Evidence that the VκIII Gene Usage Is Nonstochastic in Both Adult and Newborn Peripheral B Cells and that Peripheral CD5+ Adult B Cells Are Oligoclonal

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Abstract

There is evidence that in certain situations the expressed antibody repertoire is dominated by small subsets of V gene segments. They include fetal, CD5+, and autoantibody-forming B cells as well as low grade B cell malignancies. For instance, inside the VκIII family of ~ 10 members, only 3 (*humkv*325, 328, and *Vg*) are used recurrently for autoantibody production. However, the significance of this recurrence is difficult to interpret without a clear vision of the actual repertoire in normal subjects. To address this, we have sequenced and compared two sets of rearranged VκIII genes generated by cDNA PCR amplification from a normal newborn, a normal adult, and from CD5+ B cells of the same adult donor. The results show that: (*a*) only four VκIII gene segments are used by neonatal and total adult B cells (*humkv*325, *humkv*328, *Vg*, and *kv*305), *humkv*325 being overexpressed in both repertoires; (*b*) there is no significant difference in terms of VκIII gene usage between the adult and newborn repertoires; (*c*) regarding the junction regions, there is a favored use of the most 5′ J_K gene segments (Jk1–Jk2); ~ 20% of the newborn and adult junction sequences was characterized by one or two additional codons, most probably resulting from a nontemplate addition of nucleotides; (*d*) adult clones, in contrast to most newborn clones, show sequence divergences from prototype sequences with patterns which suggest antigen-driven diversity; (*e*) regarding the adult CD5+ B cell library, it is most probable that the 78 clones analyzed derived from no more than nine different V_K–J_K rearrangements. *Humkv*325 is used by at least six of them, and most of the expressed V genes were in exact or very near germline configuration.

Collectively these results suggest that the expressed antibody VκIII repertoire in the adult represents only a fraction of the potential genetic information and that it resembles the preimmune repertoire of the neonate. The data, which also suggest that the adult peripheral blood CD5+ B cell population may be dominated by a small number of B cell clones, are discussed with regards to the VκIII usage in pathological situations. (*J. Clin. Invest.* 1994. 93:2093–2105.) Key words: immunoglobulin • variable region genes • diversity

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Introduction

The large number of available V, D, and J germline (GL)[1] genes, the random rearrangements between them, the imprecision of junction mechanisms, and the random pairing of functional H and L chains provide a vast potential preimmune repertoire estimated at ~ 10^{11} antibodies (Ab) in humans. Subsequently, further diversification occurs because of the antigen-induced hypermutation mechanism. However, how the immune system uses this potential genetic information to generate the actual Ab repertoire is largely unknown. With regard to the V gene families, previous studies using EBV-transformed B cell clones or in situ hybridization with V_H probes (1–3) showed a fairly good correlation between the estimated genomic complexity of each family and its level of expression in adult peripheral B lymphocytes. For instance, the V_H3 gene family contains ~ 40% of the V genes, and V_H3 gene products were detected in ~ 50% adult peripheral B cells examined by in situ hybridization (2). However, the same studies did not find such a correlation for the V_K families. Thus, this type of interfamily comparison suggested that a stochastic process could create the expressed V_H family repertoire, but that an unknown mechanism could be responsible for the expressed V_K family repertoire.

If we now consider particular V gene segments, the picture is not so clear: (*a*) recent data (4) suggest that the V_H3-expressing molecules are coded mainly by a small number of the V_H3 genes; (*b*) there is evidence that in certain well studied situations, the expressed Ab repertoire is dominated by a small subset of V genes; it includes fetal and neonatal B cells (5–8), CD5+ B cells (for reviews see references 9, 10), autoantibody-forming B cells (for reviews see references 11–13), and low-grade malignant B cell lymphoproliferations (14–18). The latter studies suggested that unique combinations of highly conserved V genes could account for autoreactivity in many instances and that the high frequency of self-reacting B cells in early ontogeny was either a direct consequence of a genetically programmed repetitive use of these genes or a consequence of a positive selection of the B cells expressing these same genes. For instance, the case of the VκIII family seems very interesting. This family is expressed by ~ 30% of adult B cells (2) and is made up of six functional members (19–21). One of these, *humkv*325 is expressed by 7% of fetal splenic B cells at 23 wk of gestation (22) and by 25% of kappa light chain–expressing B cell chronic lymphocytic leukemia (CLL) (17). Only three of the VκIII genes are used recurrently for autoantibody production (*humkv*325, *humkv*328, and *Vg*) (3, 23–25). *Humkv*325

1. *Abbreviations used in this paper:* Ab, antibody; CLL, chronic lymphocytic leukemia; FRW, framework; GL, germline; RF, rheumatoid factor.

encodes 40% of anti-I cold agglutinins (26), 30–50% of monoclonal rheumatoid factors (RF) arising during macroglobulinemia or mixed cryoglobulinemia (27–29), and a large proportion of polyclonal RF during RA (23). Thus, it is tempting to speculate that these unusual frequencies are related to the autoantibody activity of the VκIII-expressing molecules. However, we showed recently that this model minimizes the role of the developmentally acquired heavy chain CDR3 in autoantibody activity (30) and overestimates the role of peculiar gene segments.

Clearly, such data are difficult to interpret without a picture of the actual repertoire of the different VκIII gene segments in normal subjects. This approach seems necessary to validate the comparisons with the pathological situations. cDNA amplification by PCR can be used to address the expressed VκIII Ab repertoire because available universal primers can avoid biased amplification of specific V segments. Using this method, we have analyzed and compared the VκIII repertoires expressed by the peripheral blood total and CD5+ B cells of a normal adult and by the cord blood cells of a normal newborn to answer the following questions: (*a*) are the expressed genes, within the VκIII family, representative of the random usage of the available genomic information? (*b*) are there any differences between the three analyzed B cell populations and, if so, how can they be interpreted? (*c*) what is the physiologic frequency of long kappa chain CDR3 in these B cell populations? and (*d*) how can the present data shed light on the previously reported abnormal usage of VκIII genes during the above-mentioned situations?

Methods

Origin of the cell populations. Mononuclear cells were isolated by Ficoll-Hypaque gradient from newborn cord blood and from the peripheral blood of an healthy adult (J.L.).

Cytofluorometric analysis of newborn and adult B cells. The newborn cord blood and the adult mononuclear cells contained, respectively, 3 and 5% of CD19 and surface Ig double-positive cells. 5×10^4 surface Ig and CD5 double-positive cells were sorted from the adult peripheral blood mononuclear cells using a cell sorter (ATC-3000; ODAM, Wissembourg, France) and resuspended in 1 ml PBS containing 1% BSA.

RNA preparation. Cells from the newborn sample, from the nonsorted adult sample, and from the CD5+ sorted adult B cells were washed three times in PBS, pH 7.3, and then resuspended in cold lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 2 mM $MgCl_2$) containing 10% NP-40. RNA was prepared as described (31), ethanol precipitated, and stored in sterile water.

cDNA synthesis and PCR. The three RNA preparations used to prepare the cDNAs were extracted from the same number of B cells (10,000). cDNA corresponding to kappa chain mRNA was obtained using a C_K oligonucleotide primer (MX25: 5′ CGGACGACTTATTGAAGATAGGT 3′) as described previously (18) in a final volume of 10 μl. A first set of PCR was performed with 1 μl of cDNA mixed with oligonucleotide primers NH96 (5′ CG**AAGCTT**CTGGCGGGAAGATGAAGACAGATGGTGCAGCCACAGT 3′) and PS243 (5′ **TTGAATTC**GTCGACATGGAARCCCCAGCGCAGCTT 3′), corresponding, respectively, to human C_K- and VκIII family-specific leader regions. The PCR conditions were: 25 cycles of 94°C for 1 min, 40°C for 2 min, and 72°C for 3 min. 1 μl of the first PCR product was used for a second set of PCR with NS15 (5′ GG**GAATTC**ACCCTGTCT[GT]TGTCTCCAGGGGAAAGAGCC 3′), specific for the first VκIII framework region, and NS78 (5′ CC**AAGCTT**CTGGCGGGAAGATGAAGACAGATGGTGCAGCCACAGT 3′), specific for the C_K proximal region. The conditions were 25 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min. The last two primers contained protected cloning sites. Both Vg and Vh VκIII genes contained one mismatch with PS243; Vh also contained a single mismatch with NS15. Under our PCR conditions, these changes did not affect the amplification efficiency of these genes (data not shown).

Cloning and sequencing. The PCR products were then digested with EcoRI and HindIII and ligated into Bluescript™ (Stratagene, La Jolla, CA). Double stranded dideoxynucleotide chain termination sequencing was performed using Sequenase™ (United States Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions.

Sequence analysis. The sequences were compared with known germline sequences contained in the European Molecular Biology Laboratory data base (release 31.0) using MacDNASIS Pro analysis software, version 02-00 (Hitachi Software Engineering America, Ltd., San Bruno, CA).

Results

By using an equivalent number of unstimulated B cells for the RNA preparations, it was possible to use the PCR method to compare the three VκIII repertoires expressed by the B cell populations.

Analysis of the VκIII repertoires expressed by total B cells in adult peripheral blood and in newborn cord blood

50 adult and 40 newborn clones were picked randomly out of the PCR-amplified C_K cDNA libraries and then sequenced. We found two identical sequences originating from separate clones in each library (clones JL6/10, JL78/55, Z6/39, and Z25/40). Similarly, several sequences with identical V_K–J_K junctions shared some mutations, suggesting clonal relationships (JL 63/1 and JL 13/16); for V_K and J_K gene segment frequencies and for mutation rates, the duplicate or clonally related sequences were counted only once. All but one of the sequences corresponded with functional rearrangements. The sequences are shown in Figs. 1 and 2.

Characterization of the V_K segments. The human kappa locus has been analyzed extensively and it is thought that most, if not all V_K genes and pseudo-genes, have been sequenced (20, 32). The VκIII family consists of 10 members, of which only 6 seem functional. When compared with prototype GL sequences, each clone could be assigned to four of the six functional GL genes; three of them (*humkv*325, *humkv*328, and *Vg*) represented > 90% of the expressed repertoire in both adult and newborn (Table I). These results cannot be attributed to our amplimers since *kv*305 and *Vg″* are perfect matches with the VκIII family–specific primers and *Vh*, as well as *Vg*, differs by only one nucleotide. Surprisingly, there was no difference in the occurrence frequency of each VκIII gene segment between the adult and the neonatal expressed repertoires. When compared with the theoretical utilization frequency (i.e., if the repertoire was a random representation of the GL V gene potential), only *kv*325 was statistically overexpressed in both repertoires; both *Vg″* and *Vh* were underexpressed in adult and newborn repertoires. The underexpression of *kv*305 was only statistically significant in the adult repertoire ($P < 0.05$).

J_K gene segment usage. The results presented in Fig. 3 indicate a preference for *Jk*1 utilization in the neonatal VκIII repertoire ($0.06 < P < 0.07$). The distribution of J_K usage in the adult library was not statistically different from the number of J_K gene segments; however, there was still a favored use of 5′J_K genes, *Jk*1 and *Jk*2 representing 59% of the clones compared

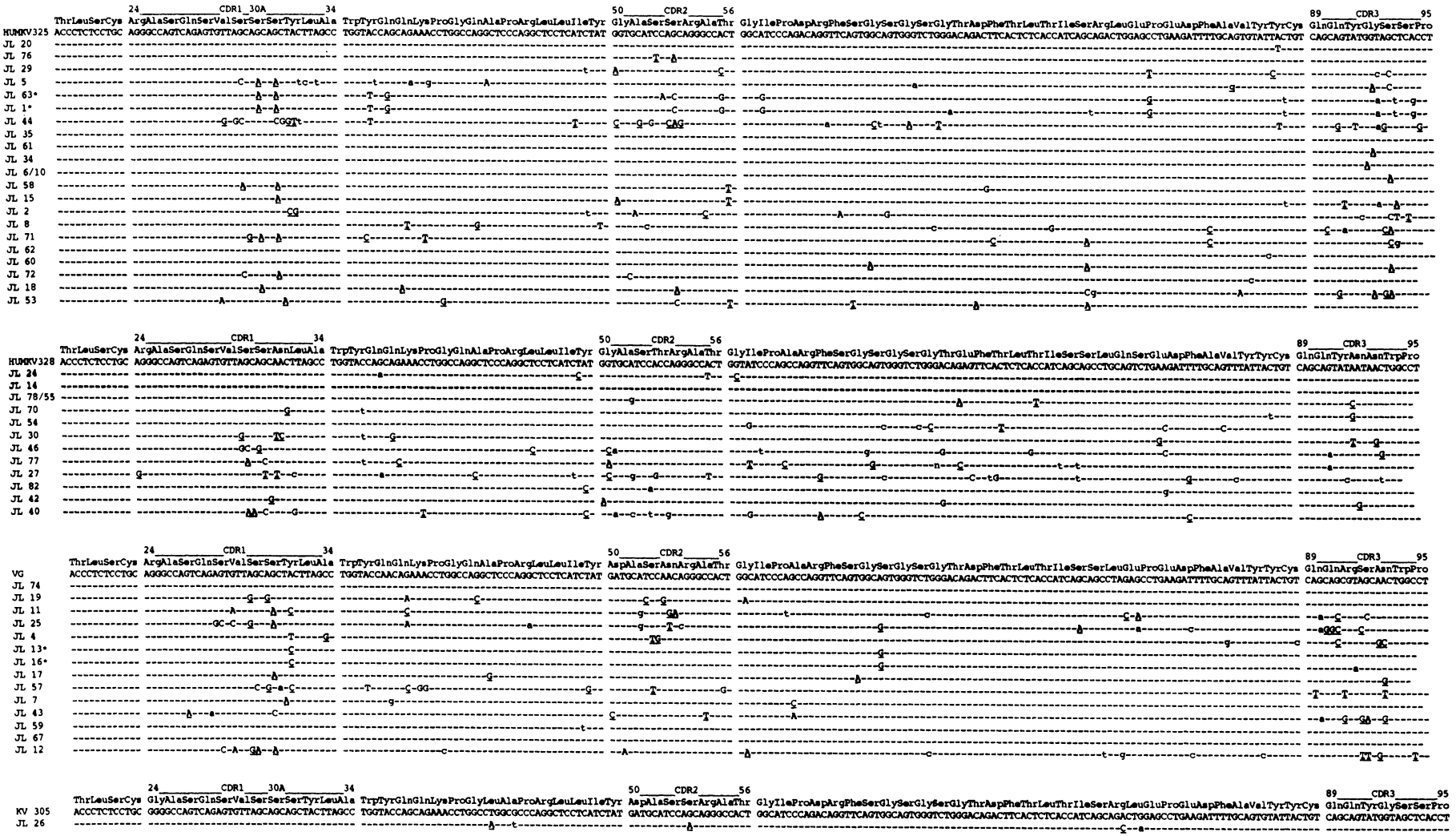


Figure 1. Nucleotide sequences of VL regions of rearranged VκIII clones in the nonsorted B cell adult library. Sequences are aligned under the most homologous prototype GL sequences. *Humkv*325 and *kv*305 are from reference 47, *Vg* is from reference 45, and *humkv*328 is from reference 46. Conserved positions are indicated by dashes. Silent changes are indicated by lowercase letters, and nonconservative changes are indicated by capital underlined letters. CDR are also indicated, as well as the amino acid transcription. *JL63/1 and JL16/13 were considered to be clonally related (see text). Amino acid numbering is according to Kabat et al. (56).

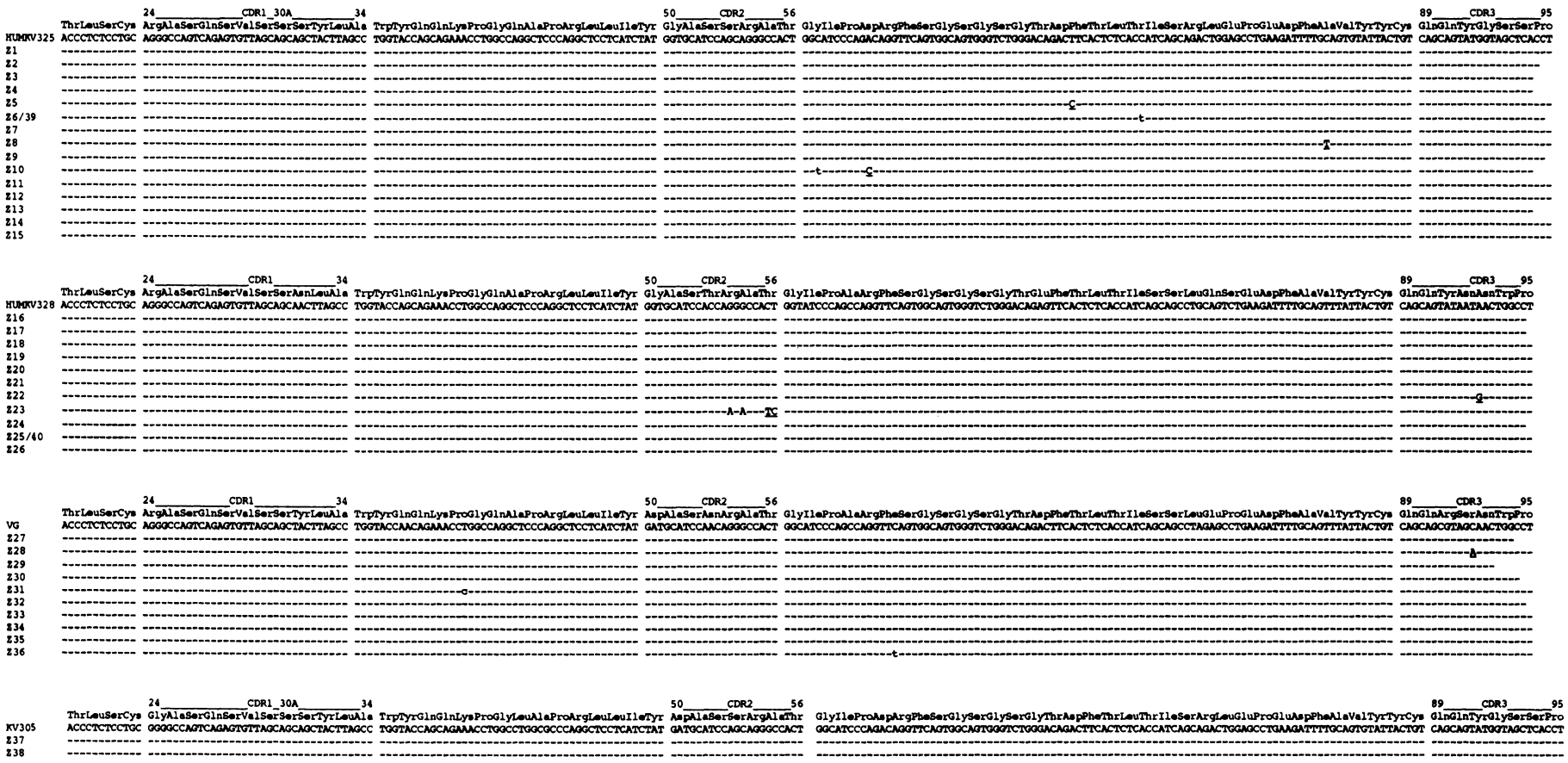


Figure 2. Nucleotide sequences of VL regions rearranged VκIII clones in the newborn cord blood library. Signs are identical to those of Fig. 1.

Table I. VkIII Gene Segment Frequency

Gene segment	Newborn	A	Adult	B	C
humkv325	15 (39%)	$P < 0.03$	20 (43%)	$P < 0.01$	NS
humkv328	11 (29%)	$P = 0.2$	12 (26%)	$P = 0.27$	NS
Vg	10 (26%)	$P = 0.3$	13 (28%)	$P = 0.09$	NS
kv305	2 (5%)	$P = 0.22$	1 (2%)	$P < 0.05$	NS
Vg''	0	$P < 0.03$	0	$P < 0.02$	NS
Vh	0	$P < 0.03$	0	$P < 0.02$	NS
Total (*n*)	38		46		

A and *B*: Statistical significance between the observed and theoretical frequency. *C*: Statistical significance between the observed frequencies in the newborn and adult libraries.

Table II. Frequency of J_K Gene Segment Usage among Newborn and Adult Sequences

		Jκ1	Jκ2	Jκ3	Jκ4	Jκ5	*n*
		%	%	%	%	%	
Humkv325	Newborn	33	20	20	20	7	15
	Adult	30	35	10	20	5	20
Humkv328	Newborn	73*	27	0	0	0	11
	Adult	8	67*	8	8	8	12
Vg	Newborn	20	20	0	20	40	10
	Adult	31	8	23	15	23	13
Kv305	Newborn	0	0	100	0	0	2
	Adult	0	0	100	0	0	1
Total	Newborn	40	21	13	13	13	38
	Adult	24	35	15	15	11	46
Stochastic usage		20	20	20	20	20	

The results are expressed as percentage of the analyzed sequences. * Statistically significant differences ($P < 0.05$).

with 61% in the newborn library (Fig. 3 and Table II). The main difference between the two libraries concerned the relative occurrence frequency of *Jk*1 and *Jk*2; this difference results mainly from a preferential rearrangement between *kv*328 and *Jk*1 in the newborn and between this same V_K gene and *Jk*2 in the adult. There was no other significant preferential rearrangement.

Heterogeneity of the VκIII–J_K junctions. The V_K–J_K junction sequences in both libraries were highly diverse (Fig. 4) and could be subdivided into three groups. In the first one, the rearrangement resulted from the simple juxtaposition of the V_K and J_K gene segments in their full coding capacity (this type of rearrangement was rare: three adult and two newborn clones). In the second group, some nucleotides of the last V_K codon and/or of the first J_K codon were nibbled away (9 newborn and 18 adult clones); in most instances, the subsequent recombination involved noncoding GL nucleotides flanking the intact gene segment. It is worth noting that we could add to this group some rare cases where several nucleotides in codons 95/96 were not readily explicable by recombinations between GL sequences. For instance, the Gln 96 in the JL24 clone could imply two substitutions, G → A in the first *Jk*1 codon or N addition (see Discussion). In the other cases, the nucleotide deletions were not compensated for nucleotides flanking the V_K or J_K segments and resulted in a shorter CDR3 than usual kappa chains. In contrast, in the third group, 16 out of 38 (42%) neonatal clones and 16 out of 46 (35%) adult clones

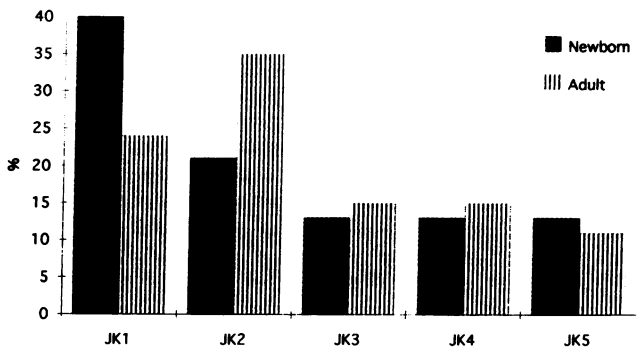
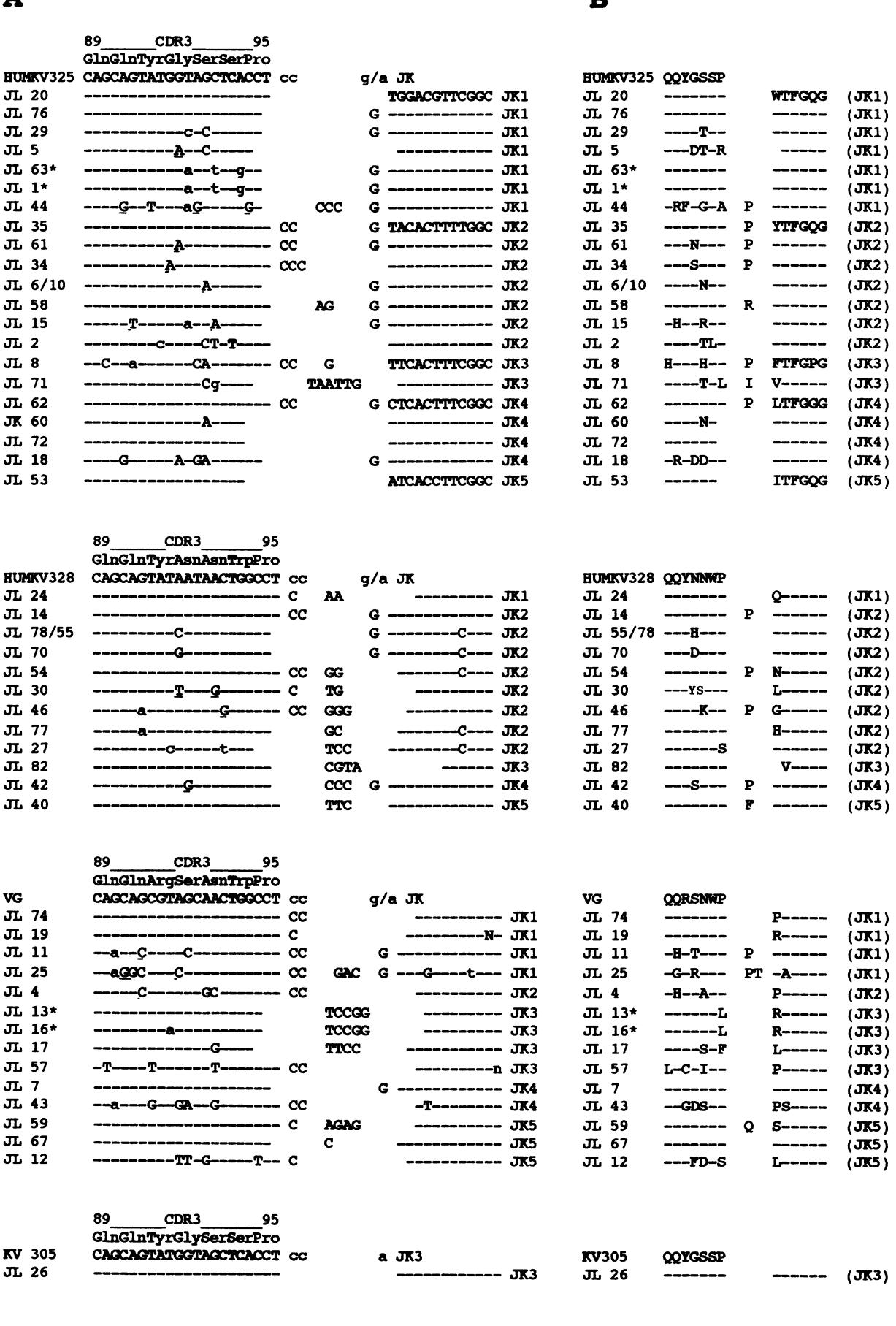


Figure 3. Graphic comparison of J_K utilization in the 46 VκIII randomly isolated clones from adult library and in 38 clones from the newborn library. The occurrence frequency of each J_K segment gene is plotted.

contained one or two additional codons inserted between V_K and J_K segments (positions 95A/B). The resulting amino acid was a proline in 12 out of 16 (75%) adult clones and in 11 out of 16 (69%) neonatal clones; the other amino acids were Arg (three sequences), Leu, Glu, and Ser (two sequences each), Ile, Phe, and Gly (one sequence each). In 41% of the sequences, the additional codon could have been generated by recombinations between GL nucleotides commonly found at the 3′ end of the V_K and at the 5′ end of the J_K genes. To give an example: in the JL14 clone, the codon 95A CCG could have been generated by a recombination between the two Cs immediately flanking the 5′ end of *kv*328 and the G flanking *Jk*2. However, in 59% of the sequences (10 out of 16 adult clones and 9 out of 16 newborn clones) the additional codons were not explicable by recombinations between GL sequences (for instance, see clone JL17) and were probably the result of an N addition process.

Expressed VκIII genes in circulating B cells from the adult but not from the neonate are somatically mutated. Fig. 2 shows that most expressed VκIII genes isolated from the cord blood were unmutated copies of GL genes. In contrast, most of the sequences from the adult differed from each other and from the prototype sequences of GL VκIII genes. Since there seems to be little polymorphism in the VκIII family and since clones JL35, JL14, and JL74 are 100% homologous to the prototype sequences of *kv*325, *kv*328, and *Vg*, respectively, most of these nucleotide differences probably resulted from somatic mutations. It is also unlikely that these base changes were due to infidelities of the Taq polymerase. Given the estimated error rate of the enzyme (10^{-4} bp/cycle) and the length of our sequences, the theoretical error rate would be 1.29 per sequence; this does not significantly modify our results. The minimal mutation rates, excluding the shared mutations of probably clonally related sequences, were estimated from the ratio between the number of base changes occurring within each region and the total number of nucleotides in the same region. The analysis of these mutation rates (Table III) shows that the CDRs were generally more mutated than the framework (FRW) regions. The frequency of base changes ranged from 3.83 to 8.77% in the CDR compared with 1.56 to 2.59% in the FRW regions. Evaluation of the R:S ratios is shown in Table


A
B
89_____CDR3_____95
GlnGlnTyrGlySerSerPro
HUMKV325 CAGCAGTATGGTAGCTCACCT cc g/a JK
JL 20 ---------- TGGACGTTCGGC JK1
JL 76 ---------- G ---------- JK1
JL 29 ----------c-C------ G ---------- JK1
JL 5 ----------A--C----- ---------- JK1
JL 63* ----------a--t--g-- G ---------- JK1
JL 1* ----------a--t--g-- G ---------- JK1
JL 44 ----G--T---aG-----G- CCC G ---------- JK1
JL 35 ---------- CC G TACACTTTTGGC JK2
JL 61 ----------A---------- CC G ---------- JK2
JL 34 ----------A---------- CCC ---------- JK2
JL 6/10 ----------A------ G ---------- JK2
JL 58 ---------- AG G ---------- JK2
JL 15 -----T-----a--A----- G ---------- JK2
JL 2 --------c----CT-T---- ---------- JK2
JL 8 --C--a------CA------- CC G TTCACTTTCGGC JK3
JL 71 ----------Cg---- TAATTG ---------- JK3
JL 62 ---------- CC G CTCACTTTCGGC JK4
JK 60 ----------A---- ---------- JK4
JL 72 ---------- ---------- JK4
JL 18 ----G-----A-GA------ G ---------- JK4
JL 53 ---------- ATCACCTTCGGC JK5
HUMKV325 QQYGSSP
JL 20 ------- WTFGQG (JK1)
JL 76 ------- ------ (JK1)
JL 29 ----T-- ------ (JK1)
JL 5 ---DT-R ----- (JK1)
JL 63* ------- ------ (JK1)
JL 1* ------- ------ (JK1)
JL 44 -RF-G-A P ------ (JK1)
JL 35 ------- P YTFGQG (JK2)
JL 61 ---N--- P ------ (JK2)
JL 34 ---S--- P ------ (JK2)
JL 6/10 -----N-- ------ (JK2)
JL 58 ------- R ------ (JK2)
JL 15 -H--R-- ------ (JK2)
JL 2 ----TL- ------ (JK2)
JL 8 H---H-- P FTFGPG (JK3)
JL 71 ----T-L I V----- (JK3)
JL 62 ------- P LTFGGG (JK4)
JL 60 ----N- ------ (JK4)
JL 72 ------ ------ (JK4)
JL 18 -R-DD-- ------ (JK4)
JL 53 ------ ITFGQG (JK5)
89_____CDR3_____95
GlnGlnTyrAsnAsnTrpPro
HUMKV328 CAGCAGTATAATAACTGGCCT cc g/a JK
JL 24 ---------- C AA --------- JK1
JL 14 ---------- CC G ---------- JK2
JL 78/55 ---------C---------- G --------C--- JK2
JL 70 ---------G---------- G --------C--- JK2
JL 54 ---------- CC GG -------C--- JK2
JL 30 ---------T---G------ C TG --------- JK2
JL 46 -----a--------G------ CC GGG --------- JK2
JL 77 -----a---------- GC -------C--- JK2
JL 27 --------c-----t--- TCC --------C--- JK2
JL 82 ---------- CGTA ------ JK3
JL 42 ----------G--------- CCC G ---------- JK4
JL 40 ---------- TTC ---------- JK5
HUMKV328 QQYNNWP
JL 24 ------- Q----- (JK1)
JL 14 ------- P ------ (JK2)
JL 55/78 ---H--- ------ (JK2)
JL 70 ---D--- ------ (JK2)
JL 54 ------- P N----- (JK2)
JL 30 ---YS--- L----- (JK2)
JL 46 -----K-- P G----- (JK2)
JL 77 ------- H----- (JK2)
JL 27 ------S ------ (JK2)
JL 82 ------- V---- (JK3)
JL 42 ---S--- P ------ (JK4)
JL 40 ------- F ------ (JK5)
89_____CDR3_____95
GlnGlnArgSerAsnTrpPro
VG CAGCAGCGTAGCAACTGGCCT cc g/a JK
JL 74 ---------- CC ---------- JK1
JL 19 ---------- C ---------N- JK1
JL 11 --a--C----C---------- CC G ---------- JK1
JL 25 --aGGC---C---------- CC GAC G ---G----t--- JK1
JL 4 -----C------GC------- CC ---------- JK2
JL 13* ---------- TCCGG --------- JK3
JL 16* --------a--------- TCCGG --------- JK3
JL 17 ----------G---- TTCC ---------- JK3
JL 57 -T----T------T------- CC ---------n JK3
JL 7 ---------- G ---------- JK4
JL 43 --a---G--GA--G------- CC -T-------- JK4
JL 59 ---------- C AGAG ---------- JK5
JL 67 ---------- C ---------- JK5
JL 12 ---------TT-G-----T-- C ---------- JK5
VG QQRSNWP
JL 74 ------- P----- (JK1)
JL 19 ------- R----- (JK1)
JL 11 -H-T--- P ------ (JK1)
JL 25 -G-R--- PT -A---- (JK1)
JL 4 -H--A-- P----- (JK2)
JL 13* ------L R----- (JK3)
JL 16* ------L R----- (JK3)
JL 17 ----S-F L----- (JK3)
JL 57 L-C-I-- P----- (JK3)
JL 7 ------- ------ (JK4)
JL 43 --GDS-- PS---- (JK4)
JL 59 ------- Q S----- (JK5)
JL 67 ------- ------ (JK5)
JL 12 ---FD-S L----- (JK5)
89_____CDR3_____95
GlnGlnTyrGlySerSerPro
KV 305 CAGCAGTATGGTAGCTCACCT cc a JK3
JL 26 ---------- ---------- JK3
KV305 QQYGSSP
JL 26 ------- ------ (JK3)

C

	89_____CDR3_____95 GlnGlnTyrGlySerSerPro	g/a JK	
HUMKV325	CAGCAGTATGGTAGCTCACCT cc		TGGACGTTCGGC (JK1)
Z1	----------	TT	---------- (JK1)
Z2	----------	TT	G ---------- (JK1)
Z3	----------	ACAGGA	---------- (JK1)
Z4	----------		---------- (JK1)
Z5	---------- CC		G TACAGTTTTGGC (JK2)
Z6/39	----------	GAG	G ----G------- (JK2)
Z7	---------- CC	AA	-G------- (JK2)
Z8	---------- C	GGGG	TTCACTTTCGGC (JK3)
Z9	----------	TT	A ---------- (JK3)
Z10	----------	CT	A ---------- (JK3)
Z11	----------		G CTCACTTTCGGC (JK4)
Z12	---------- CC		---------- (JK4)
Z13	----------		---------- (JK4)
Z14	----------		G ATCACCTTCGGC (JK5)
Z15	---------- CC		

	89_____CDR3_____95 GlnGlnTyrAsnAsnTrpPro	g/a JK	
HUMKV328	CAGCAGTATAATAACTGGCCT	GCC	---------- (JK1)
Z16	----------	GCCGAGC	---------- (JK1)
Z17	----------		G ---------- (JK1)
Z18	---------- CC	A	G ---------- (JK1)
Z19	---------- C	GA	---------- (JK1)
Z20	---------- C		---------- (JK1)
Z21	---------- C		G ---------- (JK1)
Z22	----------G-------- CC		---------- (JK1)
Z23	---------- C		G ---------- (JK2)
Z24	----------		G ---------- (JK2)
Z25/40	---------- CC		----G------- (JK2)
Z26	---------- CCC		

	89_____CDR3_____95 GlnGlnArgSerAsnTrpPro	g/a JK	
VG	CAGCAGCGTAGCAACTGGCCT	GGA	---------- (JK1)
Z27	----------		G ---------- (JK1)
Z28	----------A--------- CC	C	GG ----G------- (JK2)
Z29	----------	A	G ----G------- (JK2)
Z30	----------	GC	GG ---------- (JK4)
Z31	---------- CC		G ---------- (JK4)
Z32	----------	T	G ---------- (JK5)
Z33	---------- C	GTC	G ---------- (JK5)
Z34	---------- CC		---------- (JK5)
Z35	----------	TC	G ---------- (JK5)
Z36	----------		

	89_____CDR3_____95 GlnGlnTyrGlySerSerPro	g/a JK	
KV305	CAGCAGTATGGTAGCTCACCT		---------- (JK3)
Z37	---------- C	G	---------- (JK3)
Z38	---------- CC		

D

	QQYGSSP		
HUMKV325	QQYGSSP		WTFGQG (JK1)
Z1	-------		------ (JK1)
Z2	------L		------ (JK1)
Z3	------L		G----- (JK1)
Z4	------T		P----- (JK1)
Z5	-------		YTFGQG (JK2)
Z6/39	-------	PR	-S---- (JK2)
Z7	------- PR		QS---- (JK2)
Z8	-------	G	FTFGPG (JK3)
Z9	------- G		------ (JK3)
Z10	------L	L	------ (JK3)
Z11	------- L	P	LTFGGG (JK4)
Z12	------- P		------ (JK4)
Z13	------		out of frame
Z14	-------	P	ITFGQG (JK5)
Z15	------- P		

	QQYNNWP		
HUMKV328	QQYNNWP		P----- (JK1)
Z16	-------	P	S----- (JK1)
Z17	------- P	P	------ (JK1)
Z18	------- P	Q	------ (JK1)
Z19	------- Q		R----- (JK1)
Z20	-------		R----- (JK1)
Z21	-------	P	------ (JK1)
Z22	----D-- P		R----- (JK1)
Z23	-------		------ (JK2)
Z24	-------	P	------ (JK2)
Z25/40	------- P	P	-S---- (JK2)
Z26	------- P		

	QQRSNWP		
VG	QQRSNWP		----- (JK1)
Z27	------G	P	------ (JK1)
Z28	---R--- P		-S---- (JK2)
Z29	-----R		-S---- (JK2)
Z30	------Q	PR	------ (JK4)
Z31	------- PR		------ (JK4)
Z32	-------	L	------ (JK5)
Z33	------- L	PS	------ (JK5)
Z34	------- PS		------ (JK5)
Z35	-------	S	------ (JK5)
Z36	------- S		

	QQYGSSP		
KV305	QQYGSSP		L----- (JK3)
Z37	-------		P----- (JK3)
Z38	-------		

Figure 4. (*A*) Nucleotide sequences of the light chain junction regions of rearranged VκIII clones in the nonsorted B cell adult library. GL nucleotides between the coding sequences and the heptamer and possibly involved in the V_K–J_K rearrangements are represented by lowercase letters upstream of J_K and downstream of V_K GL segments. Gaps were introduced to facilitate alignment of the sequences and to distinguish nucleotides not directly explicable by recombinations between GL sequences (see text). Signs are otherwise identical to those of Fig. 1 and J_K locus is from reference 57. (*B*) Amino acid transcriptions of the sequences presented in *A;* gaps have been introduced to distinguish additional amino acid residues in positions 95A/B. Amino acid numbering is according to Kabat et al. (56). (*C*) Nucleotide sequences of the light chain junction regions of rearranged VκIII clones in the newborn cord blood library. Signs are identical to those in *A*. (*D*) Amino acid transcriptions of the sequences presented in *C;* signs are identical to those in *B*.

Table III. Percent Mutation Rates in the Adult

	CDR1	FR2	CDR2	FR3	CDR3
Humkv325	3.83	1.69	5.22	1.79	8.33
Humkv328	4.55	2.59	5.95	2.28	4.10
Vg	5.84	2.22	5.10	1.56	8.77
Kv305	—	—	—	—	—
Global	4.48	2.13	5.36	1.89	7.01

IV. R:S ratio is a valuable index of nucleotide mutations in determining the structurally or functionally important domains of a protein. Because of the genetic code, the theoretical value for mutations occurring randomly is ~ 2.9; with regard to antibodies, high CDR R:S ratios reflect the positive selection mediated by the antigen, whereas low FRW R:S ratios reflect the structural constraints of these regions. As expected, R:S ratios were consistently < 2.9 in the FRW regions, whereas they were mostly > 4 in the CDR. Exceptions are the CDR3 of *kv*325 and *kv*328 (2.67 and 1.5, respectively) and most notably the CDR2 of *kv*328 (0.87).

Analysis of the VκIII repertoire expressed by adult CD5+ peripheral blood B cells

A total of 78 randomly picked clones was sequenced. The V_K regions of the cloned VJ segments are shown in Fig. 5, and the V–J junction regions are shown in Fig. 6. Surprisingly, many sequences were noted repeatedly. The V–J sequences of 52 clones could be classified in nine groups, each one represented only once with the sequence numbers in brackets (JLCD5/10[2]; /1[10]; /603[4]; /405[8]; /9[3]; /40[4]; /100[14]; /416[5]; and /406[2]). These clones are most probably derived from no more than nine different V_K–J_K rearrangements and possibly less. Indeed, the groups JLCD5/405[8] and JLCD5/9[3] are probably clonally related on the basis of a nearly identical sequence across the V_K–J_K junction. The same observation can be made for the groups JLCD5/1[10] and /603[4]. The remaining 26 sequences occurred only once in the library. However, all but three (JLCD5/34, /400, and /8) could be assigned to one of the seven different V_K–J_K rearrangements mentioned above on the basis of strong homology in the V segment and identical V_K–J_K junction. Each clone contained unique base changes in addition to shared mutations. Finally, JLCD5/8 arose probably from JLCD5/400 from which it differs by two nucleotide substitutions. Collectively these data suggest that the 78 library clones analyzed are actually derived from nine different B cell clones (referred to as clones A–I). With regards to V_K gene usage, only three members of the VκIII family were detected, and although the actual number of B cell clones analyzed was low, there appeared to be a preference for *kv*325 GL gene expression. Most of the sequences are highly homologous to known germline genes. However, JLCD5/1[10] shows only 92% homology with kv325 and may have derived from an allelic variant or from a new unreported V_K gene.

Table IV. R/S Ratios in the Adult

	CDR1	FR2	CDR2	FR3	CDR3
Humkv325	6.25	2.2	22	2	2.67
Humkv328	17	1.33	0.87	2.08	1.5
Vg	12.5	2.5	4	0.91	5.23
Kv305	—	—	—	—	—
Global	9.57	1.87	3.5	1.66	3

The analysis of the nine different V_K–J_K junctions (Fig. 6) shows that five are not explicable by recombinations between GL sequences (B cell clones A, C, D, E, F); only one has additional codons in positions 95A and 95B. It is noteworthy that the B cell clone C may have been artificially created. If the G in the codon 95 present in clones JLCD5/9[3], /2, and /24 resulted from a T → G mutation, these sequences belong to this B cell clone. However, if this G resulted from N addition, these sequences originated from a different B cell clone.

Discussion

To better understand all the available data concerning gene usage in the VκIII family made by fetal B cells, autoantibody-forming cells, and low malignant tumor B cells, we have used the cDNA/PCR cloning method to examine the usage of VκIII members in peripheral blood total and CD5+ B cells of one healthy adult and cord blood B cells of a normal newborn. The procedure does not introduce significant bias in the V gene sampling since four of the six GL VκIII genes are perfect matches with the VκIII-specific amplimers used, and two (*Vh* and *Vg*) differ by only one nucleotide. One cannot totally exclude the possibility of somatic variants with mutations in the hybridization region of the primers introducing a bias. However, this seems very unlikely and should not affect the comparative expression frequencies of each V gene segment. All three libraries were prepared from the same number of B cells. The cells were not stimulated and should therefore reflect the actual repertoire of circulating B cells in their native state. Since the libraries were prepared with cDNA, one expects to preferentially sample cells that have been activated in vivo. Consequently, our results may not represent the exact total repertoire. However, PCR is more sensitive than in situ hybridization and should cover a larger sample of B cell population (2).

Adult and newborn libraries

Nonrandom features of the VκIII repertoires. The usage of V_K genes was clearly not random in both adult and infant. Among the six functional members of the VκIII family, three (*kv*325, *kv*328, and *Vg*) represented > 90% of the expressed repertoire, and one member, *humkv*325, was statistically overrepresented in both libraries. Since, *humkv*325 is expressed by ~ 7% of fetal splenic B cells at week 23 of gestation (22), the bias observed in the neonatal library was expected, but the absence of difference in the occurrence frequencies of each V gene segment between the adult and the neonatal expressed repertoires was surprising. Examples of nonrandom V_H gene usage in normal adults have been published recently. They include *VH*26 (33, 34), *VH*4.21 (35), *VH*51*p*1 (36), and *VH*6 (37, 38). *Humkv*325 has also been identified by its idiotypic marker in 3.8% of tonsillar B cells (36) and is found recurrently in antibodies to exogenous antigens (39–41). It is striking to note that many of these overexpressed genes are also known to encode autoantibodies and/or to be expressed preferentially in the early ontogeny and during CLL. Even though our results concern only two individuals, the data clearly indicate that the "unusual" frequencies of expression of individual genes during

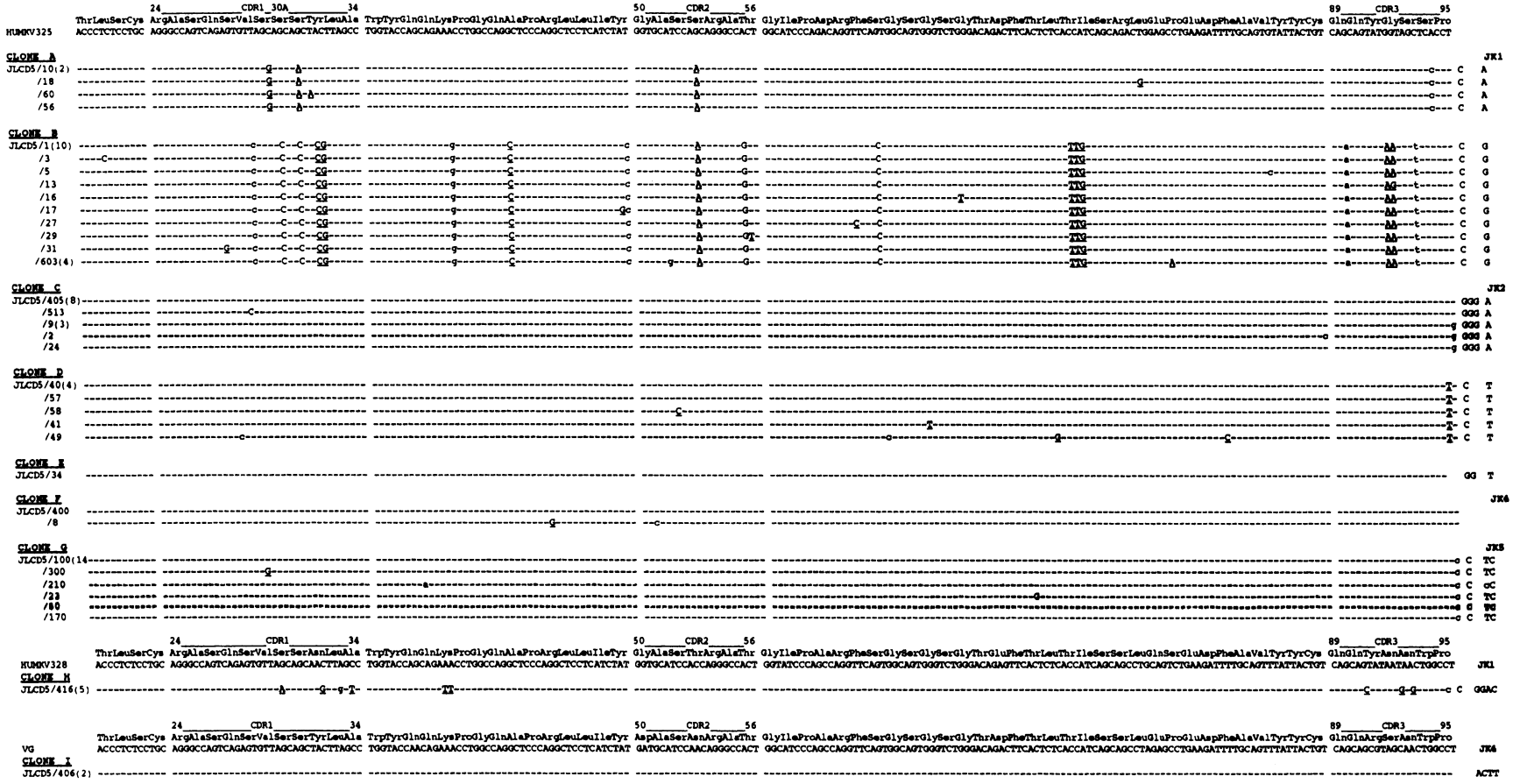


Figure 5. Nucleotide sequences of VL regions of rearranged VκIII clones in the CD5+ B cell adult library. Signs are identical to those in Fig. 1.

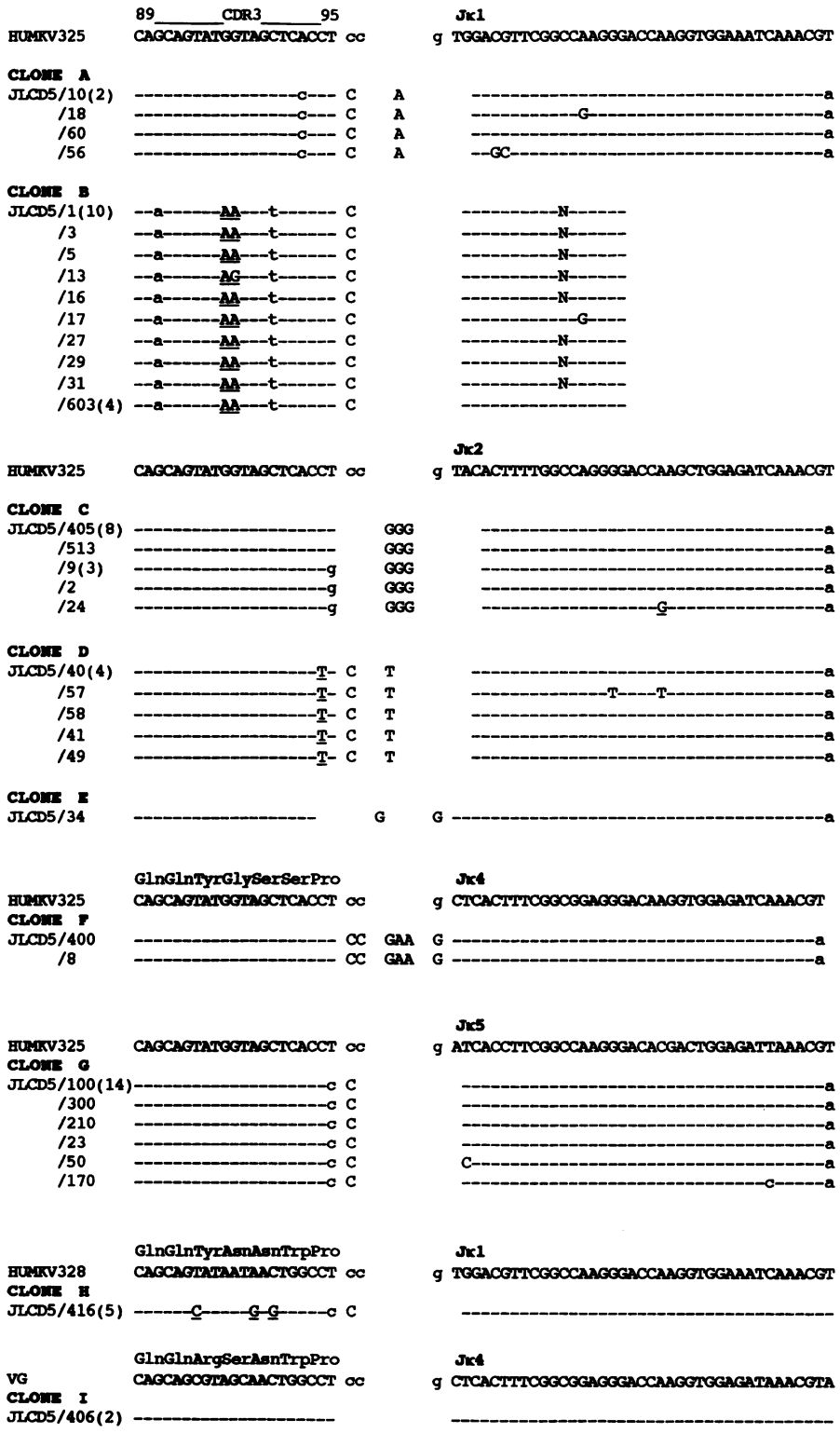


Figure 6. Nucleotide sequences of the light chain junction regions of rearranged VκIII clones in the CD5+ B cell adult library. Signs are identical to those in Fig. 4 *A*. See text for the naming of clones A–I.

pathological situations must be compared with the actual frequency of expression of these genes in normal conditions. For instance, the reported frequency of *kv*325 usage during K-expressing CLL ranged from 4 (32) to 25% (17) and should be compared with our actual frequency in normal B cells (close to 13% of the K-expressing B cells) before any interpretation can be suggested.

Common hypotheses for overrepresentation of certain GL genes include chromosomal position, number of gene copies, gene specific regulatory sequences (42), preferential pairing with H chains, and preferential selection on the basis of antigen binding or idiotypic specificities. *Humkv*325 is present in one copy in the haploid genome (13, 32), is highly conserved in the human species (43), and its chromosomal position does not

directly explain its overrepresentation (21). At that stage, we have no direct argument to distinguish between the last three hypotheses. Further studies will be required to determine whether this VκIII gene distribution is stable over periods of time and whether it is found recurrently in other unrelated individuals.

J_K gene segment usage. The results show a preference of *Jk*1 utilization in the neonatal repertoire, whereas the expression of J_K segments was fairly random in the adult repertoire. More striking was the repeated recurrence of particular V_K–J_K combinations, namely *humkv*328–*Jk*1 in the newborn and *hum*328–*Jk*2 in the adult. With regard to the neonatal repertoire, the data are not sufficient to be able to make the distinction between genetic mechanisms (namely chromosomal location and short homologies at the breakpoints of recombination) and selection. However, it is worth noting that most *humkv*328-encoded RF derive from a rearrangement between this gene and *Jk*1 (44). The preferential association between *humkv*328 and *Jk*2 in the adult library more probably may be due to antigenic selection. Indeed, when looking at the junction regions involving *humkv*328 in the adult and newborn libraries, the overrepresentation of *humkv*328–*Jk*2 is hardly explicable by the above-mentioned genetic mechanisms.

Diversity of the V_K–J_K junctions. In contrast to the restricted utilization of VκIII genes in both libraries, there was a great diversity of the V_K–J_K junctions. Diversity at the V–J junction classically results from exonuclease trimming at either V or J coding ends, followed by recombination frequently involving noncoding GL nucleotides flanking the intact gene segment. This results in a strict maintenance of the kappa chain CDR3 size, which stands in contrast with the variability of the heavy chain CDR3 length. Surprisingly, 42% of the neonatal clones and 35% of the adult clones contained one or two additional codons inserted between the V_K or J_K segments (positions named 95A/B), resulting in unorthodoxically long CDR3. Recently, we described a cDNA/PCR library of VκIII–J_K rearrangements expressed by RF-producing B cells in the peripheral blood and synovial liquid from a patient with RA (23, 44). We found that 55% of the analyzed clones had the aforementioned abnormally long kappa chain CDR3. We also demonstrated that the additional codons resulted frequently from a mechanism of nontemplate nucleotide addition probably mediated by the terminal deoxynucleotidase. As other published examples were rare, we proposed the possibility of it being a feature of pathogenic RF during RA. Our present results clearly demonstrate that this is not the case. In many cases the additional codons could have been generated by recombinations between GL nucleotides commonly flanking the VκIII and J_K exons (JL14 represents one such example). This probably accounts for the overrepresentation of proline residues, since the most common nucleotides flanking the 3′ end of *humkv*325/328 and *Vg* are two Cs (see Fig. 4) (44–47). Finally in 56 and 63% of the neonatal and adult clones, respectively, the additional codons were not readily explicable by recombinations between GL sequences. Whichever mechanisms operate, our data demonstrate that the potential of diversity of V_K–J_K junction is much greater than previously thought (at least for the VκIII family). Moreover, this potential seems to be fully operating at birth and does not increase with age.

Somatic mutations. As expected, most of the sequences from the neonatal library were highly homologous to GL genes. In contrast, there was a pattern of considerable V_K sequence diversity in the adult library mainly because of somatic mutations. Indeed, most of the observed nucleotide differences result, most probably, from somatic mutations whose patterns suggest antigen selection. Indeed, recent data indicate that most peripheral B cells in adult mice may be ligand selected (48).

Collectively, our data indicate that the repertoire of VκIII genes expressed in peripheral B cells may be much more restricted than anticipated in view of the genetic information available. Recent data indicate that this feature may be generalized to the entire repertoire of expressed human V genes (4, 33, 34). Moreover our results suggest that an important fraction of the adult repertoire arises through clonal selection from the fetal Ab substrate which is highly polyspecific.

CD5+ B cell library

Since most of the B lymphocytes in cord blood express CD5 (49), it was interesting to compare the VκIII expression during the neonatal period with that of CD5+ B cells isolated from the adult peripheral blood. Surprisingly, we found that the 78 clones sequenced derived most probably from no more than nine different V_K–J_K rearrangements. By extension, these data suggest that the adult peripheral blood CD5+ B cell population may be dominated by a small number of B cell clones. These results are not linked to any artifact since: (*a*) the adult CD5+ library was prepared under the same conditions and with the same number of B cells as the newborn library; (*b*) a second library prepared with PBL drawn from the same donor 3 mo later gave similar results (data not shown). Although the number of B cell clones analyzed is consequently low, the adult CD5 B cell VκIII repertoire seems to parallel the newborn one in terms of V gene usage and somatic mutations. Indeed, *kv*325 is overrepresented, and most of the sequences are highly homologous to the prototype GL genes. Exceptions are represented by clones B and H. The accumulation of somatic mutations in V genes expressed by human CD5+ B cells has been described already but seems to be rare (50). B and H may represent clones that had ceased the somatic mutation process and had undergone a period of clonal expansion afterwards.

How can these data be interpreted in the context of current knowledge regarding CD5 B cells (for reviews see references 9, 10, 51)? CD5+ B cells (B1a) seem to constitute a separate lineage of B cells that: (*a*) predominates in early life; (*b*) expresses a limited set of V genes with few mutations; (*c*) produces IgM antibodies that react frequently with self antigen and that are highly interconnective (as demonstrated in the mouse); and (*d*) has the capacity of self-renewal. These features have suggested important roles for these cells in the early exoantigen-free development of the immune system. However, their potential role later in life is much more elusive. Hardy and Hayakawa (52) have demonstrated that in the mouse B1a cells arise from precursors different from those of conventional B cells; progenitors for B1a cells are abundant in fetal liver and are rare in the adult bone marrow. Conceivably, the restricted diversity that we observed could be a direct consequence of the rarefaction of the precursors with age. Alternatively, most of the B1a cells in the adult could have been generated during early life. In that case, the restriction in the repertoire may at least in part reflect selection by different antigens and/or idiotypes. For instance, B1a cells committed to RF production

could be maintained throughout life because they are exposed continuously to their antigen. Indeed, B1a cells are enriched in RF-producing cells during both neonatal and adult periods (9, 53).

A participation of B1a cells in primary immune responses has been hypothesized because of the polyreactivity of their surface receptors for antigen. Indeed, Ueki et al. (54) demonstrated that most of the B cell clones involved in the primary response to antirabies virus vaccine were CD5+. Because B1a cells use a limited set of poorly mutated V genes, they do not include a large number of clones committed to the production of antibodies able to bind a given antigen. The results are consistent with such an hypothesis and would in that case only represent a snapshot of such responses. The capacity of self-renewal and the longevity of B1a cells make them good candidates for the maintenance of immunological memory. The survival of memory B cells seems to depend on the persistence of antigen.

A last possibility suggested by our data is that most B1a cells in the adult peripheral blood could represent remnants of the early differentiation pathways that have undergone some kind of transforming events. Accordingly, chronic lymphocytic leukemia, which may be considered a malignancy of B1a cells, is the most frequent leukemia, and some data indicate that the first transforming event in this disease occurs probably early in life (55).

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