA-DP, anti-HLA-DQ, streptavidin allophycocyanin (APC) along with purified CD34(HPCA-1) were obtained from Becton Dickinson Monoclonal Center, Mountain View, CA. Anti-glycophorin (10F7) was a kind gift from Dr R. Langlois (Lawrence Livermore National Laboratory, Livermore, CA) CD19(Leu-12) and CD10(CALLA) (Becton Dickinson) were coupled with biotin, following the protocol of Billingsley. Anti-human IgM-FITC, anti-human IgD-FITC and F(ab')2 goat anti-mouse Ig-FITC were obtained from Tago, Burlingame, CA.

Cell preparation and immunofluorescence. Bone marrow aspirates were obtained from consenting normal adult volunteers. Collection of samples was performed after being approved by the Institutional Review Board, as approved by the Department of Health and Human Services. The low-density leukocytes (1.077 g/cm³) Ficoll-Hypaque, Pharmacia, Piscataway, NJ) were washed...
in RPMI 1640 containing 10% fetal bovine serum (FBS) and aliquoted for immunofluorescent staining. The cells were suspended at a concentration of 10^7/mL. In the experiments in which both antibodies were directly conjugated to the fluorochrome, the first antibody was added to 10^6 cells using an amount of antibody that gave maximal fluorescence. The cells were incubated on ice for 20 minutes. The cells were then pelleted and washed with buffer (at least five times the staining volume) before the second antibody was added. After 20 minutes on ice, the cells were again washed with buffer.

For three-color analysis, cells were reacted with FITC-conjugated, PE-conjugated, and biotin-conjugated antibodies for 20 minutes and were then washed. APC-conjugated streptavidin was then added, and the cells were incubated for an additional 20 minutes and washed and fixed in 1% paraformaldehyde.

Three-color analysis using CD34 was performed as follows: Cells were incubated with CD34 on ice for 20 minutes, washed and incubated with F(ab')2 goat anti-mouse Ig for 20 minutes, and washed. Cells were further incubated with 10% normal mouse serum to block any free binding sites of the second step antibody. After being washed, the cells were reacted with PE-conjugated and biotin-conjugated antibodies, followed by the APC-conjugated streptavidin. Unstained cells, IgG1-FITC, IgG2-PE, and cells labeled with streptavidin-APC but without biotin conjugate were included as controls.

**Flow cytometry.** Quantitative fluorescence analysis was performed using a five-parameter FACS 440 (Becton Dickinson Immunocytometry Systems). The optical configuration and filter combinations for three-color immunofluorescence using FITC, PE, and APC have been described previously.20-22 The argon-ion laser was operated at 488 nm using 200 mW of power. FITC emissions were collected with a 530/30 bandpass (BP) filter, and PE emissions were collected with a 585/42 BP filter. The laser beam was focused using a 2-inch focal length spherical lens. Three-color immunofluorescence was performed using HeNe laser of 633 nm, 40 mW, together with an argon laser as described.23 APC emission was collected with a 660/20 BP filter. Fluorescence signals were processed using a four-decade logarithmic amplifier. Light scattering signals were processed by linear amplification.

A minimum of 30,000 events for each sample was collected in list mode on a Consort 30 Data Management System, Becton Dickinson. This permitted reanalysis of the data to demonstrate the correlation between three colors of immunofluorescence for populations of cells identified by forward-angle and right-angle light scattering. In the assessment of class II and later B lymphoid antigens of the marrow B lineage cells, the data were pre gated on light scattering and CD19 fluorescence. The contour lines in Figs 1 through 7 were drawn at levels of 2, 4, 8, 16, 32, 64, and 128. The fluorescence correlations in Figs 2 through 7 were gated on the light-scattering window depicted in Fig 1A.

**Cell sorting.** Viable cells, stained for two colors of immunofluorescence, were sorted based on the gates indicated in the appropriate Figs. Replicate cytocentrifuge slides were prepared for each sorted fraction of cells. These slides were coded, stained for nuclear TdT (rabbit anti-TdT, a kind gift from Dr F. Bollum) using an immunoperoxidase procedure (Supertechs, Bethesda, MD) or Wright's, and differential counts were made for each sample.24

**RESULTS**

**Bone marrow B lymphocytes have distinctive light-scattering properties.** Normal human bone marrow cells were heterogeneous in their forward- and right-angle light-scattering characteristics (Fig 1A). The CD19+ B cells were confined to a well-defined cluster, which was previously classified as "lymphoid" (Fig 1B) with low to moderate forward-angle and low right-angle light-scattering properties.25 All subsequent multicolor immunofluorescence analyses were performed by gating on this light-scattering population, as shown in the box in Fig 1A. More than 95% of the CD19+ cells in whole un gated marrow were included within this gate.

We used two-color immunofluorescence to confirm that CD19 bound only to B lineage cells and not to cells of other lineages in normal human bone marrow. Normal marrow cells were reacted with CD19 in combination with either CD3 (T cells), CD16 (natural killer [NK] and neutrophils), CD14 (monocytes), CD11b (NK, monocytes and neutrophils), or anti-glycoporphin (erythroid cells).26-28 Quantitative fluorescence analysis on the flow cytometer demonstrated that the cells labeled with CD19 were not double labeled with the other antibodies (data not shown); thus, CD19 and the other antibodies identified mutually exclusive cell populations in bone marrow.

**CD20 and CD10 define distinct stages of B lymphocyte development.** The CD20 antigen is expressed late during B lymphocyte development, whereas CD10 has been associated with early B lymphocytes.18-13 To define more precisely when, during development, B lymphocytes lose CD10 and acquire CD20, we performed three-color immunofluorescence experiments using CD10 and CD20 in correlation with CD19.

The two-color correlated expression of CD20 and CD19...
on marrow cells (Fig 2A) confirmed that CD20 was expressed on a subset of the CD19+ cells; all CD20+ cells were also CD19+. No CD20+ cells were outside the lymphoid light scattering window. CD10 was also found on a portion of the CD19+ cells (Fig 2B). CD10+ CD19+ marrow cells were identified but these cells fell outside the lymphoid light-scattering window and were granulocytes (data not shown).

The correlation between CD20 and CD10 (Fig 2C) indicated that these two antibodies identified almost non-overlapping B lymphoid subpopulations: Most CD20+ cells did not express CD10 and vice versa. A small subset of cells, however, expressed both markers. The proportions of cells within marrow that expressed CD19, CD20, and CD10 are presented in Table 1. CD19 stained ~6.5% of all marrow cells, whereas CD20 labeled ~4.5%. A slightly smaller proportion of marrow cells, 3.5%, were CD10+. Of the CD19+ cells in marrow, ~60% expressed CD20, whereas only 40% expressed CD10. Only ~20% of the CD20+ cells were also CD10+, and a similar proportion of all the CD10+ cells in this light-scattering window were double labeled with CD20. Together, CD10 and CD20 identify almost all CD19+ B lineage cells. A few marrow B lineage cells (<5% of CD19+ cells) lack both CD10 and CD20, a phenotype consistent with plasma cells.

Early marrow B lineage cells co-express CD19, CD10, and CD34. CD34 was expressed on a subset of B lineage ALL cases, as well as on a small population of immature normal marrow cells, including all in vitro assayed hematopoietic progenitor cells, but was not expressed on mature normal cells.4 All TdT+ cells in normal marrow were previously found in the CD34+ fraction.31 Because TdT expression is a characteristic of early lymphoid cells, we used three-color immunofluorescence to investigate whether the B cell antigens identified by CD19 and CD10 could be found on any CD34+ cells.

In the correlation between CD19 and CD34, a small population of dim CD19+ cells was also labeled with CD34 (Fig 3A). Most of the CD19+ cells, however, were negative for the CD34 marker (Table 2). Less than one-half of the CD34+ cells were CD19+.

In a similar correlation between CD10 and CD34, a population of cells was double labeled. The cells that were most intensely stained with CD10 were the cells that also bound CD34 (Fig 3B). In three-color experiments, these few CD10+, CD34+ cells were the ones that also expressed low amounts of CD19 (Fig 3A). These CD34+, dim CD19+, bright CD10+ cells constituted ~1% of total marrow cells (Table 2).

To characterize this minor cell population further, marrow cells were reacted with either (a) CD34 and CD19, or (b) CD34 and CD10. The cell subpopulations identified were then (FACS) sorted, and cytocentrifuge slides were prepared from the sorted fractions (Fig 3A and B shows sorting windows). These slides were examined for morphology (Wright's stain) or for nuclear TdT expression24,31 (Table 3).

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Table 1. Proportion of B Lymphoid Populations Identified by CD19, CD20, CD10 in Normal Bone Marrow

<table>
<thead>
<tr>
<th>Cell Surface Phenotype†</th>
<th>Total Marrow Cells (%)‡</th>
<th>CD19* Marrow Cells (%)§</th>
<th>CD20* Marrow Cells (%)∥</th>
<th>CD10* Marrow Cells (%)¶</th>
</tr>
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<tbody>
<tr>
<td>CD19*</td>
<td>6.5 (4.3; 53)</td>
<td>NA**</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CD20*, CD19*</td>
<td>4.7 (2.9; 29)</td>
<td>59.1 (20.0; 8)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CD10*, CD19*</td>
<td>3.1 (3.1; 15)</td>
<td>38.6 (17.9; 15)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CD10*, CD20*</td>
<td>0.9 (1.0; 9)</td>
<td>10.3 (8.1; 9)</td>
<td>22.0 (12.3; 9)</td>
<td>20.0 (8.1; 9)</td>
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*Percentages are reported with (in parentheses) SD and total number of independent samples (n) used in the calculation.
†Cell surface phenotypes were determined using the monoclonal antibodies discussed in the Materials and Methods section.
‡Mean percentage of positive cells calculated for all nucleated cells (30,000) in each marrow sample.
§Mean percentage of positive cells calculated as a percentage of CD19+ cells in each sample.
∥Mean percentage of positive cells calculated as a percentage of CD20+ cells in each sample.
¶Mean percentage of positive cells calculated as a percentage of CD10+ cells in each sample.
**Not applicable.
Fig 3. Immunofluorescence analysis of bone marrow cells labeled with CD34/HPCA-1 and either CD19/Leu-12 or CD10/CALLA. (A) The correlation between CD19 and CD34 indicates that a few double-labeled cells were identified. (B) A similar correlation between CD10 and CD34 also showed a double-labeled population of cells. Boxes in A and B illustrate the windows used for sorting the cell populations assessed for morphology and TdT expression (Table 3).

The most immature cells by morphology were in the CD34, CD19', CD10' fraction. This population contained many "undifferentiated" blast cells, without definite lymphoid features. A minor percentage of these cells stained perceptibly for intranuclear TdT. The double-labeled CD34', CD10' or the CD34', CD19' cells were somewhat more mature by morphology and could be characterized as lymphoblasts or lymphocytes. Most of these cells were intensely labeled (intranuclear pattern) with the TdT stain (Table 3). In contrast, the CD34', CD10', or CD34', CD19' cells contained a greater number of mature lymphocytes recognized by morphology that were negative for TdT staining.

The cells that did not stain with either set of antibodies (ie, double negative, CD34', CD10'; or CD34', CD19') were a mixture of lymphocytes and developing erythroid cells from the middle to late normoblast stage. These cells did not contain detectable intranuclear TdT.

To determine whether CD10 was expressed before CD19 on these early B lineage cells, the correlation of these two antigens on only the CD34 cells was determined (Fig 4). Although a few cells (~0.1%) expressed CD34 and CD10 without CD19, an equivalent number were identified expressing CD34 and CD19 without CD10 (Fig 4B). Three replicate experiments gave the same results with no clear evidence for either CD10 or CD19 to precede the other on the CD34' cells.

HLA-DR, HLA-DP, HLA-DQ class II antigens are sequentially expressed during B lymphocyte development. Two-color immunofluorescence studies have shown that normal marrow CD34' cells express low but detectable amounts of HLA-DR. Quantitative studies of HLA-DR on B cells in comparison to the CD34' cells indicated that the amount of HLA-DR expressed on the (average) CD19' cell was approximately four times greater than that on the (average) CD34' cell (Fig 5). All the CD10' lymphoid cells expressed HLA-DR, although the brightest CD10' cells (ie, the CD34', bright CD10' from above) were less intensely labeled with HLA-DR than were most CD10' cells.

Three-color immunofluorescence was used to detect the acquisition of two other class II antigens: HLA-DP and HLA-DQ on marrow B lymphoid cells. Three-color immunofluorescence was used to detect the acquisition of two other class II antigens: HLA-DP and HLA-DQ on marrow B lymphoid cells. Three-color immunofluorescence was used to detect the acquisition of two other class II antigens: HLA-DP and HLA-DQ on marrow B lymphoid cells. Three-color immunofluorescence was used to detect the acquisition of two other class II antigens: HLA-DP and HLA-DQ on marrow B lymphoid cells. Three-color immunofluorescence was used to detect the acquisition of two other class II antigens: HLA-DP and HLA-DQ on marrow B lymphoid cells. Three-color immunofluorescence was used to detect the acquisition of two other class II antigens: HLA-DP and HLA-DQ on marrow B lymphoid cells. Three-color immunofluorescence was used to detect the acquisition of two other class II antigens: HLA-DP and HLA-DQ on marrow B lymphoid cells. Three-color immunofluorescence was used to detect the acquisition of two other class II antigens: HLA-DP and HLA-DQ on marrow B lymphoid cells. Three-color immunofluorescence was used to detect the acquisition of two other class II antigens: HLA-DP and HLA-DQ on marrow B lymphoid cells.
of other cell types. (Additional class II-bearing cells were identified outside the light-scattering window in Fig 1A, since HLA-DR, HLA-DP, and HLA-DQ also labeled blasts and monocytes within the marrow.) A comparison between Fig 6A and C indicates that HLA-DP was expressed on almost all B lymphocytes, including the CD10⁺ cells, whereas HLA-DQ stained mainly the CD10⁻ B lymphocytes. The brightest CD10⁺ cells (again corresponding to the CD34⁺, CD10⁺ cells shown above) lacked both HLA-DP and HLA-DQ.

In reciprocal experiments, marrow cells were labeled with CD19, CD20, and either anti–HLA-DP or anti–HLA-DQ to assess class II expression on more mature marrow B cells. In relating the CD20 with class II antigens on only the CD19 B lineage cells (Fig 6B and D), HLA-DP reacted with some B lineage cells that were CD20⁺ (these cells were CD10⁺, as shown in Fig 6A). When CD20 and HLA-DQ were compared on the CD19⁺ cells (Fig 6D), all CD20⁺ cells were also HLA-DQ⁺ and vice versa.

**Later stages of B lymphocyte development are defined by CD20, CD22, CD21, sIgM, and sIgD.** We used three-color immunofluorescence to study the expression of other cell surface markers on these CD19⁺ marrow B lineage cells in a pairwise fashion. The data from 1 of 12 similar experiments yielding identical results are shown in Fig 7. Comparing CD20 and sIgM on the CD19⁺ cells, we found that these two markers identified the same populations (Fig 7A). However, in a correlation between CD20 and sIgD, a significant population of CD20⁺, sIgD⁻ cells was identified in addition to the CD20⁺, sIgD⁺ cells (Fig 7B).

In the correlation of CD20 with CD22 (Fig 7C), there was a population of CD20⁺, CD22⁻ cells, in addition to the CD20⁺, CD22⁺ cell population. Likewise, there was a significant CD20⁺, CD21⁺ population in addition to a population of CD20⁺, CD21⁻ cells (Fig 7D). There were clearly more B lineage cells in the marrow labeled with CD20 than were reactive with antibodies detecting sIgD, CD21, or CD22.
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In correlating sIgM with CD22 and CD21 (Fig 7D and G), sIgM was clearly expressed on more marrow B lineage cells than was either CD21 or CD22. When CD22 and CD21 binding was correlated on marrow B lineage cells, the cells identified the same population (Fig 7F). Among the marrow B lineage cells, fewer cells were labeled with sIgD than with either CD20, CD21, or CD22 (Fig 7B, E and H).

DISCUSSION

Adult bone marrow is a complex tissue that is heterogeneous with respect to the multiple lineages represented in a marrow aspirate as well as to maturational differences within each lineage. By identifying cells of the B lineage as distinct from cells of all other lineages, we were able to assess the expression of multiple cell surface markers on cells that differed only in their degree of maturity. The appearance of these cell surface antigens during the maturational process was deduced by comparing in a pairwise fashion the populations labeled by the antibodies.

In these studies, the B lymphoid cells were identified by a combination of light-scattering characteristics (forward and right angle) and the binding of CD19. The light-scattering window used in these studies included essentially all CD19+ cells but excluded cells of both the monocytic and granulocytic lineages. The light-scattering gates were necessary, since the autofluorescence of myeloid cells interfered with the discrimination of the minor subpopulations identified by immunofluorescence. Two-color immunofluorescence showed that CD19 did not label either mature or immature cells of the myeloid lineages. The CD19+ cells classified in this light-scattering window could all be identified as either T, NK, or erythroid cells. In addition, all cells that bound CD19 were identifiable as lymphocytes by morphology. These data are consistent with the studies of non-T ALL in which CD19 was identified as the most precise available antigenic marker of the B lineage.

Three-color immunofluorescence was used to identify the cell surface expression of antigens on the B lymphoid cells in marrow; one color identified the CD19+ B lymphoid cells, and the other two colors compared pairwise the reactivity of additional antigens. The CD19+ population was split into two, almost non-overlapping fractions by the combination of CD10 and CD20. The more mature B lineage cells were identified by the CD20 antigen, whereas the immature B lymphoid cells were CD10+. The CD19+ cells were essentially all double labeled by either CD10 or CD20, suggesting that all the B lymphoid cells were identified in these two populations.

The immature CD10+ B lymphoid cells could be further subdivided by using CD34. An average of 0.6% of marrow cells coexpressed CD19, CD10, and CD34. As found by Ryan and co-workers using lymphocyte-enriched fractions from normal marrow, CD10+ expression was brighter on the CD34+ cells as compared with the remaining CD10+ lymphocytes. Most cells within this population were positive for nuclear TdT.

Class II antigens were not uniformly expressed on marrow B lymphoid cells. HLA-DR was found on all CD19+ cells in marrow but was significantly dimmer on the bright CD10+, CD34+ cells as compared with the more mature CD20+ cells. HLA-DP was not expressed on the brightest CD10+ cells but was found on all other B lymphoid cells. In contrast, HLA-
DQ was found only on the CD20+ cells and not on the less mature CD10+ cells.

The B lymphoid antigens expressed at later stages of maturation reacted only with the CD20+ cells in marrow. Cell surface IgM and HLA-DQ identified the same populations as CD20. In contrast, CD20 reacted with more B lymphoid cells than did CD21, CD22, and anti-sIgD, suggesting that CD20 is expressed before these other antigens.18

The data identifying the antigens expressed on B lineage cells can be used to develop a model for the sequential acquisition of cell surface antigens during B lymphocyte development (Fig 8). Our data indicate that the sequence of display of cell surface antigens is highly controlled during development of B cells, with coordinated expression of multiple cell surface antigens at distinct stages during the maturational process. These stages (I, II, III, and IV) are each identified by three or more essentially simultaneous changes in cell surface phenotype.

The most immature cells identifiable in bone marrow express CD34 as well as HLA-DR.32 CD34 antigen is found on all assayed progenitor cells including CFU-“blast,” pre-CFU-GEMM, CFU-GEMM, CFU-GM, CFU-MIX, BFU-E, and CFU-E.33 In the current studies and those of Ryan et al, CD34 is found on the earliest recognizable B lineage cells (CD19+, bright CD10+).32 This evidence links the CD34 progenitor cell antigen to the B lymphocyte lineage, as well as to the myeloid lineages. HLA-DR was found on these earliest B cells, another characteristic shared by erythroid and myeloid progenitor cells.32 At this early CD19+, CD34+, HLA-DR+ B cell stage, nuclear TdT and cell surface CD10 are intensely expressed. Because of the presence of TdT in this CD19+, CD34+, bright CD10+ fraction of cells, we speculate that the rearrangement of Ig genes occurs at this stage.34

At the beginning of stage II, maturing B lineage cells lose cell surface CD34 and nuclear TdT. At this time, the density of HLA-DR increases fourfold, while the amount of CD10 decreases by a factor of 3. These changes occur with no detectable change in cell size (as assessed by forward light scattering). HLA-DP is detected on cells at approximately this same time.

The beginning of stage III is marked by the acquisition of CD20, HLA-DQ, and sIgM. The intensity of staining of cells for sIgM, CD20, and HLA-DR is brighter than for HLA-DP, HLA-DQ, or CD19.

The acquisition of two more cell surface markers, CD21 and CD22, and the loss of CD10 identify stage IV. These newly acquired cell surface antigens are not brightly labeled, being expressed in similar amounts as CD19 or HLA-DP. Studies of B lymphoid antigens on peripheral blood indicate that sIgD is expressed on significantly fewer cells than any of the other antigens examined (M.R. Loken and V.O. Shah, unpublished observations). This may explain the data observed in Fig 7B, E, and H and is consistent with stage IV representing peripheral blood contamination of the bone marrow sample. The sIgD− cells are depicted as more immature cells (as compared with the sIgD+ cells) in Fig 8 to correspond to studies on fetal tissue.39 Because sIgD is lost after antigenic stimulation, however, the sIgD− cells observed in blood and marrow may represent the cells that have already been exposed to antigenic stimulation.40

The scheme presented in Fig 8 assumes a linear sequence of events in the development of B lymphocytes. The mature B lymphocyte population, as identified in peripheral blood, is assumed to be homogeneous. The lack of expression of sIgD on all peripheral blood B lymphocytes indicates that this assumption is not entirely valid. Other studies must be completed to define where different sublineages of B lymphocytes may deviate from the linear model.

The developmental scheme in Fig 8 is inferred from coordinated expression of cell surface antigens on single samples. Confirmation of the progression from one stage to the next requires the isolation of a particular stage with subsequent induction to the next stage in vitro.

There are differences between the stages of B lymphocyte development identified in this study and those deduced from studies of non-T cell ALL by Nadler and co-workers.23 The most notable difference is in the conclusion from the leukemia studies that CD19 precedes the acquisition of CD10. Nadler et al identified a small group of ALL cases expressing CD19+ and HLA-DR+ but lacking CD10+. In contrast, in our studies on normal B cells, detailed analysis of the CD34+ cells for CD10 and CD19 expression indicated that these antigens were expressed at essentially the same time.33 Similar conclusions were drawn from the studies of Ryan et al. Similar conclusions were drawn from the studies of Ryan et al. Whether the leukemic cells classified in this group lost the CD10 antigen during the development of the leukemia or if the normal counterpart of this cell is so infrequent (<2% of marrow B cells) that it was not detectable in our studies is not known.

A second difference between our model and that of Nadler et al is that the frequencies of non-T cell (“B lineage”) ALL cases classified in the four stages do not appear to reflect the relative proportions of similar populations observed in normal marrow. Almost one-half of the B lineage leukemias had the CD19+, CD10+, CD20+ phenotype, whereas this phenotype constitutes a minor group of normal cells. Although this may be the result of relatively small numbers of patients studied, it is also possible that the leukemic event does not occur randomly during the developmental process.

**Fig 8.** Schematic representation summarizing the characteristics of B lineage cells as they mature in normal human bone marrow. Relative quantities of the cell surface antigens are shown on the ordinate; relative maturation of the cells increases is shown along the ordinate. Four stages of development (I through IV) can be assigned based on multiple changes in cell surface antigen expression that occur at essentially the same time during the maturational process.

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and that the proportion of leukemias classified into each group does not reflect the proportion of the normal counterpart in the bone marrow. A third explanation may come from disorganization of expression of different cell surface antigens in the leukemic cells. During the process of leukemogenesis, mutational or other events may occur in which some antigens are deleted or others are incorrectly expressed. Such changes would splay the extrapolation from leukemic cells to normal counterparts.

The data presented in this article illustrate that multiparameter flow cytometry can be used to identify a single lineage of cells as distinct from all others in a heterogeneous mixture. By comparing the antigens expressed on only these cells, the composition of that defined population can be assessed. These techniques can be extended to examine the sequential acquisition of antigens in other lineages. In addition, the multiparameter approach can be used to identify populations of cells with abnormal cell surface and physical characteristics that may be indicative of particular disease states or hematopoietic abnormalities.

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