

Unusual Patterns of Immunoglobulin Gene Rearrangement and Expression during Human B Cell Ontogeny: Human B Cells Can Simultaneously Express Cell Surface κ and λ Light Chains

By Mary E. Pauza, Julie A. Rehmann, and Tucker W. LeBien

From the Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

Summary

Immunoglobulin gene rearrangement during mammalian B cell development generally follows an ordered progression, beginning with heavy (H) chain genes and proceeding through κ and λ light (L) chain genes. To determine whether the predicted $\kappa \rightarrow \lambda$ hierarchy was occurring *in vitro*, we generated Epstein-Barr virus-transformed cell lines from cultures undergoing human pre-B cell differentiation. A total of 143 cell lines were established. 24 expressed cell surface μ/λ by flow cytometry and were clonal by Southern blotting. Surprisingly, two of the μ/λ -expressing cell lines contained both κ alleles in germline configuration, and synthesis/expression of conventional λ L chains was directly proven by immunoprecipitation/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in one of them. Thus, human fetal bone marrow B lineage cells harbor the capacity to make functional λ L chain gene rearrangements without rearranging or deleting either κ allele. A third unusual cell line, designated 30.30, was observed to coexpress cell surface κ and λ L chains associated with μ H chains. The 30.30 cell line had a diploid karyotype, a single H chain rearrangement, both κ alleles rearranged, and a single λ rearrangement. Immunoprecipitation/SDS-PAGE confirmed that 30.30 cells synthesized and expressed κ and λ L chains. Multiparameter flow cytometry was used to demonstrate the existence of κ^+/λ^+ cells in fetal bone marrow and fetal spleen at frequencies of 2–3% of the total surface Ig^+ B cell population. The flow cytometry data was confirmed by two-color immunofluorescence microscopy. The existence of normal human B cells expressing cell surface κ and λ refutes the widely accepted concept that expression of a single L chain isotype is immutable. The κ^+/λ^+ cells may represent transients undergoing L chain isotype switching.

Ig gene rearrangement during mammalian B cell development generally follows an ordered progression beginning with H chain genes, and proceeding to κ and then λ L chain genes (1–3). The outcome of a functional rearrangement at both H and L chain loci is an immature B cell expressing a clonotypic cell surface Ig receptor. Functional rearrangements of H and L chain genes are probably essential for survival of most developing B cells, since surface Ig^- B lineage cells with nonfunctional Ig gene rearrangements have never been found in secondary lymphoid tissues. Functional Ig gene rearrangements and subsequent differentiation of B cell precursors are regulated by recombination activating genes 1 and 2 (RAG-1 and -2)¹ (4), and external factors (e.g., cytokines and B cell precursor–stromal cell interactions) in the bone marrow microenvironment (5, 6). A number of

studies have also suggested that μ H chain proteins can regulate Ig gene rearrangement, through both suppression of continuing H chain gene rearrangement, and activation of L chain gene rearrangement (7, 8).

Although the H chain \rightarrow κ L chain \rightarrow λ L chain hierarchy is the predominant rearrangement sequence during B cell development, exceptions have been described. For example, Kubagawa et al. (9) characterized four EBV-transformed human fetal bone marrow (FBM) pre-B cell lines that synthesized κ L chains, but no μ H chains. Two of the four had both H chain alleles in germline, indicating that H chain gene rearrangement and expression is not an absolute requirement for κ L chain gene rearrangement and expression. Exceptions to the $\kappa \rightarrow \lambda$ hierarchy have also been reported. Several mouse cell lines have been described with functional λ rearrangements and both κ alleles in germline (10, 11), as have rare cases of human leukemic cells with λ rearrangements and germline κ alleles (12, 13).

We have developed a short-term culture that supports func-

¹ Abbreviations used in this paper: PI, propidium iodide; RAG-1, recombination activating gene 1.

tional L chain gene rearrangement and differentiation of normal human pre-B cells in vitro (14). As part of an effort to determine whether the conventional $\kappa \rightarrow \lambda$ rearrangement hierarchy was occurring in vitro, we generated EBV transformants from cultures undergoing pre-B cell differentiation. Several of these cell lines are shown to manifest patterns of L chain gene rearrangement and expression that have not been described in human B cell development. Evidence is presented that normal, nontransformed human B cells coexpressing cell surface κ and λ L chains exist in vivo.

Materials and Methods

Cells. Cells were isolated from 18–21-wk gestational age human fetal bone marrow in accordance with the guidelines of the University of Minnesota Committee on the Use of Human Subjects in Research. CD10⁺/surface L chain⁻ B cell precursors were isolated by magnetic bead depletion as described in detail elsewhere (14, 15). This enriched B cell precursor population (90–95% CD19⁺ cells) contained ~30% cytoplasmic μ^- pro-B cells, and ~70% cytoplasmic μ^+ pre-B cells. After magnetic bead depletion, the cell viability was 99% as assessed by trypan blue exclusion. The enriched CD10⁺/surface L chain⁻ cells were resuspended in RPMI-1640, 10% FCS, 100 U penicillin/ml, and 100 μ g streptomycin/ml (complete medium) at a concentration of 10⁶ cells/ml. They were immediately transformed with EBV (day 0) as described below, or incubated at 37°C, 5% CO₂ for 1 or 3 d before EBV transformation (day 1 or 3). Viability of the CD10⁺/surface L chain⁻ cells cultured for 1–3 d was 66–84%. In some experiments, the entire fetal bone marrow B lineage population was isolated by leaving out the anti- μ mAb in the magnetic bead depletion. These cells were used to determine whether B cells coexpressing κ and λ L chains exist (see below).

Fetal spleen cell suspensions were prepared by pressing small pieces of tissue through a sterile, stainless steel mesh. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation, and the adherent cells were depleted by a 1–2-h incubation on plastic tissue culture flasks. T cells were then eliminated by magnetic bead depletion using anti-CD2 and -CD7 mAb. The resulting population was >95% CD19⁺.

Antibodies. Hybridoma cells obtained from the American Type Culture Collection (Rockville, MD) were used to produce murine mAb recognizing human μ H chains (HB57), and κ L chains (HB61). HB61 was directly conjugated to FITC for use in some experiments (16). The mAb were purified from ascites using standard protein A-Sepharose chromatography (Pharmacia LKB Biotechnology, Piscataway, NJ). F(ab')₂ goat anti-human κ -FITC and F(ab')₂ goat anti-human λ -PE were used as a dual staining cocktail in two-color flow cytometry along with control F(ab')₂ goat Ig conjugated to FITC and PE. These reagents were purchased from Tago Immunologicals (Burlingame, CA). Goat anti-human κ -FITC and goat anti-human λ -tetramethyl-rhodamine isothiocyanate (TRITC) were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL), as were unconjugated and biotinylated mouse anti-human λ (product 9180-01 and 9180-08) used in immunofluorescence and immunoprecipitation assays. Murine IgG1 myeloma protein (Cappel Products, Durham, NC) was FITC conjugated or biotinylated using standard methods (16). Streptavidin-PE was purchased from Caltag Laboratories (South San Francisco, CA).

Immunofluorescent Staining, Flow Cytometry, and Fluorescence Microscopy. Cells were washed twice with fluorescence buffer (PBS,

2.5% newborn calf serum, 0.02% NaN₃) and stained with F(ab')₂ goat anti-human κ -FITC and λ -PE for 20–30 min on ice. Controls consisted of F(ab')₂ goat Ig conjugated to FITC or PE. An alternative staining protocol used mouse anti-human κ -FITC and biotinylated mouse anti-human λ plus streptavidin-PE. Controls in this protocol were mouse IgG1-FITC and biotinylated mouse IgG1. Cells were washed twice in fluorescence buffer and fixed in 1% paraformaldehyde before analysis on a FACScan[®] using Consort 30 software (Becton Dickinson & Co., Mountain View, CA). Some experiments incorporated propidium iodide (PI) (Sigma Chemical Co., St. Louis, MO) to electronically gate out nonspecific fluorescent staining of dead cells. Cells in these experiments were not fixed in paraformaldehyde. A FACStar[®] equipped with the FACStar PLUS[®] Pulse Processing Option (Becton Dickinson & Co.) was used to measure the pulse width of forward angle light scatter (17), in order to formally exclude cell doublets from some analyses. Potential Fc receptor bound Ig was disassociated by washing the cells in pH 4.0 acetate buffer for 1 min at 4°C (18).

Immunofluorescence microscopy was used to directly analyze normal B lineage cells for coexpression of cell surface κ and λ L chains. CD10⁺ fetal bone marrow B lineage cells (containing ~25% B cells expressing cell surface κ and λ) were stained on ice for 30 min with saturating concentrations of goat anti-human κ -FITC and goat anti-human λ -TRITC, washed three times in fluorescence buffer, and fixed in 1% paraformaldehyde. Fixed cells were cytocentrifuged onto microscope slides, and cell dots were mounted in one drop of 1 mg/ml *p*-phenylenediamine (Sigma Chemical Co.) in pH 8.0 glycerol. The slides were examined using a fluorescent microscope (Zeiss, Oberkochen, Germany) equipped with ploom epillumination and selective barrier filters to distinguish green and red fluorescence. ASA 400 film (Ektachrome; Eastman Kodak, Rochester, NY) was used for phase/fluorescent photomicrographs.

EBV Transformation and Culture Conditions. CD10⁺/surface L chain⁻ cells were incubated at 37°C, 5% CO₂ for 3 d with EBV obtained from culture supernatants of the B95-8 marmoset cell line. Cells were then cultured in complete medium in 96-well microtiter plates (Costar, Cambridge, MA) containing 4,000 or 10,000 irradiated (6,000 rad) fetal bone marrow fibroblasts. Seeding densities ranged from 10 to 10⁵ B cell precursors/well. One half of the culture supernatant was replaced twice weekly with fresh complete medium. Stable EBV transformants emerged in 2–4 mo.

Southern Blotting. Cells were digested at 37°C for 18 h in TNE (10 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM EDTA, pH 8.0), 2 mg/ml proteinase K, and 1% SDS. DNA was extracted in ~6 M NaCl, and precipitated in 2 vol of cold absolute ethanol. Dry DNA pellets were dissolved in 50–100 μ l TE (10 mM Tris, pH 7.6 and 1 mM EDTA, pH 8.0), and digested with EcoRI, BamHI, or BamHI-HindIII (GIBCO BRL, Gaithersburg, MD) for 4–5 h at 37°C. 2–10 μ g of DNA were loaded per lane of a 0.6 or 0.8% agarose gel and electrophoresed in TBE (0.089 M Tris, 0.089 M boric acid, and 0.002 M EDTA). Transfer of DNA to nylon membranes (GeneScreenPlus; NEN Research Products, Boston, MA) was facilitated by 10 \times SSC using vacuum blotting with PosiBlot[®] (Stratagene, La Jolla, CA) following the manufacturer's protocol. The membrane was dried at room temperature or UV crosslinked using 1,200,000 joules (UV crosslinker; Stratagene). Membranes were prehybridized in 10% dextran sulfate (Sigma Chemical Co.), 1% SDS, and 1 M NaCl. Hybridization was conducted at 65°C for 18 h with α -³²P]dCTP-labeled probes (see below). The membranes were washed sequentially with 2 \times SSC, 0.5% SDS at room temperature for 10 min; 1 \times SSC, 0.5% SDS at 63°C for 15–30 min; and, when needed, 0.1 \times SSC, 0.5% SDS

at 63°C for 15–30 min. Membranes were then exposed to a final wash with 2× SSC at room temperature for 10 min before autoradiography using X-Omat AR film (Eastman Kodak) at –70°C. Exposure times ranged from 3 to 5 d.

Probes were labeled with α -[³²P]dCTP to sp act of $\sim 2.0 \times 10^9$ dpm/ μ g using random sequence hexanucleotide primers in a DNA polymerase I (Klenow fragment) catalyzed reaction (19), according to the manufacturer's recommendations (Multiprime DNA Labeling Systems; Amersham Corp., Arlington Heights, IL). Four probes were used to assess the status of the Ig genes; a 0.8-kb germline BamHI–HindIII fragment containing the C λ_1 gene (20), a 2.5-kb germline EcoRI C κ probe (21), a 2.5-kb HindIII–BamHI fragment of the κ deleting element (κ de) (22, 23), and a 2.4-kb Sau3a genomic J μ fragment (24).

Biosynthetic Labeling, Immunoprecipitation, and SDS-PAGE. Log phase cells were biosynthetically labeled for 3 h at 37°C with L-[³⁵S]cysteine (Amersham Corp.) as previously described (25). Labeling was halted by three washes in cold PBS, and the cells were lysed in 0.1 M Tris (pH 8.1), 0.9% NaCl, 0.5% NP-40 (Particle Data Inc., Elmhurst, IL), 1 mM EDTA, 1% aprotinin, 1.0 μ g/ml leupeptin, 0.7 μ g/ml pepstatin, and 2 mM PMSF (Sigma Chemical Co.). Debris was pelleted by centrifugation at 10,000 *g* for 30 min at 4°C. Lysates from biosynthetically labeled cells were precleared twice (2 h and overnight) with 10% protein A–Sepharose CL-4B (Pharmacia LKB Biotechnology) at 4°C. 50 μ l aliquots of precleared lysate were incubated with 25 μ g of purified antibody for 3 h at 4°C. Immune complexes were precipitated with 75 μ l of 10% protein A–Sepharose containing 1 mg/ml BSA for 1 h at 4°C, and washed four times in lysis buffer containing 0.5% deoxycholate (Sigma Chemical Co.). Immune complexes were eluted, reduced in sample buffer (80 mM Tris, pH 6.8, 3% SDS, 15% glycerol, 0.01% bromophenol blue, and 5% 2-ME), and resolved on 13% SDS-PAGE gels. Gels were submerged in Amplify (Amersham Corp.) for 30 min and visualized using Kodak X-Omat AR film at –70°C.

Results

Characterization of In Vitro Differentiated Pre-B Cells Rescued by EBV Transformation. We have described a short-term culture that supports functional L chain gene rearrangement and differentiation of normal human pre-B cells (14). To determine whether the conventional $\kappa \rightarrow \lambda$ rearrangement hierarchy was occurring in vitro, we generated EBV transformants from cultures undergoing pre-B cell differentiation. CD10⁺/surface L chain[–] B cell precursors containing 60–70% pre-B cells were isolated from six donors and cultured for 1 or 3 d. As shown in Table 1, L chain gene rearrangement and expression occurred in all six. Consistent with our previous findings (14), λ expression predominated over κ in four of the six cultures (Table 1). EBV transformation of these six cultures, as well as fresh CD10⁺/surface L chain[–] cells isolated from three of the donors, culminated in the establishment of 143 cell lines. Two-color flow cytometric analysis revealed that 38 of the 143 cell lines expressed cell surface λ L chains. 24 of these cell lines were clonal by Southern blotting utilizing J μ , C κ , κ de, and C λ probes. 21 of the 24 λ -expressing clonal cell lines had rearranged the κ locus, as would be predicted from earlier studies (1–3, 26). Of the 21, 15 had both κ alleles deleted, three had one κ allele deleted and the other in germline, two had one κ allele rearranged

Table 1. Percent Analysis of κ and λ L Chain Cell Surface Expression on CD10⁺/Surface L Chain[–] B Cell Precursors Before and After Culture

Sample No.	Day 0		Day 1 or 3	
	κ^+	λ^+	κ^+	λ^+
	%			
FBM-28	0*	2	13	22
FBM-30	0	1	6	14
FBM-34	3	5	10	10
FBM-35	1	1	9	21
FBM-36	1	2	13	19
FBM-41	1	0	14	12

CD10⁺/surface L chain[–] B cell precursors were isolated as described in Materials and Methods, and analyzed for expression of cell surface κ and λ by two-color flow cytometry. The cells were then cultured in RPMI-1640/10% FCS for 1 or 3 d, and reanalyzed for expression of cell surface κ and λ .

* Percentage of κ (or λ) positive cells calculated by subtracting background staining.

and the other in germline, and one had both κ alleles rearranged. Three cell lines (35.30, 36.31, and 30.30) had unexpected patterns of L chain rearrangement–expression, and were studied in greater detail.

Identification of λ -Expressing EBV-transformed Cell Lines That Retain Germline κ Genes. The EBV-transformed cell lines 35.30 and 36.31 demonstrated a unique pattern of L chain gene rearrangement: their λ genes were rearranged but their κ genes were germline. These cell lines were derived from independent donors, with 35.30 established from the fresh CD10⁺/surface L chain[–] cells of FBM-35, and 36.31 established from the day 1 culture of FBM-36. On repeated two-color flow cytometric analyses, both cell lines expressed cell surface λ (>64%) with no cell surface κ (Fig. 1). Southern blots performed on DNA isolated from cell line 36.31 are shown in Fig. 2. A clonal H chain gene rearrangement was detected as a single 3.8-kb BamHI–HindIII fragment. The clonality of 36.31 was also supported by additional Southern blotting of BamHI-digested DNA hybridized with the J μ probe (data not shown). C κ and κ de were in germline configuration. The 36.31 cell line had a single 11.0-kb λ rearrangement (Fig. 2), consistent with the flow cytometric data in Fig. 1. Therefore, 36.31 cells have a functional λ rearrangement, with no evidence of rearrangement or deletion of C κ . The 35.30 cell line differed only by the presence of two λ rearrangements (data not shown). Metabolic labeling with L-[³⁵S]cysteine and immunoprecipitation provided direct evidence that 36.31 cells synthesized μ H chains and λ L chains (Fig. 3). The presence of a 29-kD λ L chain (Fig. 3) also eliminated the possibility that our results could be explained by the synthesis of surrogate λ L chains, which have molec-

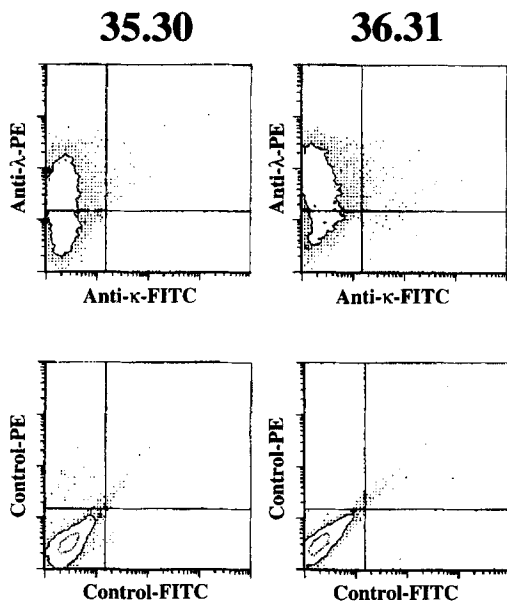


Figure 1. Surface L chain expression of EBV-transformed cell lines 35.30 and 36.31. Representative two-color flow cytometric analyses, utilizing F(ab)₂ goat anti-human κ-FITC and λ-PE, reveal that cell line 35.30 contains 64% λ⁺ cells (top left), and cell line 36.31 contains 76% λ⁺ cells (top right). (Bottom) Results of staining with preimmune F(ab)₂ goat Ig-FITC and -PE. Events appearing in the double L chain⁺ (top right) quadrants are likely due to nonspecific fluorescence of dead cells.

ular weights of 16,000, 18,000, and 22,000 (26–28). Cell line 35.30 was lost in culture, and could not be examined for λ L chain synthesis.

Identification of an EBV-transformed Cell Line That Coexpresses κ and λ L Chains. A highly unusual cell line, designated 30.30, was identified while screening the 143 EBV-transformed cell lines for surface L chain expression. This cell line origi-

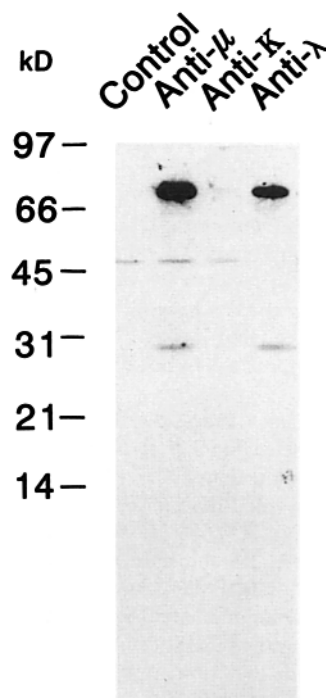


Figure 3. SDS-PAGE analysis (reducing conditions) of immunoprecipitated μ and λ proteins from L-[³⁵S]cysteine metabolically labeled 36.31 cells. (Left) The relative molecular mass (kD) of protein standards. Lysates immunoprecipitated with purified mouse IgG1 myeloma protein served as a control. Exposure time was 3 d.

nated from the day 1 culture of FBM-30, and initial two-color flow cytometric analysis revealed that 49% of the cells coexpressed κ and λ L chains (Fig. 4). Identical staining results were obtained using FITC and biotinylated mouse mAb against κ and λ L chains (data not shown). 30.30 cells have a normal diploid karyotype and express the following phenotype: CD5⁺, CD7⁻, CD10⁻, CD19⁺, CD20⁺, CD21⁺, CD23⁺, and CD40⁺. Attempts to isolate a stable population of κ⁺/λ⁺ cells by FACS[®] have been unsuccessful, with sorted κ⁺/λ⁺ cells reverting to a mixed κ⁺/λ⁺ and κ⁺/λ⁻ phenotype after several weeks in culture.

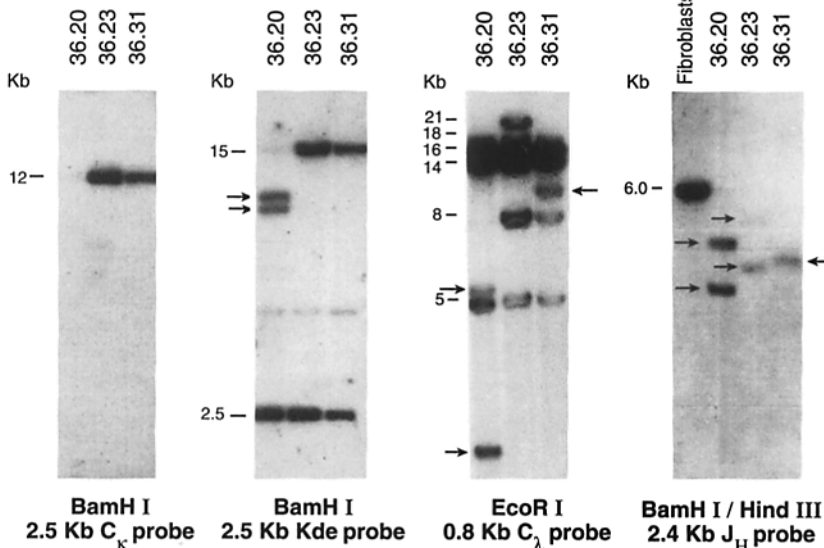


Figure 2. Ig gene rearrangements in 36.31 cells assessed by Southern blot analysis using DNA digested with the indicated restriction endonucleases and hybridized with probes specific for C_κ, k_{de}, C_λ, and J_H genes. All analyses utilized DNA isolated from EBV-transformed cell lines derived from FBM-36, with the exception of the fibroblast DNA in the first lane of the J_H blot, which was used to define the germline J_H fragment size. (Left) Germline fragments defined on the original blot with fibroblast DNA. The 2.5-kb strongly crosshybridizing fragment present in all lanes of the k_{de} blot does not map to the k_{de} locus (30). The crosshybridizing 5-kb fragment present in all lanes of the C_λ blot maps to a pseudogene on chromosome 18 (31). (Arrows) All rearrangements. Cell line 36.23 contains κ and λ genes in germline, and serves as a λ germline control to define λ rearrangements in 36.20 and 36.31. Cell line 36.20 DNA typifies a conventional λ-expressing cell line that has deleted both κ alleles, and rearranged both k_{de} alleles (~8.4 and 9.5 kb), both λ alleles (~2.4 and 5.5 kb), and both H chain alleles (~3.5 and 4.0 kb). Identical amounts of DNA were loaded per lane in each blot, except in the J_H blot where 5 μg were loaded in the first two lanes and 2.5 μg in the last two lanes.

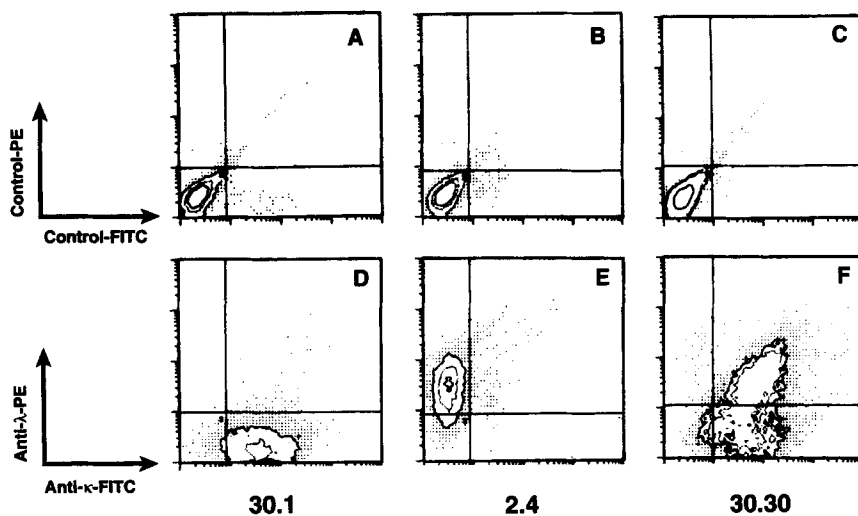


Figure 4. Two-color flow cytometric analysis showing κ/λ coexpressing 30.30 cells. Cells were stained with preimmune F(ab')₂ goat Ig-FITC and -PE (A-C) or F(ab')₂ goat anti-human κ -FITC and λ -PE (D-F). EBV-transformed cell lines demonstrating κ (30.1; 94% κ^+) or λ (2.4; 91% λ^+) surface L chain expression were used as compensation controls. The 30.30 cell line contained 49% κ^+/λ^+ cells and 45% κ^+/λ^- cells. Over a 1-yr period the percentage of κ^+/λ^+ cells ranged from 22 to 58%.

Southern blot analyses of DNA from 30.30 cells showed both κ alleles rearranged (~ 7.5 and 10.5 kb), both κ de in germline, a single ~ 8.5 -kb λ rearrangement, and a single ~ 4.8 -kb H chain rearrangement (Fig. 5). A single H chain rearrangement was also detected using BamHI-digested DNA (data not shown). Rearrangement of a single H chain allele must represent the functional VDJ rearrangement encoding the μ H chain, and provides strong evidence that 30.30 cells are clonal.

L-[³⁵S]cysteine labeling and immunoprecipitation was undertaken to directly examine whether 30.30 cells synthesized κ and λ L chains. As shown in Fig. 6, the ~ 75 -kD μ H chain was immunoprecipitated with antibodies against μ , κ , and

λ . A ~ 29 -kD κ L chain was immunoprecipitated with anti- κ , a ~ 28 -kD λ L chain was immunoprecipitated with anti- λ , and both L chains were immunoprecipitated with anti- μ . The weak ~ 29 -kD protein in the anti- λ lane and a barely visible ~ 28 -kD protein in the anti- κ lane may represent immunoprecipitation of Ig molecules containing two H chains, one κ L chain, and one λ L chain. Importantly, human surrogate λ L chains (27-29) were not detectable in the anti- μ or anti- λ immunoprecipitates, thereby excluding the possibility that κ L chains were coexpressed with surrogate λ L chains.

Detection of Normal κ/λ Coexpressing B Cells. To determine whether a normal counterpart to the 30.30 cell line exists in vivo, we performed a detailed analysis of L chain

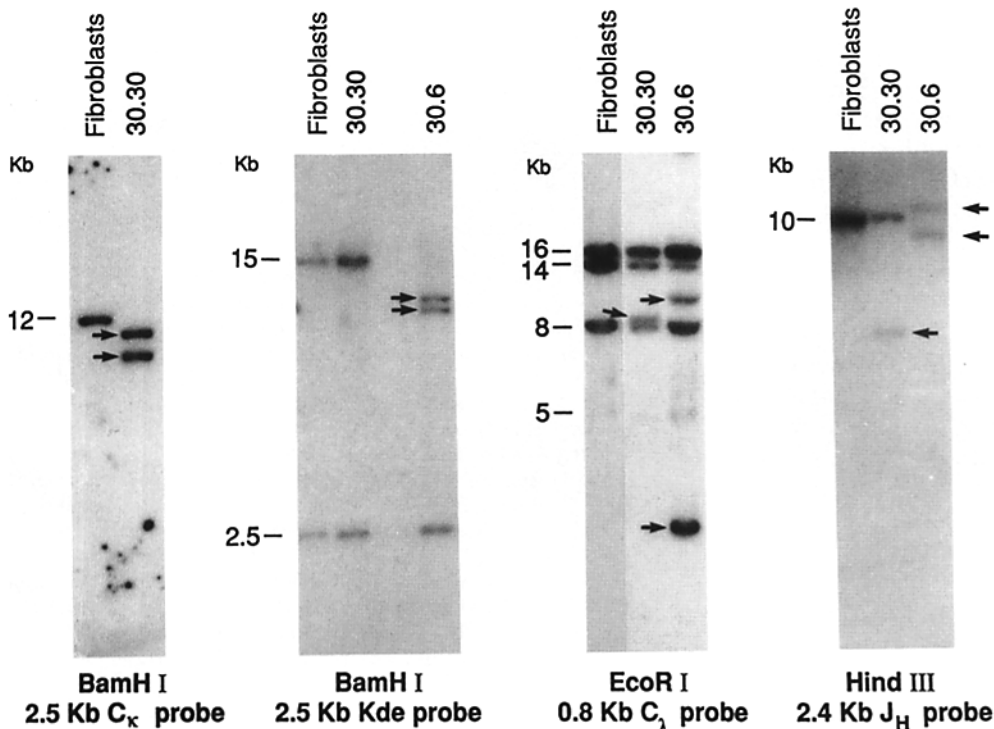


Figure 5. Southern blot analysis of Ig gene rearrangement in 30.30 cells. DNA isolated from fibroblasts, cell lines, 30.30 and 30.6 (a control λ -expressing cell line) was digested with the indicated restriction endonucleases and hybridized with probes specific for C κ , κ de, C λ , and J $_H$ genes. (Left) Germline fragments. (Arrows) Rearranged fragments.

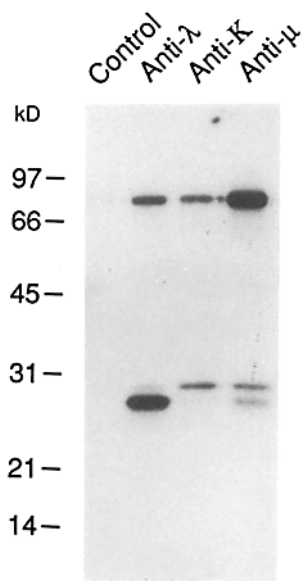
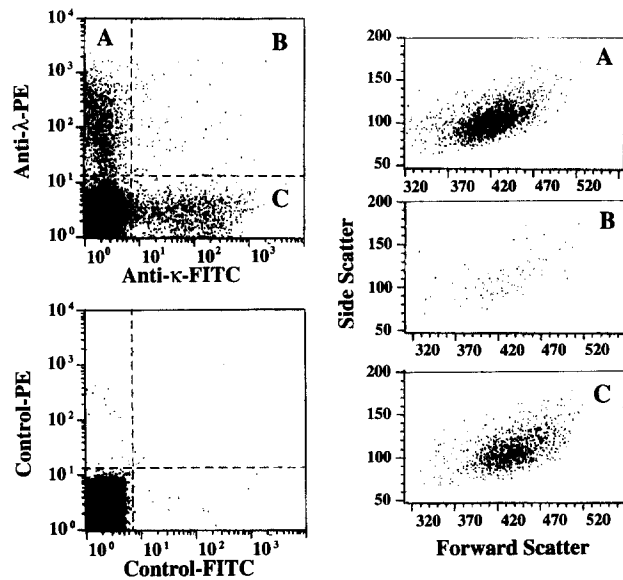


Figure 6. SDS-PAGE analysis (reducing conditions) of immunoprecipitated μ , κ , and λ proteins from [^{35}S]cysteine metabolically labeled 30.30 cells. (Left) The relative molecular mass (kD) of protein standards. Lysates immunoprecipitated with purified mouse IgG1 myeloma protein served as a control. Exposure time was 6 d.

expression on human fetal bone marrow and splenic B cells. Three-color analysis was used to assess whether κ^+/λ^+ cells exist in a PI-negative (i.e., viable) cell population. A representative experiment is shown in Fig. 7. The dot plot (top left) demonstrates the existence of κ^+/λ^+ cells (Fig. 7 B) representing 2.5% of the surface Ig $^+$ B cells in fetal bone marrow. Similarly, the spleen contained κ^+/λ^+ cells (Fig. 7 E) representing 3.1% of the surface Ig $^+$ B cells. Additional analyses revealed that κ^+/λ^+ cells comprised $2.2 \pm 0.6\%$ ($n = 13$ experiments) of fetal bone marrow surface Ig $^+$ B cells and $3.3 \pm 0.6\%$ ($n = 4$ experiments) of fetal splenic surface Ig $^+$ B cells. Three-color staining using anti- κ , anti- λ , and anti-CD19 confirmed that the κ^+/λ^+ cells were CD19 $^+$, and therefore of B lineage origin (data not shown). Fig. 7 also shows the forward and side (90°) light scatter properties of the λ^+ , κ^+/λ^+ , and κ^+ populations. The identical light scatter properties (particularly forward light scatter which is a direct measurement of cell size) of each of the three populations suggests that the κ^+/λ^+ population cannot be explained as cell doublets. The possibility that the κ^+/λ^+ population reflected a cell doublet artifact was formally excluded by pulse width analysis on 3 of the 13 fetal bone marrow donors. An analysis of cells from one donor is shown in Fig. 8. The boxed κ^+/λ^+ population in Fig. 8 A was analyzed for pulse width forward light scatter and PI characteristics (Fig. 8 B). Only 0.9% of the κ^+/λ^+ events were PI-negative doublets (top left quadrant). Similar analysis on two other donors, coupled with the donor in Fig. 8, revealed that only $0.53 \pm 0.58\%$ of the κ^+/λ^+ cells had pulse width profiles consistent with cell doublets. Importantly, analysis of the rare PI-negative doublets in the total B lineage population (Fig. 8 C, top left) revealed a random distribution amongst the κ^-/λ^- , κ^+/λ^- , κ^-/λ^+ populations (Fig. 8 D). Treatment of fetal bone marrow B cells with a pH 4.0 acetate buffer wash also had no effect on the coexpression of κ and λ L chains (data not shown).

Fetal Bone Marrow



Fetal Spleen

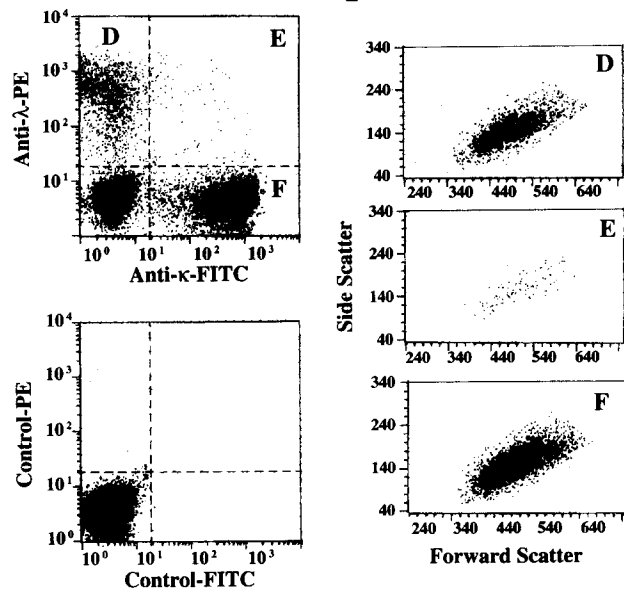


Figure 7. Three-color flow cytometric analysis of donor-matched normal fetal bone marrow and splenic B cells demonstrates κ/λ coexpression. The left column contains dot plots of the total CD10 $^+$ /CD19 $^+$ population of fetal bone marrow and spleen cells stained with F(ab') $_2$ goat anti-human κ -FITC and λ -PE, or preimmune F(ab') $_2$ goat Ig-FITC and -PE. Only PI $^-$ events collected within the lymphoid gate are shown. Fetal bone marrow λ^+ cells (A) represent 16%; κ^+/λ^+ cells (B) represent 1.2%; and κ^+ cells (C) represent 13% of the total B lineage population. Fetal splenic λ^+ cells (D) represent 34%; κ^+/λ^+ (E) represent 2.9%; and κ^+ (F) represent 59% of the total B lineage population. (Right) Forward and side (90°) light scatter characteristics of the fetal bone marrow and spleen λ^+ (A and D), κ^+/λ^+ (B and E), and κ^+ (C and F) populations. The light scatter mean channel values for the three fetal bone marrow B cell populations were: λ^+ (FSC-420, 90°-105), κ^+/λ^+ (FSC-427, 90°-112), and κ^+ (FSC-434, 90°-109). The analogous splenic values were: λ^+ (FSC-486, 90°-147), κ^+/λ^+ (FSC-514, 90°-163), and κ^+ (FSC-489, 90°-146).

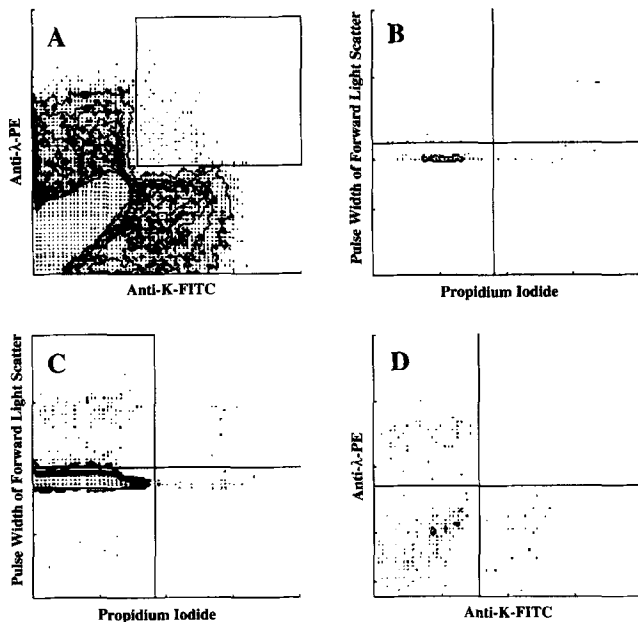


Figure 8. A representative analysis of the pulse width measurements made on the forward light scatter of κ^+/λ^+ fetal bone marrow cells demonstrates that detection of this rare cell type cannot be explained by a cell doublet artifact. Three-color multiparameter analysis of fetal bone marrow B lineage cells stained with PI and F(ab')₂ goat anti-human κ -FITC and λ -PE (A) showed 0.1% of the total B cell population were κ^+/λ^+ in this experiment. When pulse width analysis of the forward light scatter events was performed on the κ^+/λ^+ population (box, A), 89% of the events represented viable single cells (B, bottom left). The remaining events were distributed as follows: 0.9% PI⁻ doublets (B, top left), 0.9% PI⁺ doublets (B, top right), and 9.2% PI⁺ single cells (B, bottom right). Pulse width analysis of the total B lineage population in A is presented in C. This provides a clear definition of the electronic gating used to define viable single cells (bottom left) and cell doublets (top left). (D) κ and λ distribution of the viable cell doublets identified in the top left quadrant of C.

Fig. 9, B and C shows an example of a κ^+/λ^+ fetal bone marrow B cell detected by two-color immunofluorescence microscopy. Also shown are κ^+/λ^- , κ^-/λ^+ , and κ^-/λ^- lymphocytes. The intense fluorescent staining of the κ^+/λ^+ cell confirms the flow cytometry data shown in Fig. 7. Similar results were obtained when fetal splenic B cells were analyzed (data not shown).

Discussion

In a study published several years ago, we observed that normal human pre-B cells that differentiate in vitro give rise to a higher percentage of immature B cells expressing surface μ/λ than surface μ/κ (13). A possible explanation for this data is that some pre-B cells make functional λ rearrangements directly, and bypass κ rearrangements altogether. Studies originally reported by Korsmeyer et al. (26) and Hieter et al. (32) suggested an ordered progression (hierarchy) of $\kappa \rightarrow \lambda$ rearrangement in human B cells, an observation consistent with studies in the mouse (for a review see reference 3). The $\kappa \rightarrow \lambda$ hierarchy is, nonetheless, not absolute. Several mouse

cell lines have been described with functional λ rearrangements and both κ alleles in germline (10, 11), and analyses of human cells also suggest that λ rearrangement can precede κ (12, 13). However, the studies of human cells did not resolve whether the λ rearrangements gave rise to λ L chains.

In the current study, EBV transformation was used to rescue the differentiated progeny of pre-B cells, and transformants expressing μ/λ surface Ig receptors were examined for their κ gene rearrangement status. Using this approach, we produced and characterized two cell lines, designated 35.30 and 36.31, both containing functional λ rearrangements with κ alleles in germline. The germline status of the κ alleles in both cell lines was confirmed by the absence of detectable rearrangements of the κ de alleles. The H chain rearrangement profile was consistent with clonality in both cell lines, and synthesis/expression of λ L chains was directly demonstrated in 36.31 cells (Fig. 3). These collective results provide direct evidence that nonleukemic human fetal bone marrow B lineage cells harbor the capacity to make functional λ L chain gene rearrangements without rearranging or deleting either κ allele.

It is conceivable that 36.31 and 35.30 arose from fetal bone marrow B lineage cells containing germline λ genes, and λ rearrangements occurred after expansion of the EBV-transformed clone in vitro. Yet, additional Ig gene rearrangements and isotype switching are rarely observed after EBV transformation (9, 33, 34). Rearrangement of the κ de (or recombining sequence as it has been called in the mouse) has been proposed as a mechanism that serves to initiate λ rearrangement (23, 35, 36). However, studies comparing the Ig gene rearrangement status of short- versus long-term EBV-transformed cell lines (26, 32), and studies done with a murine myeloma (10), suggest that deletion of C κ can occur after λ rearrangement has been initiated. It is therefore possible that 36.31 and 35.30 represent cells rescued by EBV before rearranging or deleting one or both κ alleles.

The notion that expression of a single L chain isotype is stringently imposed at the level of individual mature B cells, first demonstrated in human cells by Bernier and Cebra (37, 38), is a widely accepted concept in immunology. Sporadic reports have appeared suggesting that exceptions to the single L chain isotype rule may exist in human B cells. Human myeloma cells coexpressing cytoplasmic κ and λ L chains have been described (39–41), as has a single case of acute lymphoblastic leukemia (42). However, none of these studies provided immunochemical evidence that the myeloma cells synthesized and expressed κ and λ L chains. The results presented in this report provide solid evidence that human B cells exist which coexpress κ and λ L chains on the surface of a single cell. The EBV-transformed cell line 30.30 provided the initial clue. Two-color flow cytometry revealed a major population of 30.30 cells coexpressing κ and λ L chains (Fig. 4). Southern blotting revealed that 30.30 had a single H chain rearrangement, both κ alleles rearranged, both κ de alleles in germline, and one λ rearrangement (Fig. 5). Direct evidence that 30.30 cells synthesize and express κ and λ L chains was demonstrated by biosynthetic labeling and immunoprecipitation/SDS-PAGE (Fig. 6). Efforts to isolate a stable, pure population of 30.30

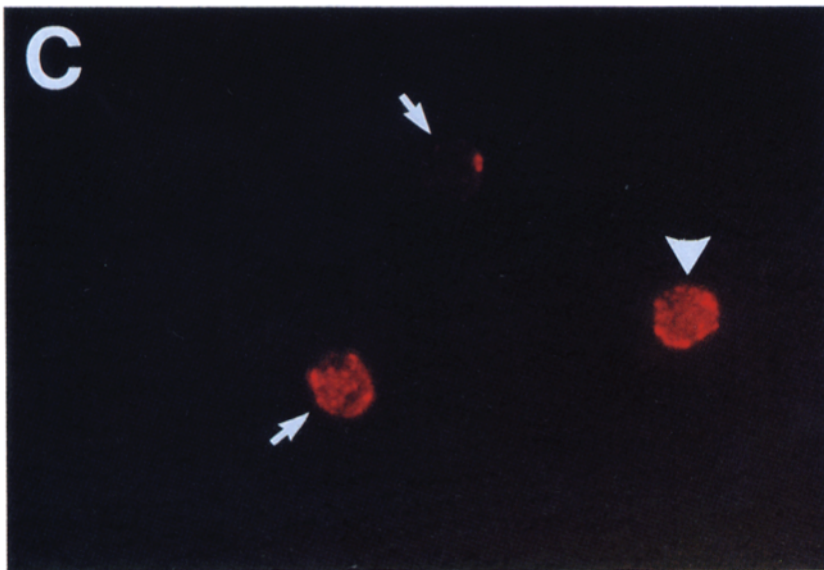
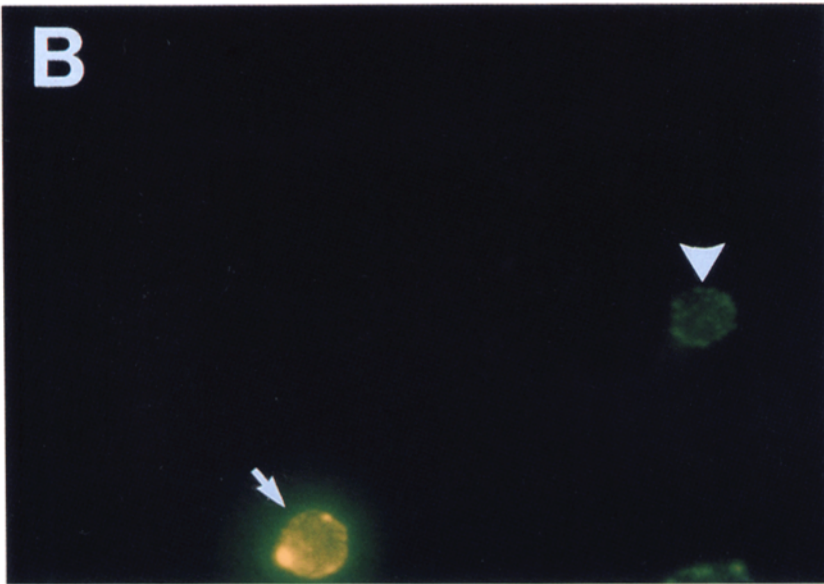
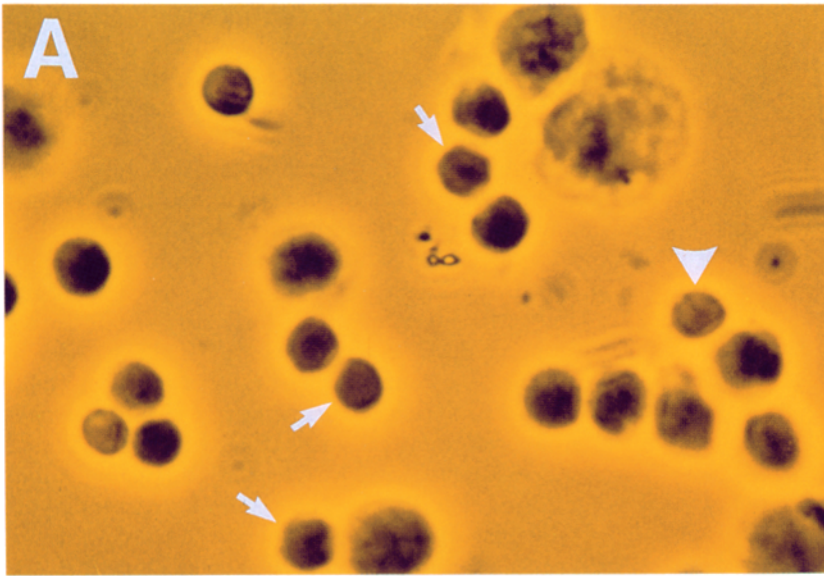


Figure 9. Detection of κ^+/λ^+ cells in normal fetal bone marrow by two-color immunofluorescence microscopy. Cells were isolated, stained, and analyzed as described in Materials and Methods. A single field of cells is visualized by phase contrast (A) or fluorescence (B, FITC; C, TRITC) microscopy. (\blacktriangledown) The κ^+/λ^+ cell. (\rightarrow) One κ^+/λ^- and two κ^-/λ^+ cells. Note also the presence of small lymphoid cells that do not stain for κ or λ (κ^-/λ^- cells).

cells coexpressing κ and λ L chains by FACS[®] were unsuccessful, and repetitive two-color flow cytometry consistently showed the fluorescence profiles presented in Fig. 4. The constant presence of a λ^- population is in some discord with the immunoprecipitation data (Fig. 6) suggesting that λ L chains are synthesized at a faster rate and/or are more stable than κ L chains. It is conceivable that all 30.30 cells express λ L chains, but the amount which exists as μ/λ cell surface Ig is variable. Similarly, κ L chains may form more stable complexes with μ H chains than λ L chains.

The establishment and characterization of the κ^+/λ^+ 30.30 cell line prompted us to search for the existence of a normal in vivo counterpart. The data presented in Figs. 7-9, provide strong evidence that κ^+/λ^+ human B cells exist in fetal bone marrow and spleen. The possibility that the κ^+/λ^+ population is an artifact attributable to dead cells, cell doublets, or passively (Fc receptor) adsorbed Ig was eliminated by propidium iodide gating to eliminate dead cells, pulse width analysis to eliminate cell doublets, and acid washing and 37°C incubation to eliminate potential passively adsorbed Ig. Most importantly, the flow cytometry data was confirmed by two-color immunofluorescence microscopy. The existence of κ^+/λ^+ cells can be explained in several ways. Pre-B cells containing κ and λ in germline could simultaneously undergo functional κ and λ V to J rearrangements, giving rise to κ^+/λ^+ cells. This developmental scenario would assume that the recombinase ensemble (e.g., RAG-1 and -2) would have equal access to the heptamer-nonamer recognition sequences in both κ and λ loci. Alternatively, μ^+/κ^+ or μ^+/λ^+ B cells could rearrange the alternate L chain gene to give rise to κ^+/λ^+ cells. Independent of how κ^+/λ^+ cells arise, they may be transients during B cell ontogeny, in the

process of switching L chain isotypes. The most likely pathway invoked by this model would be $\kappa \rightarrow \kappa\lambda \rightarrow \lambda$. In this pathway, the ultimate sole expression of λ might occur after κ deletion by the κ de. Support for a $\kappa \rightarrow \kappa\lambda \rightarrow \lambda$ pathway can be found in the study by Gollahon et al. (43), who observed λ L chain expression in hybridomas generated from mice harboring a functionally rearranged κ transgene. They also presented evidence that ~20% of λ^+ normal splenic B cells coexpressed κ L chain, and used this data to argue the existence of a so-called $\kappa\lambda$ -B cell lineage which lacks feedback inhibition of L chain gene rearrangement (43). Hardy et al. (44) also described a LPS-induced cell surface κ^+ murine Ly-1⁺ lymphoma that expressed cell surface λ L chains. A $\lambda \rightarrow \kappa\lambda \rightarrow \kappa$ pathway is also possible, since human B cells with functional λ rearrangements and germline κ alleles exist in the form of the 36.31 and 35.30 cell lines. Support for this pathway is weakened by the absence of a homologue to the κ de in the λ locus (45).

Do κ^+/λ^+ cells subserve any function in the immune response? Cohn and Langman (46) have used the theoretical argument that "doubles" (including κ^+/λ^+ and cells expressing two alleles of the same L chain isotype) must exist to maintain the evolutionary selection pressure necessary for haplotype exclusion. B cells undergoing L chain isotype switching could also contribute to Ig repertoire diversification by (presumably) changing antigen specificity. Our identification of κ^+/λ^+ B cells in normal fetal bone marrow and spleen, coupled with the recently described CD40/L cell system for growing normal human B cells in vitro (47), may facilitate characterization of V_H and V_L usage, cytokine responsiveness, and antigen specificity in these unusual cells.

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Address correspondence to Dr. Tucker W. LeBien, Department of Laboratory Medicine and Pathology, Box 609 UMHC, University of Minnesota Medical School, Minneapolis, MN 55455.

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References

1. Korsmeyer, S.J., P.A. Hieter, J.V. Ravetch, D.G. Poplack, T.A. Waldmann, and P. Leder. 1981. Developmental hierarchy of immunological gene rearrangements in human leukemic pre-B cells. *Proc. Natl. Acad. Sci. USA.* 78:7096.
2. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature (Lond.)* 302:575.
3. Blackwell, T.K., and F.W. Alt. 1989. Mechanism and developmental program of immunoglobulin gene rearrangement in mammals. *Annu. Rev. Genet.* 23:605.
4. Schatz, D.G., M.A. Oettinger, and M.S. Schlissel. 1992. V(D)J recombination: molecular biology and regulation. *Annu. Rev. Immunol.* 10:359.
5. Kincaid, P.W., G. Lee, C.E. Pietrangeli, S.I. Hayashi, and J.M. Gimble. 1989. Cells and molecules which regulate B lymphopoiesis in bone marrow. *Annu. Rev. Immunol.* 7:111.
6. Dorshkind, K. 1990. Regulation of hemopoiesis by bone

- marrow stromal cells and their products. *Annu. Rev. Immunol.* 8:111.
7. Storb, U., P. Engler, J. Hagman, K. Gollahon, J. Manz, P. Roth, C. Rudin, L. Doglio, J. Hackett, D. Haasch, et al. 1989. Control of expression of immunoglobulin genes. *Prog. Immunol.* 7:316.
 8. Rolink, A., and F. Melchers. 1991. Molecular and cellular origins of B lymphocyte diversity. *Cell.* 66:1081.
 9. Kubagawa, H., M.D. Cooper, A.J. Carroll, and P.D. Burrows. 1989. Light-chain gene expression before heavy-chain gene rearrangement in pre-B cells transformed by Epstein-Barr virus. *Proc. Natl. Acad. Sci. USA.* 86:2356.
 10. Berg, J., M. McDowell, H.-M. Jäck, and M. Wabl. 1990. Immunoglobulin λ gene rearrangement can precede κ gene rearrangement. *Dev. Immunol.* 1:53.
 11. Felsher, D.W., D.T. Ando, and J. Braun. 1991. Independent rearrangement of Ig λ genes in tissue culture-derived murine B cell lines. *Int. Immunol.* 3:711.
 12. Tang, J.-Q., M.C. Béné, and G.C. Faure. 1991. Alternative rearrangements of immunoglobulin light chain genes in human leukemia. *Leukemia (Baltimore).* 5:651.
 13. Beishuizen, A., K. Hählen, A. Hagemeijer, M.-A.J. Verhoeven, H. Hooijkaas, H.J. Adriaansen, I.L.M. Wolvers-Tettero, E.R. van Wering, and J.J.M. van Dongen. 1991. Multiple rearranged immunoglobulin genes in childhood acute lymphoblastic leukemia of precursor B-cell origin. *Leukemia (Baltimore).* 5:657.
 14. Villablanca, J.G., J.M. Anderson, M. Moseley, C.-L. Law, R.L. Elstrom, and T.W. LeBien. 1990. Differentiation of normal human pre-B cells in vitro. *J. Exp. Med.* 172:325.
 15. Wolf, M.L., J. Buckley, A. Goldfarb, C.-L. Law, and T.W. LeBien. 1991. Development of a bone marrow culture for maintenance and growth of normal human B cell precursors. *J. Immunol.* 147:3324.
 16. Goding, J.W. 1986. *Monoclonal Antibodies: Principles and Practices.* 2nd ed. Academic Press, New York. 223-233.
 17. Sharpless, T., F. Traganos, Z. Darynkiewicz, and M.R. Melamed. 1975. Flow cytofluorimetry: discrimination between single cells and cell aggregates by direct size measurements. *Acta. Cytol.* 19:577.
 18. Kumagai, K., T. Abo, T. Sekizawa, and M. Sasaki. 1975. Studies of surface immunoglobulins on human B lymphocytes: I. dissociation of cell-bound immunoglobulins with acid pH or at 37°C. *J. Immunol.* 115:982.
 19. Feinberg, A., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction fragments to high specific activity. *Anal. Biochem.* 132:6.
 20. Hieter, P., G. Hollis, S. Korsmeyer, T. Waldmann, and P. Leder. 1981. Clustered arrangement of immunoglobulin λ light chain constant region genes in man. *Nature (Lond.).* 294:536.
 21. Hollis, G., P. Hieter, O. McBride, D. Swan, and P. Leder. 1982. Processed genes: a dispersed human immunoglobulin gene bearing evidence of RNA-type processing. *Nature (Lond.).* 296:321.
 22. Hieter, P., E. Max, J. Seidman, J. Maizel, and P. Leder. 1980. Cloned human and mouse immunoglobulin constant and J region genes conserve homology in functional segments. *Cell.* 22:197.
 23. Siminovitch, K., A. Bakhshi, P. Goldman, and S. Korsmeyer. 1985. A uniform deleting element mediates the loss of κ genes in human B cells. *Nature (Lond.).* 316:260.
 24. Ravetch, J., U. Siebenlist, S. Korsmeyer, T. Waldmann, and P. Leder. 1981. Structure of the human immunoglobulin μ locus: characterization of embryonic and rearranged J and D genes. *Cell.* 27:583.
 25. Boué, D., and T.W. LeBien. 1988. Structural characterization of the human B lymphocyte-restricted differentiation antigen CD22: comparison with CD21 (complement receptor type 2/Epstein-Barr virus receptor). *J. Immunol.* 140:192.
 26. Korsmeyer, S., P. Hieter, S. Sharrow, C. Goldman, P. Leder, and T. Waldmann. 1982. Normal human B cells display ordered light chain gene rearrangements and deletions. *J. Exp. Med.* 165:975.
 27. Kerr, W., M. Cooper, L. Feng, P. Burrows, and L. Hendershot. 1989. Mu heavy chains can associated with a pseudo-light chain complex (ψ L) in human pre-B cell lines. *Int. Immunol.* 1:355.
 28. Nishimoto, N., H. Kubagawa, T. Ohno, G. Gartland, A. Stankovic, and M. Cooper. 1991. Normal pre-B cells express a receptor complex of mu heavy chains and surrogate light chain proteins. *Proc. Natl. Acad. Sci. USA.* 88:6284.
 29. Schiff, C., M. Milili, D. Bossy, A. Tabilio, F. Falzetti, J. Gabert, P. Mannoni, and M. Fougereau. 1991. λ -like and V pre-B genes expression: an early B-lineage marker of human leukemias. *Blood.* 78:1516.
 30. Graninger, W., P. Goldman, C. Morton, S. O'Brien, and S. Korsmeyer. 1988. The K-deleting element: germline and rearranged, duplicated and dispersed forms. *J. Exp. Med.* 167:488.
 31. Vasicek, T., and P. Leder. 1990. Structure and expression of the human immunoglobulin λ genes. *J. Exp. Med.* 172:609.
 32. Hieter, P., S. Korsmeyer, T. Waldmann, and P. Leder. 1981. Human immunoglobulin κ light-chain genes are deleted or rearranged in λ -producing B cells. *Nature (Lond.).* 290:368.
 33. Webb, C.F., M.D. Cooper, P.D. Burrows, and J.A. Griffin. 1985. Immunoglobulin gene rearrangements and deletions in human Epstein-Barr virus-transformed cell lines producing different IgG and IgA subclasses. *Proc. Natl. Acad. Sci. USA.* 82:5495.
 34. Kubagawa, H., P.D. Burrows, C.E. Gossi, J. Mestecky, and M.D. Cooper. 1988. Precursor B cells transformed by Epstein-Barr virus undergo sterile plasma-cell differentiation: J-chain expression without immunoglobulin. *Proc. Natl. Acad. Sci. USA.* 85:875.
 35. Durdik, J., M.W. Moore, and E. Selsing. 1984. Novel κ light-chain gene rearrangement in mouse λ light chain producing B-lymphocytes. *Nature (Lond.).* 307:749.
 36. Persiani, D., J. Durdik, and E. Selsing. 1987. Active λ and κ antibody gene rearrangement in abelson murine leukemia virus-transformed pre-B cell lines. *J. Exp. Med.* 165:1655.
 37. Bernier, G.M., and J.J. Cebara. 1964. Polypeptide chains of human gamma-globulin: cellular localization by fluorescent antibody. *Science (Wash. DC).* 144:1590.
 38. Bernier, G.M., and J.J. Cebara. 1965. Frequency distribution of α , γ , κ , and λ polypeptide chains in human lymphoid tissues. *J. Immunol.* 95:246.
 39. Bouvet, J.P., D. Buffe, R. Oriol, and P. Liacopoulos. 1974. Two myeloma globulins, IgG1- κ and IgG1- λ , from a single patient (Im). *Immunology.* 27:1095.
 40. Hopper, J.E. 1977. Comparative studies on monotypic IgM λ and IgG κ from an individual patient. IV Immunofluorescent evidence for a common clonal synthesis. *Blood.* 50:203.
 41. Barlogie, B., R. Alexanian, M. Pershouse, L. Smallwood, and L. Smith. 1985. Cytoplasmic immunoglobulin content in multiple myeloma. *J. Clin. Invest.* 76:765.
 42. Choi, Y.J., and M.S. Wong. 1981. Double light-chain produc-

- tion by leukemic cells of common clonal origin: a case report with review of pertinent literature. *Am. J. Hematol.* 11:93.
43. Gollahon, K.A., J. Hagman, R.L. Brinster, and U. Strob. 1988. Ig λ -producing B cells do not show feedback inhibition of gene rearrangement. *J. Immunol.* 141:2771.
 44. Hardy, R.R., J.L. Dabl, K. Hayakawa, G. Jager, L.A. Herzenberg, and L.A. Herzenberg. 1986. Frequent λ light chain gene rearrangement and expression in a Ly-1 B lymphoma with a productive κ chain allele. *Proc. Natl. Acad. Sci. USA.* 83:1438.
 45. Storb, U. 1990. The published data. *Immunol. Rev.* 115:253.
 46. Cohn, M., and R.E. Langman. 1990. The protection: the unit of humoral immunity selected by evolution. *Immunol. Rev.* 115:7.
 47. Banchereau, J., P. De Paoli, A. Valle, E. Garcia, and F. Rousset. 1991. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. *Science (Wash. DC).* 251:70.