**Brief Definitive Report**

**κ⁺λ⁺ Dual Receptor B Cells Are Present in the Human Peripheral Repertoire**

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**Summary**

It is a common notion that mature B lymphocytes express either κ or λ light (L) chains, although the mechanism that leads to such isotypic exclusion is still debated. We have investigated the extent of L chain isotypic exclusion in normal human peripheral blood B lymphocytes. By three-color staining with anti-CD19, anti-κ, and anti-λ antibodies we could estimate that 0.2–0.5% of peripheral blood B cells from healthy adults express both κ and λ on the cell surface. The κ⁺λ⁺ cells were sorted, immortalized by Epstein-Barr virus, and five independent clones were characterized in detail. All clones express both κ and λ on the cell surface and produce immunoglobulin M that contain both κ and λ chains in the same molecule, i.e., hybrid antibodies. Sequencing of the L chains revealed in three out of five clones evidence for somatic mutations. It is interesting to note that among a panel of single receptor B cell clones we identified two λ⁺ clones that carried a productively rearranged κ, which was inactivated by a stop codon generated by somatic mutation. These findings indicate that dual receptor B lymphocytes can be found among mature antigen-selected B cells and suggest that somatic mutation can contribute to increase the degree of isotypic exclusion by inactivating a passenger, nonselected L chain.

**I**t is generally accepted that mature B lymphocytes make antibodies with one single type of L chain, either κ or λ (1, 2). In most cases, this isotypic exclusion is due to the fact that mature B cells express only one productively rearranged L chain gene. The mechanism responsible for this phenomenon has not been definitively established. An instructive/regulated model proposes a precise order of L chain gene rearrangement starting at one chromosome, first at the κ and then at the λ locus (3, 4). A stochastic model proposes that isotypic exclusion results from the high frequency of non-productive rearrangements and by a different recombination frequency of κ and λ loci (5, 6).

In both cases, the termination of L chain genes rearrangement (coincident with downregulation of recombinant activating gene 1 [RAG1] and RAG2 gene expression) is mediated by the surface deposition of a complete Ig molecule (7–9). Although this is a necessary requirement, the production of a complete membrane (mlg) may not always be sufficient to terminate recombination. This may occur when the specificity of the antibody is inappropriate either because it is autoreactive (10–12) or because it fails to promote positive selection (13).

There are only few exceptions to the rule of isotypic exclusion. In humans, expression of both κ and λ chains on the same cell has been described in myelomas (14, 15), as well as in one EBV-transformed clone (16) and in one B cell line (17) isolated from fetal bone marrow, but not in the normal mature repertoire.

Here we report that a small but discrete fraction of human peripheral blood B cells carries both κ and λ chains as part of the same Ig molecule and can undergo somatic hypermutation. Furthermore, in κ⁺λ⁺ B cells somatic mutations may increase the degree of isotypic exclusion by inactivating one of the two L chain genes.

**Materials and Methods**

**FACS Staining and Isolation of B Cell Clones.** PBMC were stained with red 613-labeled anti CD19 (GIBCO BRL, Gaithersburg, MD), FITC-labeled anti-κ and PE-labeled anti-λ (both from Dakopatts, Glostrup, Denmark). CD19⁺κ⁺λ⁺ sorted cells were immortalized in limiting dilution cultures in Terasaki plates. The medium was complete RPMI 1640 containing 10% FCS, 30% supernatant of the EBV-producing marmoset cell line B95.8, 30 μg/ml of 90% saturated human transferrin, 500 ng/ml cyclosporin A, and 5 × 10⁴/ml irradiated (3,500 rad) allogeneic PBMC as feeder cells. After 3 wk the B cell clones were transferred to 96-well plates in the presence of irradiated feeder cells and subsequently expanded in medium alone. The B cell clones were analyzed by two-color staining using FITC-conjugated F(ab)₂ anti-κ and PE-conjugated F(ab)₂ anti-λ (Southern Biotechnology Associates, Birmingham, AL). The DNA content of the B cell clones was determined as described (18).

**Detection of Ig and Hybrid Antibodies by ELISA.** Polystyrene microplates (F-Form, Greiner, Frickenhausen, Germany) were coated overnight at 4°C with 5 μg/ml goat anti-human μ, γ, κ, or λ (Southern Biotechnology Associates) in carbonate-bicarbonate buffer, pH 9.6. The plates were washed with PBS and incubated at room temperature with saturation buffer (200 mM Tris-HCl, pH 7.5, containing 1% BSA). After 2 h the buffer was discarded and dilutions of the samples in PBS-1% FCS-0.2% Tween 20 were added for 2 h. The plates were washed and the alkaline phospho-
tase–conjugated goat anti–human κ, λ, μ, or γ was added for 1 h. After a final wash the enzyme activity bound was determined using 1 mg/ml p-nitrophenylphosphate in coating buffer.

RT-PCR and Direct Sequencing of Vκ and Vλ Chains. Total RNA was extracted from 1–3 × 10⁶ cells as described (19) and reverse transcribed using oligo d(T) and avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI) in 30 μl final volume. cDNA (1.5 μl) was PCR amplified in a thermocycler (model 9600; Perkin-Elmer Cetus, Emeryville, CA) using degenerate Vκ and Vλ oligonucleotides (kindly provided by Drs. Greg Winter and Ian Tomlinson, MRC Centre for Protein Engineering, Cambridge, U-K) and primers complementary to the CA and CX regions (20). The PCR profile was 2 min at 94°C, 30 cycles at 94°C for 15 s, 60°C for 20 s, 72°C for 35 s, and a final 10-min extension. The products were electrophoresed in 1.5% agarose gels and then allowed to enter in 0.7% NuSieve (FMC BioProducts, Rockland, ME). The product bands were excised, melted at 65°C, and directly sequenced by the cycle sequencing method (Sequitherm; Epicentre Technols. Corp., Madison, WI) with the same CA and CX oligonucleotides.

Southern Blot Analysis. High molecular weight genomic DNA was extracted from 5 × 10⁶ cells. 8 μg DNA was digested with BamHI, electrophoresed through 6% polyacrylamide gel, and transferred onto nylon filter (Hybond-N+; Amersham International, Amersham, Bucks, UK) and hybridized with a 32P-labeled pHCK probe (2.7 kb, EcoR1; 20) according to the manufacturer’s instructions.

Sequencing of Germline Vκ Genes. DNA was extracted from 10⁶ T cells and 0.5 μg was PCR amplified with oligonucleotides specific for the VκIV-subgroup member VκB3 (VκB3 5', GGC-TGTTAATTTACATGG; VκB3 3', AAAGTATTAGGAGAGACAT; VκA3 5', ATTGTCTCAGGAGACAT; VκA3 3', CAGCACCATGTTGACACTGG). The products were purified and sequenced as above.

Results

Identification of κ⁺λ⁺ B Cells and Isolation of Clones that Produce Hybrid Antibodies. To identify B cells expressing both κ and λ proteins, PBMC were stained with FITC-labeled anti-κ, PE-labeled anti-λ, and Red 613-labeled anti-CD19. Three-color analysis showed that a small proportion of the B cells were stained by both anti-κ and anti-λ (Fig. 1 A). In five different donors tested, the proportion of κ⁺λ⁺ B cells ranged from 0.2 to 0.5% of B cells in peripheral blood. The culture supernatant was tested by ELISA for the presence of Ig H and L chain determinants. All five κ⁺λ⁺ clones produce IgM and both κ and λ L chains, although at different ratios (Fig. 2 A). In addition, both κ and λ are present in the same Ig molecule, since the culture supernatants gave a

Figure 1. Isolation of κ⁺λ⁺ B cell clones from adult peripheral blood. (A) PBMC were stained with Red 613-labeled anti-CD19, FITC-labeled anti-κ, and PE-labeled anti-λ. The dot plot shows the κ/λ staining of the gated CD19⁺ cells. 10⁶ events were acquired. The sorting gate is shown with the percentage of positive cells. (B-G) κ⁺λ⁺ staining of EBV-B cell clones: (B) FSA10 (IgGa); (C) 11.3 (IgGκ); (D-G) L47, F29, L32, and G28 (IgMκ).

Figure 2. κ⁺λ⁺ B cell clones produce hybrid antibody molecules. (A) Amount of κ (empty bar) and λ (black bar) proteins in the culture supernatant of the various clones as detected by ELISA using anti-IgM as a capturing Ab and alkaline phosphatase–labeled anti-κ or anti-λ. (B) Reactivity of the supernatants in an ELISA assay in which the capturing Ab is anti-κ and the developing Ab is anti-λ. Dual receptor clones: L47 (x), G28 (+, 1.32 (•), F29 (△), and G21 (■). Single receptor clones: FSA10 (O) and G4 (∆).
positive signal in an ELISA assay in which the capturing antibody is anti-κ and the developing antibody anti-λ (Fig. 2 B).

Evidence for Somatic Mutations in κ+λ B Cells. The κ+λ+ clones were analyzed by RT-PCR using degenerate Vκ and Vλ oligonucleotides followed by direct sequencing from two independent PCR to minimize possible errors of the Taq polymerase. In all cases, the direct sequences of these products were unique, indicating that only one κ and one λ allele were expressed in each clone.

Since the human κ locus has been extensively sequenced (21), it was possible to identify the corresponding germline gene and thus the potential somatic mutations. This was more problematic for the λ sequences, since much less information is available (22–24). Three out of the five clones showed candidate mutations in both the Vκ-Jκ and, possibly, in the Vλ-Jλ segments (Fig. 3, clones L47, F29, and L32). One clone (F21) had a germline Vκ and Vλ-Jλ, but contained two mutations in the Jκ2 segment. The fifth clone (G28)
possessed a nonmutated \( \kappa \) chain, whereas V\(\lambda\) could not be clearly assigned. As already noticed, extra nucleotides were often present at the V-J junction and either could be due to an accumulation of mutations in the CDR3 or to additions by TdT (Fig. 3). A very short V\(\kappa\)-J\(\kappa\) junction lacking four amino acids were found in clone L32. However, this \(\kappa\) chain paired to the H chain and was expressed on the cell surface (Fig. 1 F).

In summary, the sequences of \(\kappa\) and \(\lambda\) L chain genes in dual receptor B cells do not show any special features that may distinguish them from those expressed by single receptor B cells. Three out of five cases show a modest degree of somatic mutation.

Identification of \(\lambda^+\) B Cells that Carry a Somatically Inactivated, but Otherwise Functional \(\kappa\) Chain Gene. Because somatic mutations can cripple Ig genes one may expect the dual \(\kappa^+\lambda^+\) B cells undergoing somatic hypermutation may often lose one VL gene and become single receptor cells. To look for this possibility we analyzed by RT-PCR and sequencing a panel of 147 \(\kappa^+\) or \(\lambda^+\) B cell clones and identified two \(\lambda^+\) clones (FSA10, IgG\(\kappa\) and RR25, IgM\(\lambda\)) that carried an in-frame rearranged Vk that was somatically hypermutated (Fig. 3). Both Vk genes involved (VkA3 and VkB3) were reported to be functional, but in these clones they were crippled by a mutation resulting in the substitution of tryptophane 35 with a stop codon (Fig. 3).

To rule out the possibility that the stop codons might represent rare allelic variants and not a somatic mutation event, the corresponding germline Vk segments were amplified and sequenced from T cell lines obtained from the same donors from which the clones were isolated. In both cases the results confirmed the somatic origin of the crippling mutation (Fig. 3).

The possibility that the \(\kappa\) sequences were due to contaminants of the clones was ruled out by two experiments. First, clones FSA10 and RR25 were subcloned and all subclones tested carried the same \(\kappa\) message (data not shown). Second, Southern blot analysis demonstrated that both FSA10 and RR25 carry one rearranged \(\kappa\) allele (Fig. 4).

Discussion

Our results demonstrate that a small fraction of human mature B cells express both \(\kappa\) and \(\lambda\) and produce hybrid antibodies. This finding supports the stochastic versus regulated model of gene rearrangement. The fact that both in B and T cells (33, 34) it is possible to find exceptions to the "one cell one receptor" rule indicates that there are no specific mechanisms that prevent the generation and selection of dual receptor lymphocytes.

What may be the origin of \(\kappa^+\lambda^+\) B cells? A first possibility is that a secondary \(\lambda\) rearrangement took place as a consequence of receptor editing. In transgenic animal models it has been shown that autoreactive B cells can edit their receptor by rearranging new VL genes (10-12). However, receptor editing must lead to loss of the autoreactive receptor, due to preferential pairing of the new L chains or possibly deletion of \(\kappa\). Because in the clones described here both \(\kappa\) and \(\lambda\) chains are expressed on the cell surface, we tend to exclude receptor editing as an explanation for \(\kappa^+\lambda^+\) B cells.

A second possibility is that in \(\kappa^+\lambda^+\) B cells the first L chain produced, although able to pair with the H chain, may have failed to terminate recombination, possibly because of failure to induce positive selection (13).

A third and more likely possibility, in the context of the stochastic model of Ig gene rearrangement, is that simultaneous rearrangements at both L chain loci may occur at low frequency and result, in some cases, in the production of \(\kappa^+\lambda^+\) B cells. This notion is supported by several published exceptions to the \(\kappa\)-\(\lambda\) hierarchy of rearrangement (35-38) and by the finding of one \(\kappa^+\) clone bearing an out-of-frame rearranged \(\lambda\) allele and one \(\lambda^+\) clone with both \(\kappa\) alleles in germ line configuration (Giachino, C., unpublished data).

Our analysis is limited to isotypic exclusion, which is set to a large extent by the asynchronous rearrangement at the two L chain loci. We suggest that there will be also cases
of dual receptor B cells generated by lack of allelic exclusion. These cases may actually be more frequent because of a more synchronous rearrangement of the two alleles at the same locus.

An intriguing possibility is that dual receptor B cells can achieve isotypic exclusion as a consequence of somatic mutation leading to inactivation of the “passenger”, i.e., nonselected L chain. This possibility is clearly illustrated by the two λ+ clones that carry an otherwise functional Vx gene and that have been somatically inactivated.

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