Differentiation of Normal Human Pre-B Cells In Vitro

By Judith G. Villablanca,* Janet M. Anderson,‡ Melinda Moseley,‡ Che-Leung Law,‡ Rebecca L. Elstrom,‡ and Tucker W. LeBien‡

*From the Departments of *Pediatrics and ‡Laboratory Medicine/Pathology, University of Minnesota Medical School, Minneapolis, Minnesota 55455*

Summary

The differentiation of surface Ig^- pre-B cells into surface Ig^+ B cells is a critical transition in mammalian B cell ontogeny. Elucidation of the growth factor requirements and differentiative potential of human pre-B cells has been hampered by the absence of a reproducible culture system that supports differentiation. Fluorescence-activated cell sorting and magnetic bead depletion were used to purify fetal bone marrow $CD10^+$/surface μ^- cells, which contain 60–70% cytoplasmic μ^+ pre-B cells. $CD10^+$/surface μ^- cells cultured for 2 d were observed to differentiate into surface μ^+ cells. Analysis by Southern blotting provided direct evidence that rearrangement of κ light chain genes occurs in culture, and flow cytometric analysis revealed the appearance of surface Ig^+ B cells expressing μ/κ or μ/λ. Unexpectedly, the κ/λ ratio in differentiated cells was the inverse of what is normally observed in adult peripheral blood. Differentiation occurs in the absence of exogenous growth factors or cytokines, suggesting that a stimulus-independent differentiative inertia might characterize pre-B cells in vivo. Future use of this model will facilitate our understanding of normal and abnormal human pre-B cell differentiation.

A complex array of cells and molecules have been implicated in regulating mammalian B cell ontogeny, and considerable progress in elucidating this complexity has been made in the bone marrow (BM)[1] of mice (1). Although a similar level of complexity almost certainly exists in human BM, delineation of the cells and molecules that regulate human B cell ontogeny is notably lacking (2). Although progress has recently been made in characterizing the developmental stages of human B cell precursors (BCP) by cell surface antigen expression using multiparameter flow cytometry (3–6), we know very little about the growth factor requirements and differentiative potential of these cells. Several reports have described the effect of various stimuli on the differentiation of human BCP. Landreth and his colleagues observed that urine preparations collected from a patient with cyclic neutropenia could induce the appearance of pre-B (cytoplasmic μ^+/surface Ig^-) and surface Ig^+ B cells in B cell–depleted adult BM (7). Hokland and his colleagues observed that phorbol ester and/or human leukocyte–conditioned medium induced the appearance of surface IgM in $CD10^+/CD20^-$ fetal BM lymphoid cells (8). Unfortunately, a thorough analysis of the active components inducing differentiation was not presented in either report. A very recent report indicated that recombinant human IL-4 could induce the appearance of both pre-B and surface Ig^+ B cells in $CD24^+$ human fetal BM lymphoid cells (9). Here we describe the establishment and characterization of a reproducible short-term culture system for analyzing the differentiation of normal human pre-B cells to surface Ig^+ B cells. Evidence is presented that active rearrangement of κ light chain genes occurs in culture, with the subsequent appearance of surface Ig^+ B cells expressing μ/κ or μ/λ. This culture system will now permit a rigorous analysis of this critical developmental transition in human B cell ontogeny.

[1] *Abbreviations used in this paper:* APC, allophycocyanin; BCP, B cell precursors; BM, bone marrow; MFI, mean fluorescence intensity.

Materials and Methods

Cell Preparation. Fetal BM from normal 21–23-wk-old fetuses was obtained in accordance with the guidelines of the University of Minnesota Committee on the Use of Human Subjects in Research. Femoral and humeral bones were flushed with RPMI-1640/5% FCS. The low density leukocytes were isolated from the cell suspension by centrifugation over Histopaque (Sigma Chemical Co., St. Louis, MO) and washed twice with RPMI-1640/5% FCS. Monocytes were depleted by adherence to a plastic tissue culture flask for 1–2 h at 37°C in RPMI-1640/10% FCS. Cells were stored overnight at 4°C in RPMI-1640/5% FCS before cell sorting.

Serologic Reagents. PE-conjugated anti-CD10 (J5-PE) and an isotype-matched IgG2a-PE control were obtained from Coulter Immunology, Hialeah, FL. Murine mAbs recognizing human μ heavy chains (HB57), human κ light chains (HB61), CD11b/C3biR (OKM1), glycophorin A types M and N, and an isotype-matched IgG1 control were produced from hybridoma cells obtained from

J. Exp. Med. © The Rockefeller University Press • 0022-1007/90/07/0325/10 $2.00
Volume 172 July 1990 325–334

the American Type Culture Collection, Rockville, MD. Anti-My8 and anti-My9 (CD33) were kindly provided by James Griffin, Dana-Farber Cancer Institute, Boston, MA. Anti-CD2 was produced in our laboratory. Antibodies were purified from ascites on protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) using the method of Ey et al. (10). Purified HB57, HB61, and IgG1 were conjugated to FITC or biotin using the methods of Goding (11). A purified IgG1 murine mAb recognizing human λ light chains was obtained from Cappel Laboratories (No. 0201-0287; Malvern, PA). $F(ab')_2$ goat anti-human κ conjugated to FITC, $F(ab')_2$ goat anti-human λ conjugated to PE, and normal goat Ig conjugated to FITC and PE were purchased from Tago Diagnostics Inc., Burlingame, CA. The specificity of all reagents recognizing heavy or light chains was confirmed using human B cell populations (B lymphoblastoid cell lines and peripheral blood B cells). FITC-conjugated goat anti-mouse Ig was purchased from Tago. FITC-conjugated goat anti-mouse IgG1 was purchased from Southern Biotechnology Associates Inc., Birmingham, AL. Streptavidin-allophycocyanin (APC) was purchased from Becton Dickinson, Mountain View, CA.

Flow Cytometry/Cell Sorting. Fetal BM cells were stained for expression of CD10 (using J5-PE) and surface μ (using HB57-FITC), and FACS-purified by two-color cell sorting on a FACS IV (donors 1-3, Table 1) or a FACStar Plus (donors 4-8, Table 1), using our previously described methods (12). Stained cells were kept on ice before sorting, and aliquots of cells were sorted in 60–75-min intervals. Cells sorted on the FACS IV were at 4°C, whereas cells sorted on the FACStar Plus were at room temperature. Only cells exhibiting low forward angle and low right angle light scattering properties (the lymphoid gate) were sorted. Three-color analysis of surface μ, κ, and λ was performed on a FACStar Plus. FITC and PE were excited at 488 nm using a 300 mW setting on an argon ion laser. APC was excited at 635 nm using a 40 mW setting on a helium/neon laser. Single-color analysis of surface μ, κ, and λ was performed on a FACScan. Analyses were conducted on cells fixed in 1% paraformaldehyde.

Magnetic Bead Depletion. Fetal BM $CD10^+$/surface μ^- lymphoid cells were also purified by magnetic bead depletion using polymer particles with magnetite-containing cores (DYNABEADS M-450; Robbins Scientific, Mountain View, CA) conjugated to goat anti-mouse IgG. Ficoll-Hypaque–isolated fetal BM cells were adjusted to 10–15 × 10^6 cells/ml in RPMI-1640/2% FCS. Cells were then incubated for 45 min at 4°C with saturating concentrations of mAb to myeloid/monocytoid cells (My8, CD11b, CD33), erythroid precursors (glycophorin A), T cells (CD2), and mature B cells (surface μ). After one wash in RPMI-1640/2% FCS, DYNABEADS were added to a final ratio of 5 beads to 1 cell. The cell/DYNABEAD mixture was incubated for 60–90 min at 4°C on a rotary shaker, and cells bound to the beads were separated from free cells by 5 min exposure to a flat plate magnet. The highly enriched (>95%) $CD10^+$/surface μ^- cells were washed once in RPMI-1640/2% FCS and then cultured as described below.

Cell Cycle Analysis. Surface μ^+ and surface μ^- lymphoid cells were analyzed for their cell cycle status using the UV excitable dye, Hoechst 33342, essentially as described by Hollander et al. (13). Briefly, fetal BM low density leukocytes were stained with HB57 (anti-μ) or IgG1 control myeloma protein, and counterstained with FITC goat anti-mouse Ig. The cells were fixed in 0.45% paraformaldehyde in PBS overnight at 4°C, and then stained with 8 μg/ml Hoechst 33342 (Sigma) in PBS containing 0.5% Tween 20 (Sigma) overnight at 4°C. Cells with lymphoid light scatter properties were then examined for surface μ expression and DNA binding of Hoechst 33342 using a FACS 440. FITC was excited at 488 nm using an argon ion laser, and detected using a 535/20 bandpass filter. Hoechst 33342 was excited by a UV-enhanced argon ion laser operating at 350–360 nm, and detected using a 435/25 bandpass filter.

Cell Culture. After cell sorting or magnetic bead depletion the $CD10^+$/surface μ^- purified cells were re-analyzed on a FACScan to assess their purity. These cells were >95% $CD10^+$, but a minor population contained low level surface μ in some of the cell sorting experiments (see Table 1). Post-sort viability was ≥93%. Cells were suspended in RPMI-1640/10% FCS supplemented with 1% wt/vol L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, to a density of 0.4 × 10^6/ml. They were then aliquoted into 96-well round-bottomed plates (200 μl/well), and cultured for 2 d at 37°C in 5% CO_2. Wells were harvested, pooled, and the cells analyzed for viability by trypan blue exclusion (range, 60–94% from 12 different fetal BM donors) before analysis of Ig gene rearrangement and surface Ig expression. The source of FCS used in these experiments was HyClone Laboratories (Logan, UT) lot 1111795 (fetal BM donors 1-5) and lot 1111807 (fetal BM donors 6–12).

Antigenic Modulation. Fetal BM cells were stained for expression of CD10 and surface μ using J5-PE and HB57-FITC, respectively. After a 30-min incubation at 4°C the cells were washed twice, divided into two aliquots, and held at 4°C or room temperature. At 15-min intervals an aliquot was removed from 4°C and room temperature and fixed in 1% paraformaldehyde. The cells were then analyzed on a FACScan by gating on lymphoid cells with low forward angle/low right angle light scatter properties.

Southern Blotting. Cells were lysed overnight at 37°C with proteinase K (0.5 mg/ml) in TNE (10.0 mM Tris, 100 mM sodium chloride, 1 mM EDTA) buffer containing 1% SDS. DNA was isolated by phenol/chloroform extraction and ethanol precipitation, and suspended in TE. DNA was digested with 30 U of BamHI (Bethesda Research Laboratories, Gaithersburg, MD) for 8 h, and digested DNA was transferred by capillary blotting to Gene Screen Plus (NEN Research Products, Boston, MA). The membranes were prehybridized in 10% dextran sulfate, 1% SDS, 1 M sodium chloride and hybridized overnight at 65°C with a ^{32}P-(d) CTP-labeled 2.4-kb genomic C_κ probe (14). The membranes were then sequentially washed in 2× SSC, 0.5% SDS at room temperature for 10 min, 1× SSC, 0.5% SDS at 65°C for 30 min, and (if needed) 0.1× SSC, 1% SDS at 65°C until the background was low. Bands were visualized by autoradiography using X-Omat AR film (Eastman Kodak, Rochester, NY) after a 3–4-day exposure at −70°C. DNA standardization was conducted with a TCRδ probe (C_α), specific for Jδ1, 5′ untranslated to Jδ1, and Cδ (15). This probe was kindly provided by Frank Griesinger and John Kersey, University of Minnesota.

Results

CD10/Surface μ Expression in Fetal BM. We initially sought to establish a reproducible experimental system for analyzing differentiation of normal human pre-B cells using fetal BM $CD10^+$/surface μ^- cells. This choice of markers was based on our observation that CD10 is a pan-B cell antigen in fetal BM, being expressed on >95% of $CD19^+$ cells and >95% of surface μ^+ cells (6). The $CD10^+$/surface μ^- fetal BM lymphoid population contains cells at multiple stages of B (and possibly T) lineage commitment, including 60–70% cytoplasmic μ^+ pre-B cells, and is depleted of mature surface μ^+ B cells. A representative two-color bivariate contour plot with the sorting gates used to separate $CD10^+$/surface μ^- (*box A*) from $CD10^+$/surface μ^+ (*box B*) cells is shown in Fig. 1.

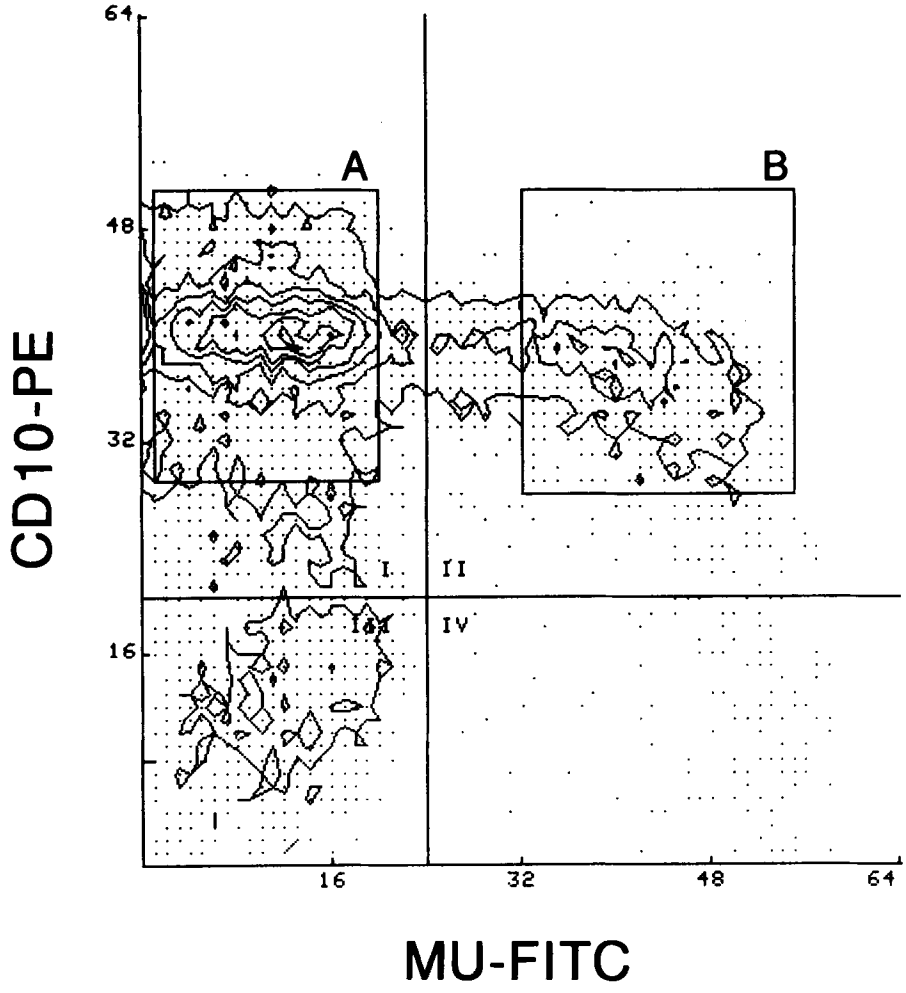

Figure 1. Two-color bivariate contour plot demonstrating the expression of cell surface CD10 (*y* axis) and surface μ (*x* axis) on fetal BM lymphoid cells from donor 7 in Table 1. Boxes define the sorting gates used to purify CD10$^+$/surface μ^- (*A*) and CD10$^+$/surface μ^+ (*B*) populations.

Consistent with data reported elsewhere (6), all surface μ^+ cells co-express CD10, whereas no CD10$^-$/surface μ^+ cells were detectable. Surface μ was expressed on 24 ± 8% (range 18–39%, n = 7) of fetal BM lymphoid cells. The small number of CD10$^-$/surface μ^- cells in the left, lower quadrant were probably erythroid precursors, since their light scattering properties are similar to lymphoid cells (16).

Acquisition of Surface μ in CD10$^+$/Surface μ^- Cells. We initially attempted to confirm a recent report that suggested that IL-4 could induce the differentiation of pre-B cells → B cells in FACS-purified CD24$^+$ fetal BM lymphoid cells (9). In experiments conducted with three individual fetal BM specimens, recombinant human IL-4 (1–100 U/ml) did not induce acquisition of cytoplasmic or surface μ in a dose-dependent manner. Surprisingly, a consistent and dramatic increase in surface μ was observed in CD10$^+$/surface μ^- FACS-purified cells cultured in RPMI-1640/10% FCS alone. Analysis of CD10$^+$/surface μ^- cells immediately following FACS-purification is shown in a representative experiment in Fig. 2. A small fraction (4%) of the post-sort cells expressed a very low level of surface μ (Fig. 2 *B*). After 2 d in culture 26% of the cells were surface μ^+ (Fig. 2 *D*), with a fourfold increase in surface μ fluorescence intensity compared with the post-sort analysis in Fig. 2 *B*. Table 1 summarizes experiments conducted with CD10$^+$/surface μ^- FACS-purified cells isolated from eight different fetal BM donors. The percent of residual surface μ^+ cells post-sort was 10 ± 6% (range 4–18%, n = 7). However, the fluorescence intensity of the residual surface μ was only 37 ± 23% (range 11–67%, n = 6), compared with surface μ^+ nonsorted cells. Acquisition of surface μ on CD10$^+$/surface μ^- lymphoid cells was completely reproducible, with 38 ± 11% (range 23–50%, n = 8) of cells expressing surface μ after 2 d in culture. It is noteworthy that this frequency of surface μ^+ lymphoid cells (38%) is greater than the frequency of surface μ^+ lymphoid cells (24%) in pre-sorted BM. Importantly, surface μ acquisition occurred using two different lots of FCS, and detection was achieved using different polyclonal and monoclonal anti-μ reagents.

Simple outgrowth or preferential survival of residual surface μ^+ cells does not explain our results. As shown in Table 1, the FACS-purified CD10$^+$/surface μ^- cells were quiescent in 2-d culture, with a viability of 75 ± 9% (range 60–91%, n = 8) on day 2. Cell yield was 95 ± 36% (range 70–167%, n = 6) compared with day 0. Furthermore, the FACS-purified CD10$^+$/surface μ^+ cells shown in Fig. 1, *box B* were 70%

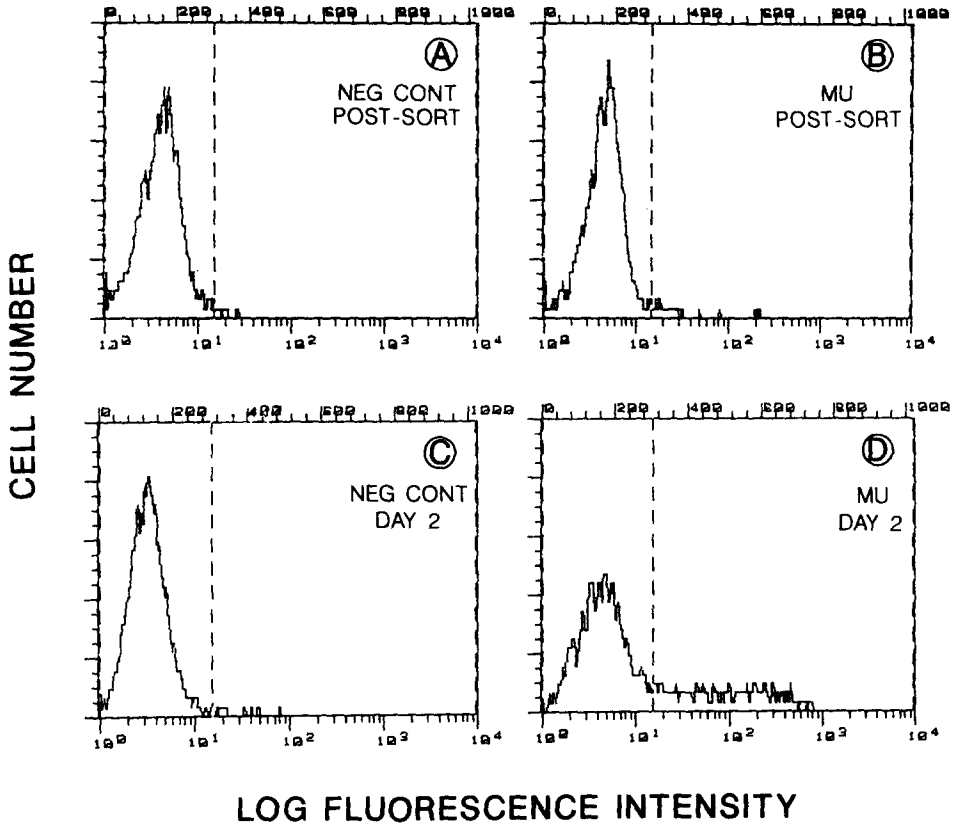

Figure 2. Acquisition of surface μ after a 2-d culture of CD10$^+$/surface μ^- FACS-purified fetal BM lymphoid cells isolated from donor 6 in Table 1. (*A*) Negative control staining (G1-FITC) of post-sort cells, (*B*) residual surface μ detected with HB57-FITC on post-sort cells, (*C*) negative control staining (G1-FITC) of cells cultured for 2 d, (*D*) surface μ detected with HB57-FITC on cells cultured for 2 d. Vertical dashed line defines background staining, with >98% of the cells found in channels to the left of the vertical line in *A* and *C*.

viable after 2 d in culture, compared with the FACS-purified CD10$^+$/surface μ^- cells shown in Fig. 1 (*box A*) which were 72% viable after 2 d in culture. A direct analysis of the proliferative status of non-sorted surface μ^+ and surface μ^- fetal BM lymphoid cells was conducted by two-color staining using HB57-FITC to detect surface μ, and Hoechst 33342 to analyze cell cycle. As shown in Fig. 3 (*box A*), surface μ^+ cells were >98% in G_0/G_1, with <2% detectable proliferating cells in S and G_2+M. In contrast, a distinct population of proliferating surface μ^- cells (~8% of total surface μ^- cells) were detectable (*box C*). These data are entirely consistent with previous analyses of human BM, B lineage cells, which concluded that surface μ^+ B cells are quiescent (13, 17). These collective data argue strongly against simple outgrowth or preferential survival of surface μ^+ cells as an explanation for our results.

Table 1. *Appearance of Surface μ on FACS-Purified CD10$^+$/Surface μ^- Cells*

	Surface μ^+		Viable	Yield
Donor	Post-sort	Day 2	Day 2	Day 2
	%		%	
1	ND	49	76	167
2	16	35	72	ND
3	18	50	60	ND
4	8	46	82	78
5	7	23	72	91
6	4	26	70	70
7	4	30	75	81
8	10	48	91	84

Surface μ values represent percent of lymphoid cells (based on low forward angle and right angle light scattering properties) expressing surface μ as determined by flow cytometry.

We also considered whether surface μ might modulate during the sorting procedure, thereby resulting in "contamination" of the CD10$^+$/surface μ^- cells with modulated surface μ^+ B cells. This was experimentally tested by sequentially examining surface μ on cells held at 4°C or room temperature. Both temperatures were studied since donors 1–3 were sorted at 4°C, whereas donors 4–8 were sorted at room temperature. As shown in Fig. 4, the percent of lymphoid cells expressing surface μ and the level of surface μ expressed as mean fluorescence intensity (MFI) did not change during a 75-min incubation at 4°C. Incubation at room temperature resulted in a slight reduction in surface μ^+ cells from 8.9 to 7.6%, and a reduction in MFI from 60 to 44. Similar results were obtained in a separate experiment. Thus, in donors 4–8 the low level modulation of surface μ during the final 30–45 min of the sort would result in an insignificant contribution (<3%) to the CD10$^+$/surface μ^- sorted cells. The dramatic increase in surface μ^+ cells in Table 1 can there-

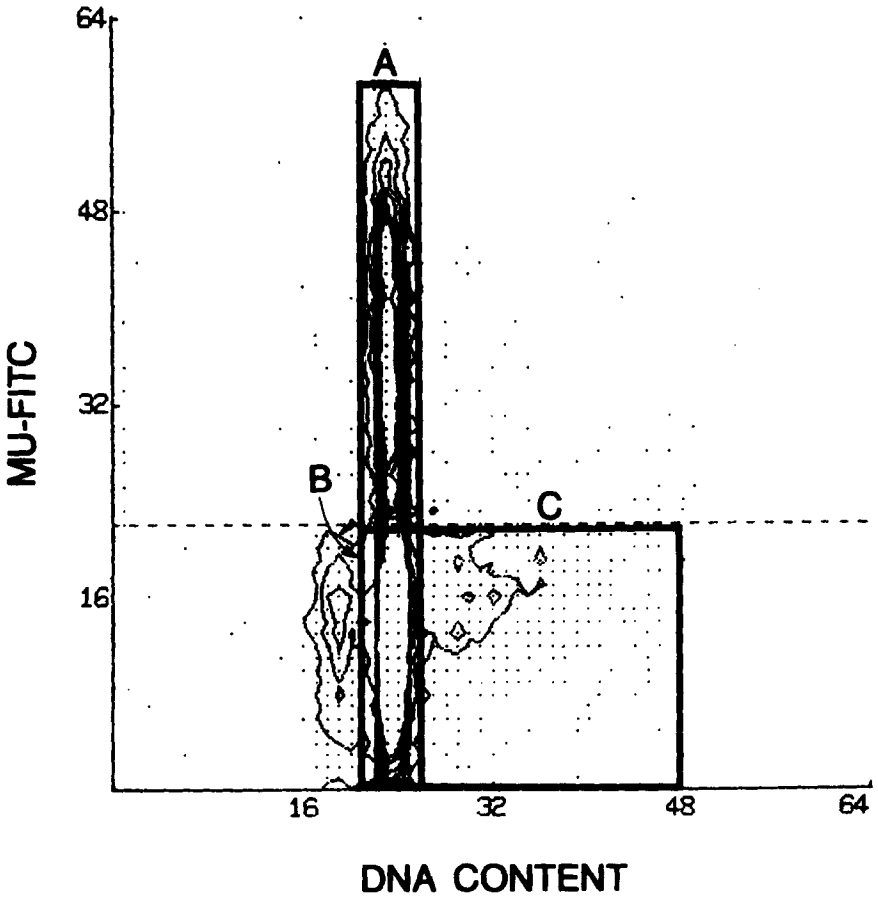

Figure 3. Analysis of DNA content (detected with Hoechst 33342) and surface μ expression (detected with HB57 + FITC goat anti-mouse Ig) on fetal BM lymphoid cells. Horizontal dashed line distinguishes surface μ^+ from surface μ^- cells, defined by control staining with an IgG1 myeloma protein. (*Box A*) surface μ^+ cells in G0/G1, (*box B*) surface μ^- cells in G_0/G_1, box C-surface μ^- cells in S and G2+M phases of the cell cycle. The contour population to the left of box B is an artifact, and probably represents a small number of cells that did not completely intercalate Hoechst 33342.

fore not be attributed to reexpression of surface μ following antigenic modulation.

Acquisition of Surface κ and λ in CD10$^+$/Surface μ^- Cells. The expression of κ and λ light chains on noncultured fetal BM lymphoid cells is presented in Fig. 5 *B*. Gating on the surface μ^+ lymphoid cells by three-color analysis reveals no significant population of μ^+/light chain$^-$ cells (Fig. 5 *C*). Analysis of fresh fetal BM lymphoid cells from several donors revealed that 11 ± 2% (range, 10–13%, n = 5) of lymphoid cells expressed κ and 12 ± 2% (range 10–14%, n = 3) expressed λ. Acquisition of surface κ and λ was examined using CD10$^+$/surface Ig$^-$ cells isolated by magnetic bead depletion. As shown in Table 2, the percent of residual light chain$^+$ cells after magnetic bead depletion was extremely low, and this method was clearly superior to FACS for depleting surface Ig$^+$ cells. When these cells were cultured in RPMI-1640/10% FCS a striking appearance of surface κ^+ (6.8 ± 3.6%, range 4–12%) and surface λ^+ (20.2 ± 9.4%, range 9–28%) was observed (Table 2). Sequential appearance of surface κ and λ in CD10$^+$/surface Ig$^-$ cells isolated from donor 10 is shown in Fig. 6. Highly efficient removal of surface Ig$^+$ cells is demonstrated by the two-color κ/λ profile at day 0. Since magnetic bead depletion was conducted at 4°C, antigenic modulation cannot account for the absence of detectable surface Ig$^+$ cells. A dramatic appearance of surface λ^+ and κ^+ cells is evident by day 1 (a >50-fold increase in sur-

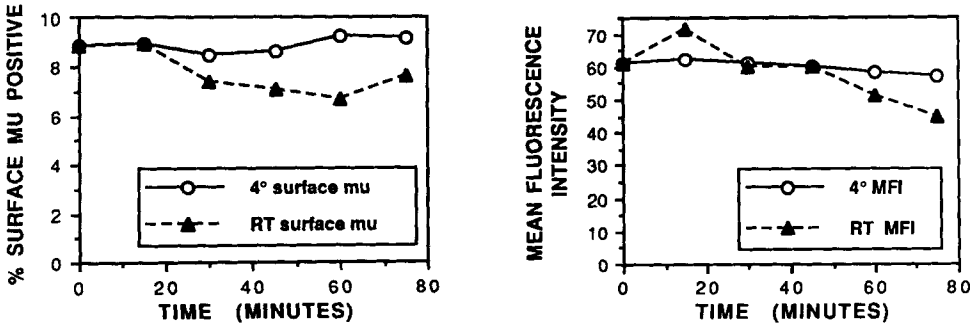

Figure 4. Stability of surface μ expression as a function of time and temperature. Antigenic modulation of surface μ was investigated at 4°C or room temperature (RT), as described in Materials and Methods, and changes in surface μ expression are presented as percent of surface μ^+ cells (*left profile*) and mean fluorescence intensity (MFI, *right profile*).

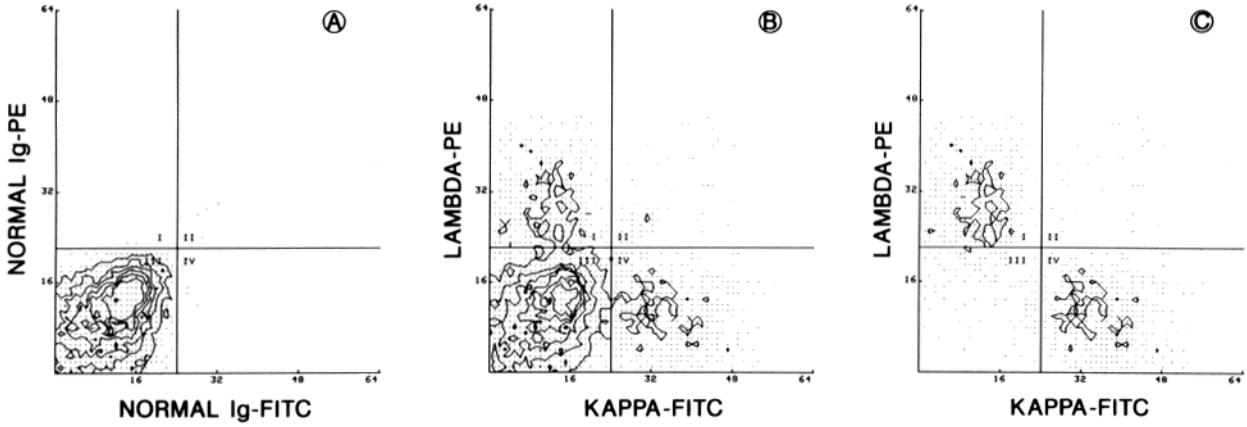

Figure 5. Three-color analysis of cell surface μ, κ, λ on Ficoll-Hypaque–isolated fetal BM lymphoid cells isolated from donor 7 in Table 1. Analyses in *A* and *B* are gated on lymphoid cells exhibiting low forward angle and low right angle light scatter. (*A*) Negative control staining of lymphoid cells, (*B*) expression of surface κ and λ on lymphoid cells, (*C*) expression of surface κ and λ gated on only those lymphoid cells expressing surface μ.

face Ig^+ cells compared with day 0), and continues to increase up to 4 d in culture. Two points are worth emphasizing regarding these experiments. First is the significant reversal of the κ/λ ratio in differentiated cells from all four donors studied. Second is the fact that differentiation occurred in the *absence* of cell-bound anti-CD10 mAb, since the purification of $CD10^+$/surface Ig^- cells by magnetic bead depletion was accomplished by negative selection.

κ Gene Rearrangement. We next determined whether acquisition of cell surface κ was due to rearrangement of κ light chain genes in vitro, or transcriptional activation of prior functional rearrangements in vivo. Fig. 7 is a Southern blot obtained by hybridizing BamHI digests with a C_κ genomic probe. Fig. 7 (*top*) shows the 12-kb germline band in the CCRF-CEM T leukemic cell line (lane *1*), and a single 7-kb rearrangement in the BET-2 B lymphoblastoid cell line (lane *4*). Lanes *2* and *3* represent $CD10^+$/surface μ^- cells post magnetic bead depletion and after 2 d in culture, respectively.

Table 2. *Appearance of Surface κ and λ on Magnetic Bead–purified $CD10^+$/Surface μ^- Cells*

	Surface κ^+		Surface λ^+		Viable	Yield
Donor	Post-deplete	Day 2	Post-deplete	Day 2	Day 2	Day 2
	%		%		%	
9	<1	6	<1	28	94	74
10	0	4	<1	9	74	70
11	<1	5	2	28	60	50
12	1	12	3	16	84	87

Surface κ and λ values represent percent of lymphoid cells (based on low forward angle and right angle light scattering properties) expressing surface κ or λ as determined by flow cytometry.

A decrease in the intensity of the 12-kb germline band and the presence of a new 20-kb rearrangement in 2 d cultured cells (lane *3*), compared with post-magnetic bead depleted cells (lane *2*), is evident. Similar results were obtained using cells from two other fetal BM donors. The 20-kb rearrangement was not present in CCRF-CEM (lane *1*) or BET-2 (lane *4*), nor has it been observed using these cell lines as controls in separate experiments. Furthermore, analysis of fibroblasts from one fetal BM donor revealed no 20-kb band (data not shown), confirming that this is a specific rearrangement and not a RFLP. Prolonged exposure of Southern blots revealed a faint 20-kb rearrangement in noncultured $CD10^+$/surface μ^- cells (data not shown). Thus, this could constitute a nontranscribed functional rearrangement that increases in frequency in cultured $CD10^+$/surface μ^- cells.

When the blot at the top of Fig. 7 was stripped and rehybridized with the TCR-δ probe C_α, identical amounts of DNA were demonstrated in lanes *2* and *3* (Fig. 7, *bottom*). The absence of detectable rearrangements in lanes *2* and *3* is also consistent with data to be reported elsewhere, indicating that TCR-δ rearrangements cannot be detected in fetal BM B lineage cells by Southern blotting (17a).

Discussion

The differentiation of surface Ig^- pre-B cells into surface Ig^+ B cells is a critical transition in mammalian B cell ontogeny. Much of our current knowledge regarding this transition is derived from murine studies using normal BM lymphoid cells, leukemic cell lines, Abelson transformants, and Whitlock-Witte cultures (reviewed in reference 18). In vitro studies conducted with normal (7–9) and fresh leukemic (19) human pre-B cells have described at least partial acquisition of surface Ig in vitro, although the culture conditions and source of cells varied between laboratories. In the current study we describe a cytokine/growth factor–independent, reproduc-

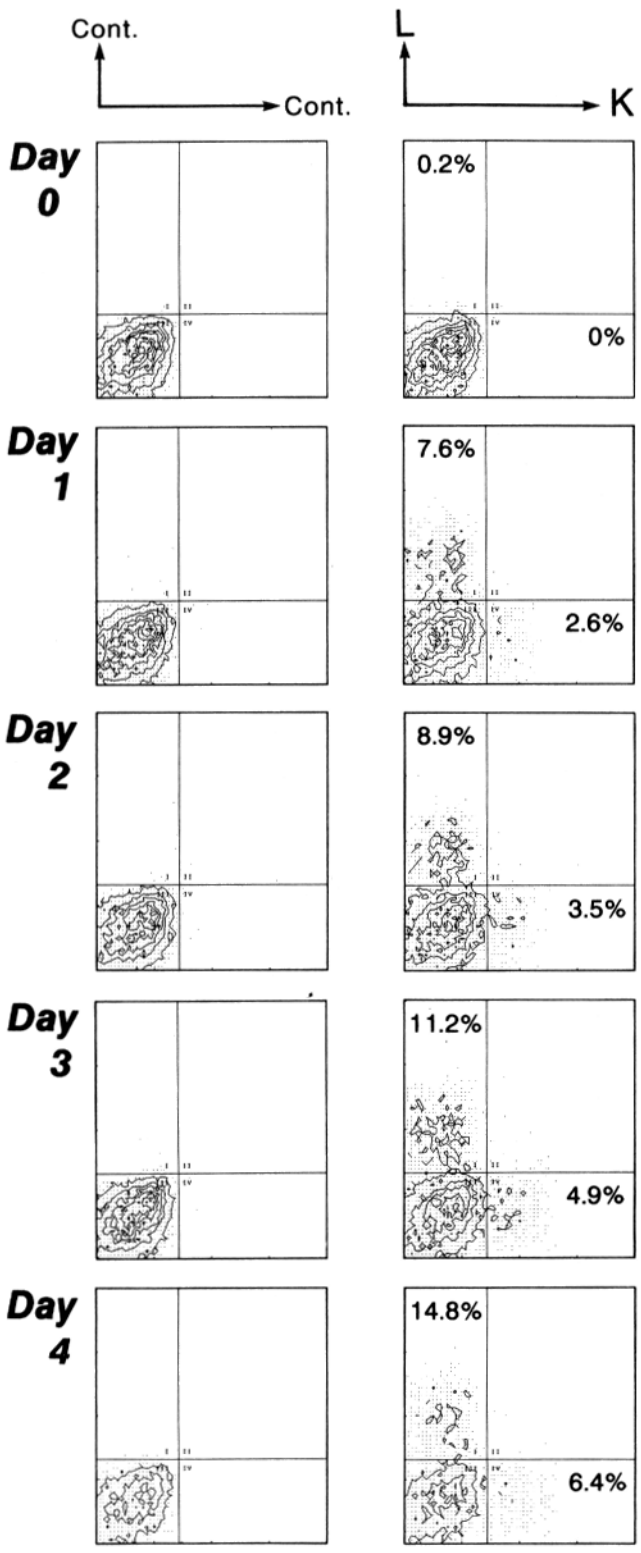

Figure 6. Two-color bivariate contour plot examining the daily levels of surface κ^+ (*x*-axis) and λ^+ (*y*-axis) using magnetic bead–purified CD10$^+$/surface μ^- cells cultured up to 4 d. The contour plots in the left column are the daily control stainings used to set the background gates for κ/λ detection. The intensity of the contour profiles on day 4 are decreased because only half as many cells were available for analysis. Fetal BM cells were from donor 10 in Table 1.

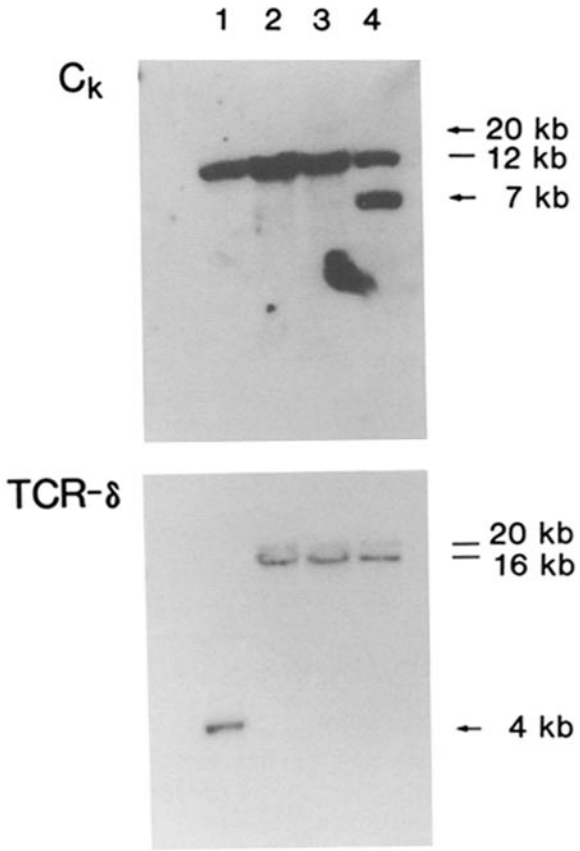

Figure 7. Southern blot analysis of κ rearrangements. DNA was digested with BamHI and hybridized with a [^{32}P]dCTP-labeled genomic C_κ probe (*top*). The blot was then stripped and rehybridized (*bottom*) with a [^{32}P]dCTP-labeled cDNA TCRδ probe, designated C_x, specific for Jδ1, 5′ untranslated to Jδ1, and Cδ. (Lane *1*) CCRF-CEM T leukemic cell line, (lane *2*) CD10$^+$/surface μ^- cells isolated by magnetic bead depletion, (lane *3*) same cells as lane *2* but cultured for 2 d in RPMI-1640/10% FCS, (lane *4*) BET-2 B lymphoblastoid cell line. The cells in lane *2* were <1% surface κ^+ and λ^+. The cells in lane *3* were 9% κ^+ and 18% λ^+. Horizontal lines identify germline fragments, horizontal arrows identify rearranged fragments.

ible culture system that supports human pre-B to B cell differentiation of normal cells. The salient characteristics of our culture system include direct evidence that: (*a*) κ light chain gene rearrangement occurs in culture, and (*b*) cells acquire cell surface μ/κ or cell surface μ/λ Ig receptors.

CD10$^+$/surface μ^- fetal BM lymphoid cells were selected as the starting population in our experiments since we have recently shown that CD10 is a pan-B cell antigen in fetal BM, being expressed on >95% of CD19$^+$ and >95% of surface μ^+ cells (6). The CD10$^+$/surface μ^- population contains cytoplasmic μ^- BCP and cytoplasmic μ^+ pre-B cells (ratio of ~1:2), but accessory cells (e.g., T cells, macrophages, or stromal cells) that may affect pre-B cell differentiation are absent. We (20) are unaware of reports implicating CD10, now known to be the enzyme neutral endopeptidase (21, 22), in regulating proliferation or differentiation of lymphoid cells. Anti-CD10 does not deliver a direct differentiative signal to pre-B cells in our culture system since CD10$^+$/surface μ^-

cells differentiate in the absence of cell-bound anti-CD10 (Fig. 6, Table 2).

Data in Figs. 2 and 6 and Tables 1 and 2 provide direct evidence that $CD10^+$/surface μ^- cells differentiate in vitro into surface Ig^+ cells expressing μ/κ or μ/λ. Additional, corroborative evidence shows that κ light chain gene rearrangements occur, based on the decrease in the intensity of the germline κ band in $CD10^+$/surface μ^- cells cultured for 2 d (Fig. 7, *top*, lane *3*). Current models of light chain gene rearrangement in both mouse (23, 24) and man (25, 26) strongly argue for a $\kappa \rightarrow \lambda$ hierarchy. Thus, the decrease in intensity of the germline κ band (Fig. 7) probably reflects functional κ rearrangements, and most likely κ deletions followed by functional (and possibly nonfunctional) λ rearrangements. Future studies that analyze V κ and V λ usage in $CD10^+$/surface μ^- cultured cells will shed more light on this issue.

An unexpected observation was the striking preponderance of surface λ^+ over surface κ^+ cells in our pre-B cell differentiation culture system. This was a consistent finding in all four donors (Table 2), and was observed sequentially up to 4 d in culture (Fig. 6). Reagent specificity does not explain this phenomenon since identical results were obtained using polyclonal and monoclonal anti–light chain reagents. Furthermore, the reagents used in this study detected κ^+ and λ^+ cells at the expected 2:1 ratio in peripheral blood. At least two other explanations can be offered. First, the high percent of λ^+ cells may include bona fide λ and the recently described ω light-chain protein (27, 28). Alternatively, a mechanism may exist in vivo for regulating the κ/λ ratio that is absent from our culture system.

Several prior studies have examined in vitro differentiation of human pre-B cells from normal BM (7–9, 29), and BM from patients following chemotherapy for treatment of leukemia (29, 30). It is difficult to directly compare our data with previous reports, since no two studies used precisely the same cell isolation procedures, culture conditions, and methods of serologic analysis. Serum source alone may not explain our observations, since pre-B cell differentiation in our culture system was reproducible in two separate lots of FCS. Hokland et al. reported some evidence of pre-B cell differentiation using fetal BM cells enriched for a $CD10^+$/$CD20^-$ phenotype and cultured in phorbol ester plus leukocyte-conditioned medium (8). Cells cultured in medium alone were not investigated. Hofman et al. utilized FACS-purified $CD24^+$ cells cultured in the presence or absence of a fetal BM–derived adherent cell feeder layer to observe pre-B to B cell differentiation in the presence of IL-4 (9). No differentiation occurred in the absence of IL-4. Our results differ substantially with Hofman et al.; we observed no obligatory or potentiating effect of IL-4. A major difference between our studies and those of Hofman et al. was the cell isolation strategy. FACS-purified $CD24^+$ fetal BM lymphoid cells (9) would encompass cells at all stages of B lineage development except plasma cells, including surface μ^+ B cells (31, 32). In contrast, FACS-purified $CD10^+$/surface μ^- cells used in our study were largely depleted of surface μ^+ cells, and magnetic bead–purified $CD10^+$/surface μ^- cells were completely depleted of surface μ^+ cells. We would emphasize that the data reported herein are the first to examine rearrangement of κ light chain genes and acquisition of surface μ/κ, surface μ/λ in normal, differentiating human pre-B cells.

The growth factor/cytokine-independent nature of normal pre-B cell differentiation described in this study suggests that a stimulus-independent differentiative inertia might characterize these cells in vivo. This "spontaneous" differentiation bears a striking resemblance to that observed in a leukemic cell line, designated BLIN-1, recently established in this laboratory (33). BLIN-1 was established from a patient with pre-B acute lymphoblastic leukemia. The hallmark characteristic of BLIN-1 is its propensity to undergo spontaneous pre-B $\rightarrow$ B cell differentiation, characterized by functional rearrangement and expression of κ light chain genes (33). Differentiation cannot be enhanced by several cytokines tested to date, but is enhanced by culturing BLIN-1 cells in low serum, or serum-free medium (33). The recombinase enzyme(s) and transcriptional factors that mediate rearrangement and expression of κ light chain genes may be under similar control in normal pre-B cells and the BLIN-1 pre-B ALL cell line. Although not directly comparable to our studies because of differences in experimental design, data derived from murine studies also support the occurrence of "spontaneous" pre-B cell differentiation in vitro. Kincade et al. observed the formation of surface μ^+ clones in semisolid agar from normal BM depleted of surface μ^+ cells (34). Surface μ^+ clones were not formed unless the BM cells were initially cultured in liquid suspension for 24 h. One notable difference between our studies and those of Kincade et al. is the accessory cell or soluble mediator dependency of murine pre-B cell differentiation.

In conclusion, we have established and characterized an experimental system for studying the differentiation of normal human pre-B cells. The existence of extensive κ light chain gene rearrangements, and the subsequent appearance of surface Ig^+ B cells expressing μ/κ or μ/λ, suggests this culture system is a legitimate model that may recapitulate pre-B cell differentiation in vivo. Future use of this model should hasten elucidation of soluble mediators that may regulate pre-B cell differentiation and analysis of light chain V gene usage, which in turn could provide insights into the etiology of immunodeficiencies or lymphoproliferative disorders involving pre-B cells.

We thank Lenise Burch and Mike Hupke for FACS analysis and sorting; John Kersey, Brian Van Ness, Martin Wolf, and Jim Greenberg for helpful discussions; and Lisa Balsis for manuscript preparation. We also thank James Griffin, Dana-Farber Cancer Institute, for providing mAb to myeloid/monocytoid antigens.

This work was supported by grants PO1 CA-21737 and RO1 CA-31685 from the National Cancer Institute/National Institutes of Health; American Cancer Society Institutional Research Grant IN-13-29-10; the Leukemia Task Force; and the Children's Cancer Research Fund. T.W. LeBien was a Scholar of the Leukemia Society of America during the course of these studies.

The current address of J.G. Villablanca is Children's Hospital of California, Division of Hematology-Oncology, P.O. Box 54700, 4650 Sunset Blvd., Los Angeles, CA 90054-0700.

Address correspondence to Dr. Tucker W. LeBien, Box 609 UMHC, Department of Laboratory Medicine/Pathology, University of Minnesota, Minneapolis, MN 55455-0315.

Received for publication 21 August 1989 and in revised form 29 March 1990.

References

1. Kincade, P.W., G. Lee, C.E. Pietrangeli, S.-I. Hayashi, and J.M. Gimble. 1989. Cells and molecules that regulate B lymphopoiesis in bone marrow. *Annu. Rev. Immunol.* 7:111.
2. LeBien, T.W. 1989. Growing human B-cell precursors in vitro: the continuing challenge. *Immunol. Today.* 10:296.
3. Ryan, D., S. Kossover, S. Mitchel, C. Frantz, L. Hennessy, and H. Cohen. 1986. Subpopulations of common acute lymphoblastic leukemia antigen-positive lymphoid cells in normal bone marrow identified by hematopoietic differentiation antigens. *Blood.* 68:417.
4. Loken, M.R., V.O. Shah, K.L. Dattilio, and C.I. Civin. 1987. Flow cytometric analysis of human bone marrow. II. Normal B lymphocyte development. *Blood.* 70:1316.
5. Kansas, G.D., and M.O. Dailey. 1989. Expression of adhesion structures during B cell development in man. *J. Immunol.* 142:3058.
6. LeBien, T.W., B. Wörmann, J.G. Villablanca, C.-L. Law, L.M. Steinberg, V.O. Shah, and M.R. Loken. 1990. Multiparameter flow cytometric analysis of human fetal bone marrow B cells. *Leukemia.* In press.
7. Landreth, K.S., D. Engelhard, M.H. Beare, P.W. Kincade, N. Kapoor, and R.A. Good. 1985. Regulation of human B lymphopoiesis: effect of a urinary activity associated with cyclic neutropenia. *J. Immunol.* 134:2305.
8. Hokland, P., J. Ritz, S.F. Schlossman, and L.M. Nadler. 1985. Orderly expression of B cell antigens during the in vitro differentiation of nonmalignant human pre-B cells. *J. Immunol.* 135:1746.
9. Hofman, F.M., M. Brock, C.R. Taylor, and B. Lyons. 1988. IL-4 regulates differentiation and proliferation of human precursor B cells. *J. Immunol.* 141:1185.
10. Ey, P.L., S.J. Prowse, and C.R. Jenkins. 1978. Isolation of pure IgG1, IgG2a, and IgG2b from mouse serum using protein A-Sepharose. *Immunochemistry.* 15:429.
11. Goding, J.W. 1986. Monoclonal Antibodies: Principles and Practice. 2nd Ed. Academic Press, New York. 276 pp.
12. Wörmann, B., T.G. Gesner, R.A. Mufson, and T.W. LeBien. 1989. Proliferative effect of interleukin-3 on normal and leukemic human B cell precursors. *Leukemia.* 3:399.
13. Hollander, Z., V.O. Shah, C.I. Civin, and M.R. Loken. 1988. Assessment of proliferation during maturation of the B lymphoid lineage in normal human bone marrow. *Blood.* 71:528.
14. Korsmeyer, S.A., A. Arnold, A. Bakhshi, J.V. Ravetch, U. Siebenlist, R.A. Hieter, S.O. Sharrow, T.W. LeBien, J.H. Kersey, D.G. Poplack, P. Leder, and T.A. Waldmann. 1983. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphoblastic leukemias of T-cell and B-cell precursor origins. *J. Clin. Invest.* 71:301.
15. Hara, J., S.H. Benedict, E. Champagne, Y. Takihara, T.W. Mak, M. Minden, and E.W. Gelfand. 1988. T cell receptor δ gene rearrangements in acute lymphoblastic leukemia. *J. Clin. Invest.* 82:1974.
16. Loken, M.R., V.O. Shah, K.L. Dattilio, and C.I. Civin. 1987. Flow cytometric analysis of human bone marrow. I. Normal erythroid development. *Blood.* 69:255.
17. Campana, D., and G. Janossy. 1988. Proliferation of normal and malignant human immature lymphoid cells. *Blood.* 71:1201.

17a. LeBien, T.W., R.L. Elstrom, M. Moseley, J.H. Kersey, and F. Griesinger. 1990. Analysis of Ig in TCR gene rearrangements in human fetal bone marrow B lineage cells. *Blood.* In press.

18. Kincade, P.W. 1987. Experimental models for understanding B lymphocyte formation. *Adv. Immunol.* 41:181.
19. Cossman, J., L.M. Neckers, A. Arnold, and S.J. Korsmeyer. 1982. Induction of differentiation in a case of acute lymphoblastic leukemia. *N. Engl. J. Med.* 307:1251.
20. LeBien, T.W., and R.T. McCormack. 1989. The common acute lymphoblastic leukemia antigen (CD10). Emancipation from a functional enigma. *Blood.* 73:625.
21. Letarte, M., S. Vera, R. Tran, J.B.L. Addis, R.J. Onizuka, E.J. Quackenbush, C.V. Jongeneel, and R.R. McInnes. 1988. Common acute lymphocytic leukemia antigen is identical to neutral endopeptidase. *J. Exp. Med.* 168:1247.
22. Shipp, M.A., J. Vijayaraghavan, E.V. Schmidt, E.L. Masteller, L. D'Adamio, L.B. Hersh, and E.L. Reinherz. 1989. Common acute lymphoblastic leukemia antigen (CALLA) is active neutral endopeptidase 24.11 ("enkephalinase"): direct evidence by cDNA transfection analysis. *Proc. Natl. Acad. Sci. USA.* 86:297.
23. Alt, F.W., V. Enea, A.L.M. Bothwell, and D. Baltimore. 1980. Activity of multiple light chain genes in murine myeloma cells producing a single, functional light chain. *Cell.* 21:1.
24. Coleclough, C., R.P. Perry, K. Karjalainen, and M. Weigert. 1981. Aberrant rearrangements contribute significantly to the allelic exclusion of immunoglobulin gene expression. *Nature (Lond.).* 290:372.
25. Hieter, P.A., S.J. Korsmeyer, T.A. Waldmann, and P. Leder. 1981. Human immunoglobulin κ light-chain genes are deleted or rearranged in λ-producing B cells. *Nature (Lond.).* 290:368.
26. Korsmeyer, S.J., P.A. Hieter, S.O. Sharrow, C.K. Goldman, P. Leder, and T.A. Waldmann. 1981. Normal human B cells display ordered light-chain gene rearrangements and deletions. *J. Exp. Med.* 156:975.
27. Pillai, S., and D. Baltimore. 1987. Formation of disulphide-linked $\mu_2\omega_2$ tetramers in pre-B cells by the 18K ω-immuno-

globulin light chain. *Nature (Lond.).* 329:172.
28. Hollis, G.F., R.J. Evans, J.M. Stafford-Hollis, S.J. Korsmeyer, and J.P. McKearn. 1989. Immunoglobulin λlight-chain-related genes 14.1 and 16.1 are expressed in pre-B cells and may encode the human immunoglobulin ω light-chain protein. *Proc. Natl. Acad. Sci. USA.* 86:5552.
29. Pearl, E.R. 1983. Pre-B cells in normal human bone marrow and in bone marrow from patients with leukemia in remission: persistent quantitative differences and possible expression of cell surface IgM in vitro. *Blood.* 61:464.
30. Paolucci, P., N.T. Rapson, L. Layward, and A.R. Hayward. 1981. Growth of pre-B cells in cultures of bone marrow from children with acute lymphoblastic leukemia and other diseases. *Clin. Exp. Immunol.* 42:143.
31. Abramson, C.S., J.H. Kersey, and T.W. LeBien. 1981. A monoclonal antibody (BA-1) primarily reactive with cells of human B lymphocyte lineage. *J. Immunol.* 126:83.
32. Bofill, M., G. Janossy, M. Janossa, G.D. Burford, G.J. Seymour, P. Wernet, and E. Kelemen. 1985. Human B cell development II. Subpopulations in the human fetus. *J. Immunol.* 134:1531.
33. Wörmann, B., J.M. Anderson, J.A. Liberty, K. Gajl-Peczalska, R.D. Brunning, T.L. Silberman, D.C. Arthur, and T.W. LeBien. 1989. Establishment of a leukemic cell model for studying human pre-B to B cell differentiation. *J. Immunol.* 142:110.
34. Kincade, P.W., G. Lee, C.J. Paige, and M.P. Scheid. 1981. Cellular interactions affecting the maturation of murine B lymphocyte precursors in vitro. *J. Immunol.* 127:255.