CONCISE REPORT

Assessment of Proliferation During Maturation of the B Lymphoid Lineage in Normal Human Bone Marrow

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Different stages of B lymphoid maturation were identified in normal bone marrow using multiple cell surface markers. The proliferation status of each of these maturation stages was determined by simultaneous quantitative DNA analysis on a flow cytometer. The technique used to quantify these parameters preserved the cell surface immunofluorescence, the light scattering properties and the stoichiometric binding to DNA. The proliferating cells were confined to a distinct population of cells expressing CD19. The number of proliferating cells in these populations was relatively constant among 12 separate bone marrow samples. The data suggest that the timing and rate of proliferation of cells within a single lineage may be a preprogrammed aspect of normal maturation.

MATERIALS AND METHODS

Bone marrow aspirates were obtained from consenting normal adult volunteers. Collection of samples was performed after approval by the Institutional Review Board as required by the Department of Health and Human Services. Low density bone marrow cells were obtained and stained for indirect immunofluorescence as previously described. Fluorescein conjugated (FITC) and/or phycoerythrin-conjugated (PE) CD19 (Leu-12), CD20 (Leu-16), CD10 (CALLA), CD34 (HPCA-1), and IgG, negative control were obtained from Becton Dickinson Monoclonal Center, Mountain View, CA. F(ab')2 goat anti-mouse Ig-FITC was obtained from Tago, Burlingame, CA and was used as a second step reactive with CD34.

RESULTS AND DISCUSSION

Marrow B lymphoid cells were identified specifically in bone marrow by combining the light scattering characteristics of the cells with the expression of CD19. The maturation stage of each B lineage cell was determined on the basis of co-expression of other cell surface antigens (CD34, CD10, and CD20). The UV excited dye, Hoechst 33342, was used to quantify the DNA content of the cells, without interference with the detection of immunofluorescence. Although Hoechst 33342 has been used in the past to stain viable cells, bone marrow cells in our experiments were first fixed with paraformaldehyde in order to preserve two colors of cell surface immunofluorescence as well as cellular light scattering characteristics. These cells were then permeabilized using a detergent in order to obtain quantitative DNA staining. Using this technique the position of the G0/G1 peak of the DNA stain was identical for cells recognized as erythroid, monomyeloid, or lymphoid based on cell surface antigen expression (data not shown). The coefficient of variation (CV) for the G0/G1 peak of the DNA stains averaged 5.0% with a range of 3.5% to 6.5%.

The quantitative DNA analysis of different populations of B lineage cells identified by a single cell surface antigen is shown in Fig 1. These data were gated on the right angle light scattering characteristics of the marrow cells in order to exclude maturing neutrophils. The percent of cells with

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greater than a 2n (diploid) amount of DNA within a population of cells was defined as the "proliferation index." In this representative experiment, the entire marrow B lineage population (as identified by CD19+ expression) had a proliferation index of 6%. The more mature B lymphoid cells (CD20+) had a proliferation index of 2.8% while the less mature (CD10+) cells had consistently more cells in the S and G2 + M phases of the cell cycle (in this experiment the proliferation index was 15%). The progenitor cells identified by CD34+ had a proliferation index of 5%. In comparison, the mature T and NK marrow cell populations recognized by CD3 and CD16, respectively, each had a proliferation index of <1%.

The cell populations identified by the single monoclonal antibody labeling cell surface described in Fig 1 overlap to varying degrees. In order to more precisely define the stage dependence of the B lymphoid cell proliferation, two color immunofluorescence was combined with the DNA stain. The previously characterized correlation between CD34 and CD10 on a representative bone marrow sample is shown in Fig 2. The proliferation indices of three populations of cells identified by these markers is also illustrated. The CD34+,CD10+ progenitor cells had a lower proliferation index (3%) as compared with the committed stage I, CD34+,CD10+ B lymphoid population (21%) and the more mature, stage II CD34+,CD10+ (15%) B lymphoid population. Similar two color immunofluorescence studies were performed on the same marrow sample, correlating CD10 and CD20 as previously described. The proliferation indices of the CD10+,CD20− (stage II) and the CD10+,CD20+...
(stage III) were 15% and 22%, respectively, much higher than the proliferation index of the CD10⁺,CD20⁺ mature stage IV population (2%), data not shown.

The results shown in Figs 1 and 2 were consistent in other experiments as demonstrated in Fig 3. Figure 3 summarizes the results of separate experiments on 12 different bone marrow samples correlating the two color cell surface antigen expression with cellular DNA content. The progenitor cells identified as CD34⁺,CD10⁻ as well as the mature B lymphocytes with the phenotype of CD10⁺,CD20⁺ had very low rates of proliferation. The cells with the phenotypes of CD34⁺,CD10⁺; CD34⁺,CD10⁻; and CD10⁺,CD20⁺ (characteristics of the maturational stages of immature B lineage cells as described previously4) all had higher proliferation indices.

The results suggest that in a distinct maturation stage, identified by CD10 expression, B lymphoid cells have relatively high indices of proliferation. This agrees with the studies of purified CD10⁺ cells using BrdU. The proliferating cells identified within either the CD34⁺ or the CD20⁺ populations (Fig 1) were almost totally included within the minor subpopulations of these cells that also expressed CD10. The data indicate that the B cell expansion begins shortly after commitment to this lineage (on the cells which express CD10, CD19, and CD34). The expansion of cells continues through the late stage of maturation (where both CD10 and CD20 are expressed). The proliferative index of these dividing CD10⁺ cells was relatively constant as they matured throughout the CD10⁺ stages.

Previous studies have shown that the majority of the CD34⁺,CD10⁻ cells are also terminal deoxynucleotidyl transferase (TdT) positive. Since these same CD34⁺,CD10⁺ cells are proliferating, the postulated mutagenic effect of TdT

**Fig 2.** Detection of proliferation of cells identified by two cell surface markers, CD10 and CD34. Normal bone marrow cells were stained for two color immunofluorescence as described followed by fixation and DNA staining outlined in Fig 1. The bivariate contour plot was obtained from 50,000 cells gated on low right angle light scatter. The vertical and horizontal lines used to detect the positively labeled cells were established using appropriate isotype matched negative controls. The DNA histograms of the three positive populations were then generated from the list mode data. The proliferation index of the subpopulation of cells identified by the two immunofluorescent stains is presented with the histograms.

**Fig 3.** Proliferation indices for cells with different phenotypes among the B lineage in normal human bone marrow. The results were obtained from 12 separate experiments using the two color immunofluorescence and quantitative DNA procedures described in Figs 1 and 2. The error bars are SD. The total number of analyzed cells ranged from 50,000 to 200,000. The population of CD34⁺,CD10⁻ cells constituted approximately 0.5% of nucleated marrow cells, which necessitated the analysis of large numbers of cells. The phenotypes are arranged from left to right as increasing maturational stages among the B lineage cells as postulated by Loken et al.⁴
might be expected to be higher than if these cells were quiescent.

It was interesting to find that the same populations of cells were proliferating and that the proliferation index for each population was similar from individual to individual. This constancy of proliferation among individuals implies that proliferation is a strictly controlled component of normal B lymphoid maturation. The rate of proliferation may be a preprogrammed aspect of the normal maturational process.

The technique to assess proliferation on cells with different cell surface antigenic phenotypes may provide a means to carefully study the normal regulation of production of blood cells. For example, the mechanism of action of growth factors such as erythropoietin, G-CSF, GM-CSF, and IL-3, may be open to examination. In addition, characterization of the proliferating component within a leukemic cell population may now be assessed as a part of the diagnosis and monitored to determine the effectiveness of chemotherapeutic regimes.

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Z Hollander, VO Shah, CI Civin and MR Loken