NORMAL HUMAN B CELLS DISPLAY ORDERED LIGHT CHAIN GENE REARRANGEMENTS AND DELETIONS

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The maturation of a pre-B cell to an immunoglobulin (Ig)-bearing B cell requires an effective recombination of a light chain variable (V_L)[1] and joining (J_L) gene segment (1–11). Man uses both light chain gene classes appreciably, expressing κ light chain in ~60% and λ light chain in 40% of mature B cells. Despite their nearly equal usage, however, the rearrangement of human Ig light chain genes may occur in a preferential order in which κ gene rearrangements precede those of λ (12–15). Specifically, we observed that κ-bearing B cell lines or leukemias had retained their λ genes within the germ line configuration, whereas comparable λ-bearing B cells had no remaining germ line κ genes (12). In addition, other leukemic lymphocytes representing precursor stages of B cell development deleted their κ genes, whereas their λ genes remained untouched in the germ line state (15). These surprising findings, which suggested a sequential order for light chain gene rearrangements, were observed on either transformed B cell lines or leukemic lymphocytes. Thus, the question arose as to whether this apparent ordering of light chain gene rearrangement was a normal, functional event or simply a consequence of the transformation process. This uncertainty required investigation, as the specific chromosomal translocations that occur in malignant human B cells frequently involve the Ig-bearing chromosomes (heavy chain genes are on chromosome 14, κ light chain genes are on chromosome 2, and λ light chain genes are on chromosome 22) (16, 17). Such translocations might well occur within the Ig gene loci and could themselves produce some of the gene recombinations and deletions we observed. To resolve this issue, we isolated the circulating λ-bearing B cells from a normal individual and determined the state of their collective κ and λ genes. Over 95% of the κ genes in these nontransformed λ B cells were no longer in their germ line form, with the majority (60%) being deleted and the remainder being present but in a rearranged state. Furthermore, the incidence of κ gene deletion was greater in long-term λ B cells than in these normal λ B cells or freshly transformed λ B cell lines. Such κ gene deletions may therefore result from a second mechanism that eliminates aberrantly rearranged genes and their products. Thus, despite the nearly equal usage of κ and λ light-chain genes in man, there appears to be a sequential order to their rearrangement during normal B cell ontogeny in which κ rearrangements precede those of the λ genes.

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[1] *Abbreviations used in this paper:* C, constant region; EBV, Epstein-Barr Virus; GH, growth hormone; J, joining segment; kb, kilobase; L, light chain; TNP, trinitrophenyl; V, variable region.

Materials and Methods

Polyclonal Nontransformed λ-bearing B Cell Preparation.   A three-unit leukopheresis was obtained from a single normal individual and subjected to Ficoll-Hypaque separation. Preliminary studies revealed that because only 2–3% of the 2.7×10^9 mononuclear cells obtained were λ-bearing B cells, numerous, rigorous purification steps would be required before a final positive selection by electronic cell sorting (Fig. 1 a). The adherent cell population plus cells with avid Fc receptors were depleted by two incubations on antigen-antibody complex-coated plastic surfaces (18). In this procedure, mononuclear cells were incubated in 25-cm^2 plastic culture flasks coated with trinitrophenyl (TNP) -anti-TNP immune complexes at 2.8×10^7 cells/flask. The nonadherent cells were gently removed by pouring, and the entire procedure was repeated. The two incubations lead to a decrease in the proportion of cells that reacted with the monoclonal antibody OKM₁ (Ortho Pharmaceutical, Raritan, NJ), which identifies monocytes as well as certain other cells, to <3% without significantly removing B cells (18). The remaining 1.3×10^9 nonadherent cells were depleted of T cells by incubation with neuraminidase-treated sheep erythrocytes on ice and separation of the T cell rosettes by centrifugation through Ficoll-Hypaque. These purified T cells were saved and used as a control source of cells from this individual. The remaining 2.35×10^8 non-T, nonadherent, B cell-enriched cells were incubated at 37°C, 5% CO_2, for 40 min to allow the cells to shed cytophilic Ig. This population was then depleted of κ-bearing B cells by passage over a sheep anti-human κ chain, specific antibody column. Of the 5×10^7 cells that passed through this column, ~45% were stained by a monoclonal mouse anti-human λ light chain antibody (9490; Bethesda Research Laboratories, Rockville, MD) whereas only 13% reacted with a combination of monoclonal antibodies that included OKT₃ (Ortho Pharmaceutical), which identifies most peripheral blood T cells, OKM₁, as well as a mouse anti-human κ light chain antibody (9470; Bethesda Research Laboratories) (20). Thus, a large proportion of the remaining cells had no cell surface antigens identified by these reagents. Therefore, it was necessary to perform a positive selection by electronic cell
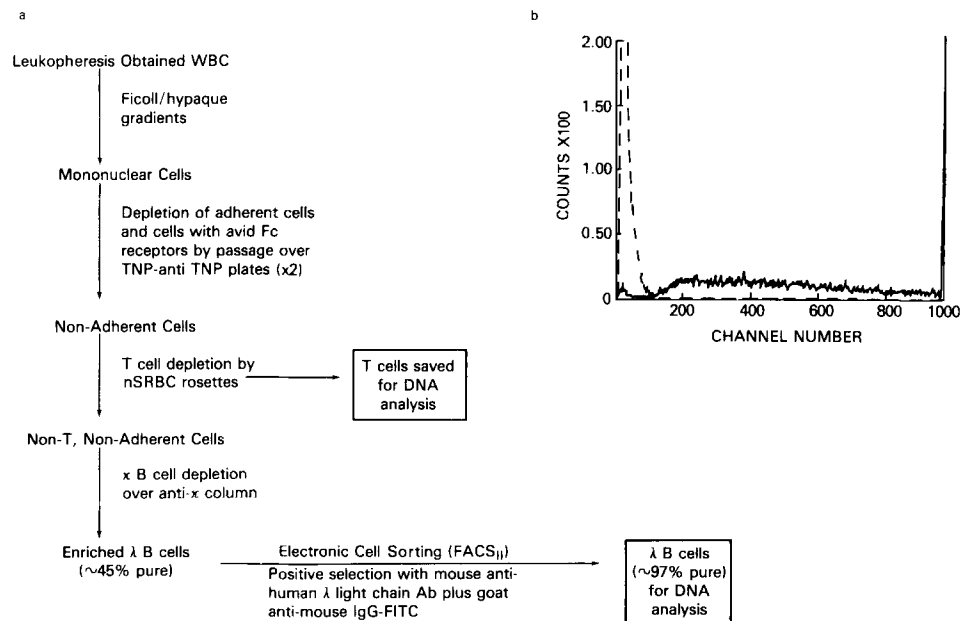

Fig. 1.   (a) Preparative procedure used to purify nontransformed, polyclonal λ-bearing B cells from a normal individual. (b) Analysis by flow microfluorometry of the positively selected λ B cells obtained by electronic cell sorting on a FACS II using a monoclonal mouse anti-human λ light chain antibody and a FITC-labeled second antibody of goat anti-mouse IgG (———). The nonfluorescent pool of cells from the preparative cell sorting (- - -) is shown to illustrate the purity of the λ-bearing B cells.

sorting to further purify the λ-bearing B cells. The 5×10^7 λ B cell-enriched cells were incubated with a monoclonal mouse (G_1) anti-human λ light chain antibody (9490; Bethesda Research Laboratories), followed by an incubation with goat antibody to mouse IgG, which was labeled with fluorescein. The appropriate fluorescence windows for electronic cell sorting were determined, and cells were separated using a fluorescence-activated cell sorter (FACS II; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) at 2,000 light scatter signals/s and collected in tubes containing RPMI 1640 with 25% fetal calf serum (FCS) (21–23). The procedure yielded 13×10^6 λ-bearing B cells in the positively selected pool. Both the positively selected λ B cells and the fluorescence negatively selected pool of cells were reanalyzed by flow microfluorometry on the FACS II to test for their purity (Fig. 1b).

Short-Term Epstein-Barr Virus (EBV) λ B Cell Clones. Peripheral blood lymphocytes from a single individual were transformed in culture with the B95-8 EBV strain (24). As soon as colonies were noted to be growing, the transformed cells were cloned by limiting dilution onto irradiated rat fibroblast feeder layers (25). Aliquots of cells arising from single wells were screened for κ and λ light chain production using a reverse hemolytic-plaque assay for κ and λ light chain secretion (26). Four separate clones of cells ($2C_8$, $2F_4$, $2F_9$, $2E_9$) were identified as making λ light chain and were expanded immediately to 2×10^8 cells for DNA extraction.

DNA Analysis. High molecular-weight DNA was extracted by a citric acid nuclei isolation technique from the nontransformed λ B cells, T cells, and the four EBV λ B cell lines (14). These genomic DNA were digested to completion with BamHI or $EcoR_1$ restriction endonuclease, size fractionated over agarose gels by electrophoresis, and transferred to nitrocellulose paper. These nitrocellulose blots were hybridized with ^{32}P nick-translated DNA probes with specific activities of 200–400 cpm/pg, and autoradiograms were developed (27–29). The normal polyclonal λ B cells yielded ~15 μg of DNA. Aliquots of this DNA were digested with $EcoR_1$ or BamHI and then electrophoresed on gels side by side with multiple concentrations of the same normal individual's T cell DNA as well as placental DNA. To accurately quantitate the gene dosage in the DNA of the normal B cells as compared with the normal T cells, the same nitrocellulose blots were reused by eluting one hybridizing DNA probe and then rehybridizing the blot with a different probe (30). Comparisons of the intensity of bands were made by a Quick Scan Jr. densitometer (Helena Laboratories, Beaumont, TX). Three or more films with different exposure times within the linear dose-response range of the film were developed for each hybridization.

Human DNA Probes. A 2.5-kilobase (kb) $EcoR_1$ embryonic $C_κ$-containing fragment was hybridized with BamHI digests of the DNA from the various cells. This probe recognizes a uniform 12.0 kb BamHI $C_κ$-containing fragment within germ line tissue (Fig. 2) (12). The recombined $VJ_κ$ probe used consisted of a 0.8 kb BamHI-Xho_1 fragment containing an entire recombined subgroup II $V_κ$ region gene as well as the $J_{κ1}$ segment and its 3' flanking sequence.

The $C_λ$ probe used consisted of a combination of a 1.2-kb embryonic BamHI-$EcoR_1$ fragment containing the Kern⁻ Oz⁻ $C_λ$ gene and a 0.8-kb embryonic BamHI-$Hind_{III}$ fragment containing the Mcg $C_λ$ gene (Fig. 3) (14). A human growth hormone (GH) containing clone designated chGH800 (the generous gift of Dr. Howard Goodman, Department of Genetics, Harvard Medical School) was used as a non-Ig gene probe to quantitate the amounts of hybridizable DNA on each nitrocellulose blot (31).

Results

Purity of the Nontransformed λ-bearing B Cells. To accurately assess the proportion of κ genes remaining in the germ line configuration in normal λ-bearing B cells, it was crucial to prepare a very pure population of λ B cells. Any contaminating T cell, natural killer, macrophage, or cells other than λ-producing B cells would contribute two copies of germ line κ genes and thus give erroneous results. To insure sufficient purity, numerous preparative steps were undertaken to deplete such contaminating cells (Fig. 1a). Immediately before the final purification step of preparative electronic cell sorting, the λ B cells had been enriched to ~45% of the population. The final positively selected λ-bearing B cells, when renanalyzed by flow microfluorometry on
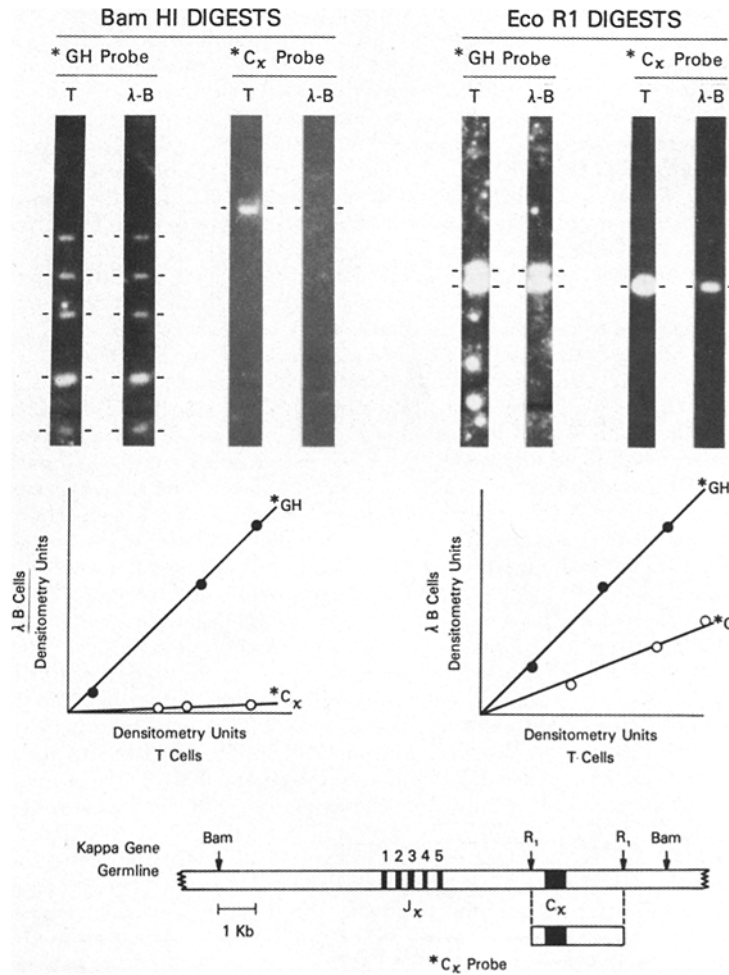
Fig. 2.   Autoradiograms comparing the amount of hybridizable specific gene segments within control T cells (T) and the nontransformed polyclonal λ-bearing B cells (λ_B). A human GH probe, chGH800 (*GH) and a C_κ probe consisting of the embryonic 2.5-kb EcoR₁ fragment (*C_κ) were hybridized against the same lanes of BamHI- and EcoR₁-digested T cell and λ B cell DNA. Graphic scales comparing the gene dosage of the GH-containing fragments (●) and the C_κ-containing fragments (○) of the T cells and λ B cells were constructed with each point representing a different film exposure time. The abscissa and ordinate represent the intensity of hybridization as determined by densitometry tracings of the T cell and λ B cell lanes, respectively. They were scaled so that the non-Ig GH gene segment comparison was at a 45° diagonal. This served to equalize the amount of potentially hybridizable DNA available within the T cells and λ B cells for the subsequent C_κ comparison. The C_κ-containing BamHI fragments are almost entirely missing from the λ B cells, and the C_κ-containing EcoR₁ fragments are reduced by ~60%. At the bottom is a schematic representation of the germ line κ gene locus revealing the EcoR₁ and BamHI sites and illustrating the specific *C_κ probe used.

the FACS II, were shown to be ~97% pure (Fig. 1 b).

Loss of Germ Line κ Constant Region Genes Within Nontransformed λ-bearing B Cells.   The DNA from the normal λ B cells was compared with an equal amount of DNA from the normal T cells of this same individual. We previously demonstrated that human T cells do not show κ gene rearrangements, and uniformly retain both of their C_κ
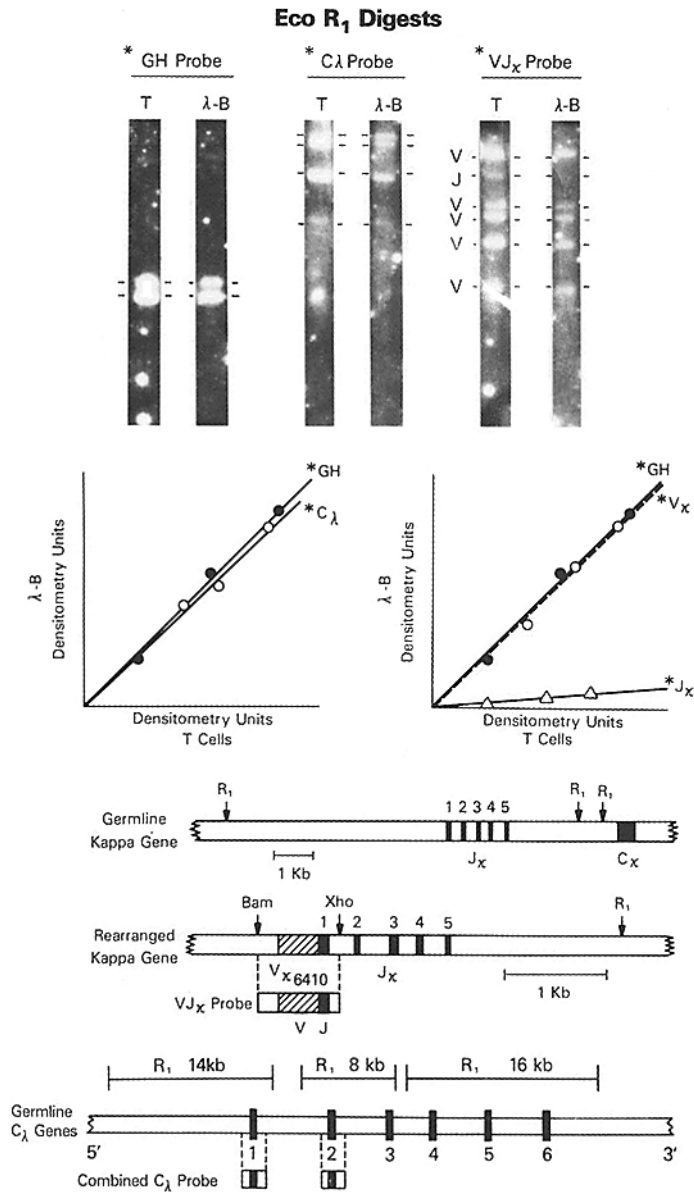
FIG. 3.   Autoradiograms of the same T cell (T) and λ B cell (λB) lanes of EcoR₁-digested DNA when hybridized with GH (*GH), constant lambda region (*Cλ), and combined variable and joining region (*VJκ) probes. Graphic comparisons of the two upper Cλ containing EcoR₁ fragments (16- and 14-kb) (O) reveal that they are present in nearly equal amounts within the λ B cells as compared with T cells. The germ line Jκ gene segments (Δ) are markedly reduced within the λ B cells, whereas the one particular Vκ gene family examined (O) is present in a full gene dosage. At the bottom, the first schematic demonstrates the EcoR₁ sites surrounding the germ line Jκ gene region. The second schematic diagrams the recombined VJκ probe (BamHI-Xho1 fragment from the rearranged allele of a κ B cell line, RPMI 1640), which was used to assess the germ line Jκ gene region and this particular Vκ gene family. The final schematic illustrates the combined Cλ probe consisting of the first two Cλ genes in the complex. The brackets identify the EcoR₁ fragments that contain the Cλ genes.

alleles in the germ line configuration (32). The T cell and λ B cell lanes, which contained roughly equivalent amounts of hybridizable DNA, were identified by hybridization with a non-Ig gene probe, human GH (chGH800) (Fig. 2). The intensity of hybridization was determined by densitometry tracings of the autoradiogram bands. This allowed construction of graphic scales comparing gene dosages within the T cells and λ B cells (Fig. 2).

After the examination of the BamHI digests with the GH probe, this probe was eluted, and the C_κ region probe was applied (Fig. 2). No appreciable amount of germ line C_κ regions remained within the nontransformed λ B cells, with loss of ~95% of the germ line C_κ alleles as determined by densitometry. Even the few remaining germ line C_κ genes (only 5% of the total dosage) might conceivably be contributed by the few contaminating cells that were not λ B cells. As the BamHI sites surrounding the germ line κ gene include one site to the 5' side of the J_κ region, the loss of the germ line C_κ band within these polyclonal cells could result from either recombinations near the J_κ locus or from deletions of the C_κ region (Fig. 2).

To determine the incidence of κ deletion as opposed to κ rearrangement in these polyclonal λ-bearing B cells, an EcoR1 digest was examined. EcoR1 sites are located just outside the borders of the C_κ region such that C_κ remains on a 2.5 kb fragment even if recombination has occurred around the J_κ region (Fig. 2). Within the EcoR1 digests of the λ B cells, ~38% of the C_κ regions were present (Fig. 2). Thus, ~60% of C_κ alleles within nontransformed λ B cells appear deleted, whereas up to 40% are rearranged, presumably in an ineffective, aberrant fashion.

The Extent of κ Gene Loss within Nontransformed λ-bearing B Cells. The boundaries of the germ line κ gene loss were examined by hybridizing the nitrocellulose blot containing the EcoR1 digested DNA with a probe containing both V_κ and J_κ sequences. As can be seen in Fig. 3, the germ line J_κ genes are almost entirely absent from these λ-bearing B cells. Either a recombination occurring near one of the five J_κ segments or a deletion extending from C_κ into the J_κ segments would produce a loss of the germ line J_κ region band. The marked loss of the germ line J_κ configuration confirms that the κ gene complex is almost always rearranged or deleted within normal λ-bearing B cells.

We previously showed (12) that λ-producing B cell lines or leukemias usually deleted their entire J_κ region as well as their C_κ region. In contrast, all members of the particular V_κ gene family examined appeared to be present in such transformed cells (12). This same variable region family (V_κ 6410) appears to have all of its members present in their total amount within the nontransformed λ-bearing B cells (Fig. 3). This indicates that the κ gene deletions that occur in these cells do not extend far enough to include this particular V_κ gene family. In addition, as κ genes are aberrantly rearranged 40% of the time in λ-bearing B cells, this particular V_κ gene family is not being utilized or deleted by these aberrant recombinations.

Status of C_λ Genes within Nontransformed λ-bearing B Cells. The normal individual examined in this study displayed C_λ gene containing EcoR1 fragments of 16-, 14-, 8-, and 5-kb sizes, previously recognized (14) as the most common polymorphic pattern (Fig. 3). Some diminution of germ line C_λ region genes might well be expected within normal λ B cells due to recombinations of the C_λ regions when forming an active light chain gene. However, very little loss of the larger (16- and 14-kb) C_λ gene fragments was demonstrable in these λ-bearing B cells (Fig. 3). This is presumably

explained by the observation that there are additional 14- and 16-kb EcoR₁ fragments recognized by our probe that lie outside of the collection of C_λ genes that recombine in λ-bearing B cells (14). These additional fragments are retained in λ-producing B cells and thus obscure any recombinational loss of the 14- and 16-kb fragments that lie within the major C_λ gene cluster. In accordance with this interpretation, the 8-kb C_λ-containing fragment, which occurs only within the major C_λ region cluster, was decreased in intensity (70% of total expected dosage) in these cells. We previously noted that λ-producing B cell lines and leukemias, which had rearranged both sets of their λ genes, frequently had deleted both copies of their 8-kb EcoR₁ fragments (12). The fact that the 8-kb band is not more profoundly reduced in intensity in the nontransformed λ B cells examined here suggests that the first attempt at λ gene recombination is frequently successful.

κ *Genes within Short-Term EBV Transformed* λ-*bearing B Cells.* The deletion of C_κ genes appears to be more frequent in long-term λ B cell lines and leukemias than in the normal λ B cells examined here. In the nontransformed λ B cells, ~60% of the C_κ alleles were deleted, whereas 40% were rearranged. In a previous study (12) we showed that within long-term B cell lines or lymphocytic leukemias producing λ light chains, ~95% of the C_κ alleles were deleted, whereas 5% were aberrantly rearranged. This difference between normal and long-term λ B cells raised the possibility that aberrantly rearranged κ genes might be subsequently deleted from the genome. Such a gene loss might conceivably occur during the process of transformation or, alternatively, might occur later during subsequent cell divisions. To differentiate between these two possibilities, four short-term λ-producing cell lines were examined immediately after EBV transformation, cloning, and a brief cell expansion. As can be seen in Fig. 4, three of the four separate short-term λ B cell clones studied retained a rearranged C_κ gene, whereas none had any remaining germ line C_κ genes. The presence of 3 rearranged C_κ alleles out of 8 possible C_κ alleles in the short-term lines contrasts with the presence of only 1 rearranged C_κ allele out of 20 possible alleles in long-term cells (12). EBV transformation itself did not seem to result in any obvious further deletion of C_κ genes when these short-term lines were compared with the nontransformed λ B cells. These findings suggest that aberrantly rearranged C_κ genes may be deleted from the genome by a second event occurring during long-term passage.
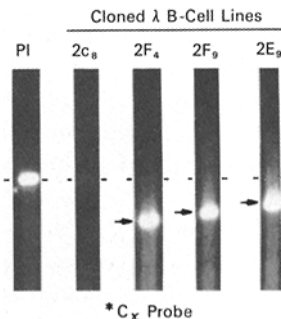

FIG. 4. Autoradiograms of the BamHI-digested DNA from four short-term EBV-transformed λ B cell lines (2C₈, 2F₄, 2F₉, 2E₉) hybridized with the *C_κ region probe. The germ line position for the C_κ gene (12.0 kb BamHI fragment) is displayed by the placental DNA control (Pl). No remaining germ line C_κ alleles (- -) exist in the four λ B cell lines, but three have a rearranged C_κ allele (→).

Discussion

The finding that germ line κ genes are missing from normal, circulating λ-bearing B cells demonstrates that this loss is a normal developmental process. These κ gene events are thus independent of the chromosomal translocations that occur in transformed B cells. In contrast, λ genes remain in their germ line configuration within κ-bearing B cells (12). The human light chain genes are located on separate nonhomologous chromosomes, with λ genes residing on the 22nd human chromosome and κ genes on the 2nd (33, 34). Therefore, deletion of any intervening DNA between joining V_λ and J_λ regions would not directly effect the configuration of the κ genes. Instead, the marked difference in the genes that code for the excluded light chain isotype in κ-bearing, as opposed to λ-bearing, B cells suggests that κ gene recombination precedes that of λ. Neither the exact mechanism nor the advantage of such a system has been defined. It is possible that the recombination of κ genes before λ is a strictly regulated system that requires κ gene recombination before λ genes can be rearranged. If so, complete deletion of the C_κ and J_κ regions does not appear to be a requirement for λ recombination, as 40% of κ genes were present in a rearranged state within normal λ B cells. The recombinational process, which attempts to join V_L and alternate J_L regions, is quite prone to error. One could imagine a cascade of light chain gene recombinations that often initially produces aberrant rearrangements and is finally terminated within a cell only when a V_L and J_L segment are effectively recombined (35). In this context, it is possible that the initial aberrant recombinations of κ genes might inactivate a negative control element, which prevents λ gene rearrangement. Alternatively, the observed order of κ before λ light chain gene recombinations in man might be based upon different inherent rates of recombination for the κ and λ genes. Perhaps aberrant recombination around the J_κ region occurs much more readily than λ rearrangements, so that any cell that ultimately produces λ light chain would almost always have rearranged its κ genes. Such a purely statistical explanation for ordered light chain gene rearrangement in man is weakened by the fact that ~40% of all light chain produced is λ in type, and that λ light chain contributes significantly to antibody diversity. In view of this, a greater frequency of κ over λ rearrangements might reflect not a gross difference in the number of available V_κ, J_κ, vs. V_λ, J_λ recombinational sites, but instead a difference in the inherent recombinational activity of such gene segments.

The occurrence of both rearranged and deleted κ genes within normal λ-bearing B cells could be accounted for by simultaneously competing events of κ rearrangement and deletion. A unitary hypothesis, however, would propose that rearranged κ genes (presumably those aberrantly rearranged) would be subsequently deleted by a second mechanism. The data here suggest that this deletional event at times may even follow λ gene rearrangements. B cells do not appear to shift their light chain class production during development, and κ B cell lines do not routinely lose production of κ light chains when cultured (36, 37). It would therefore appear that an effectively recombined κ gene is less subject to such deletion. Perhaps the aberrantly rearranged κ gene reflects a labile gene complex that is frequently disposed of within B cells. Although this might constitute a fortuitous event, it may prove to be a protective mechanism used by the cell to eliminate aberrant transcripts and prevent the synthesis of light chain fragments.

Summary

Human κ-producing B cell lines and leukemias retain their excluded λ light chain genes in the germ line configuration, whereas transformed λ-producing B cells uniformly rearrange or delete their κ genes (12). Whether the unexpected λ gene recombinations within malignant λ-producing B cells reflect a normal developmental process or are secondary to transformation and specific chromosomal translocations was uncertain. To resolve this issue, we purified circulating λ-bearing B cells from a normal individual to 97% purity by using a series of negative selection steps and a final positive selection on a cell sorter. Over 95% of the collective κ genes in these λ B cells were no longer in their germ line form, with the majority (60%) deleted and the remainder present but in a rearranged state. The chromosomal loss of the germ line κ genes included the joining (J_κ) segments as well as the constant (C_κ) region, yet the particular variable (V_κ) gene family studied was spared. In addition, the incidence of κ gene deletions was higher in long-term than in freshly transformed λ B cell lines. This implies that the deletion of aberrantly rearranged κ genes may occur as a second event. Such a mechanism would serve to eliminate aberrant transcripts and light chain fragments that might interfere with the synthesis and assembly of effective immunoglobulin molecules. Thus, despite the nearly equal usage of κ and λ light chain genes in man, there appears to be a sequential order to their expression during normal B cell ontogeny in which κ gene rearrangements precede those of λ.

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