Molecular Mechanisms and Selective Influences That Shape the Kappa Gene Repertoire of IgM$^+$ B Cells

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Abstract

To analyze the human kappa chain repertoire and the influences that shape it, a single cell PCR technique was used that amplified VκJκ rearrangements from genomic DNA of individual human B cells. More than 350 productive and 250 nonproductive VκJκ rearrangements were sequenced. Nearly every functional Vκ gene segment was used in rearrangements, although six Vκ gene segments, A27, L2, L6, L12a, A17, and O12/O2 were used preferentially. Of these, A27, L2, L6, and L12a showed evidence of positive selection based on the variable region and not CDR3, whereas A17 was overrepresented because of a rearrangement bias based on molecular mechanisms. Utilization of Jκ segments was also nonrandom, with Jκ1 and Jκ2 being overrepresented and Jκ3 and Jκ5 underrepresented in the nonproductive repertoire, implying a molecular basis for the bias. In B cells with two VκJκ rearrangements, marked differences were noted in the Vκ segments used for the initial and subsequent rearrangements, whereas Jκ segments were used comparably. Junctional diversity was generated by n-nucleotide addition in 60% and by exonuclease trimming in 75% of the VκJκ rearrangements analyzed. Despite this large degree of diversity, a strict CDR3 length was maintained in both productive and nonproductive rearrangements. More than 23% of the productive rearrangements, but only 7% of the nonproductive rearrangements contained somatic hypermutations. Mutations were significantly more frequent in Vκ sequences derived from CD5$^-$ as compared with CD5$^+$ B cells. These results document that the gene segment utilization within the Vκ repertoire is biased by both intrinsic molecular processes as well as selection after light chain expression. Moreover, IgM$^+$memory cells with highly mutated kappa genes reside within the CD5$^-$ but not the CD5$^+$ B cell compartment. (*J. Clin. Invest.* 1997. 99:1614–1627.) Key words: immunoglobulin • light chain • antigen • V(D)J rearrangement • mutation

Introduction

Antibody diversity is generated by a series of molecular events, including the random recombination of V, D, and J gene segments, the introduction of junctional diversity, and somatic hypermutation (1–3). In addition, a series of events dependent on the expression of immunoglobulin protein, such as pairing of heavy and light chains, positive and negative selection, as well as affinity maturation all influence the expressed immunoglobulin repertoire (4, 5). These mechanisms have the potential to yield a repertoire of almost unlimited antigenic specificity and provide the capacity to generate antibodies of increasing affinity in response to antigen, while avoiding the production of autoantibodies.

Immunoglobulin genes undergo rearrangement of gene segments in an ordered fashion during ontogeny (2). Heavy chain rearrangement and the expression of cytoplasmic μ chain occur first, followed by kappa light chain rearrangement. Kappa chains are encoded by ~ 40 functional V gene segments that are organized in two cassettes and span over 1,800 kb of DNA (6). They are expressed by the majority of human and mouse B cells and play a major role in determining the specificity of the immunoglobulin molecule. Despite the large number of potential VκJκ rearrangements, it appears that specific gene segments are used preferentially in the expressed repertoire. One of the eight members of the VκIII family, A27, appears to be overrepresented in both neonatal and adult human peripheral blood (7). Moreover, a preference for Jκ1 and Jκ2, compared with the other Jκ gene segments has been noted (7). Additional studies have confirmed the biased utilization of Vκ genes in the expressed repertoire. For example, in addition to A27 (8, 9), other Vκ genes have been reported to be overrepresented, including two genes belonging to the VκI family, O8/O18 and O2/O12, two genes belonging to the VκII family, A19/A3 and A17, and the single VκIV family member, B3 (8). These data indicate that despite the rather large number of genetic elements from which immunoglobulin molecules could be assembled, light chains as well as heavy chains (10) utilize specific gene segments preferentially. Neither the molecular mechanisms underlying such bias nor an evolutionary advantage of such disproportionate usage of certain genetic elements has been clearly delineated.

To date it has been difficult to identify the contributions of molecular events and those processes dependent on expression of an immunoglobulin protein on the shape of the B cell repertoire. One way to carry out this analysis would involve a comprehensive assessment of both productively rearranged and nonproductively rearranged immunoglobulin genes from the same population of B cells. Nonproductively rearranged genes are not translated into protein, and therefore, their presence does not influence the fate of the B cell expressing them. Analysis of those rearrangements permits an assessment of the molecular mechanisms that shape the repertoire. On the other

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hand, productively rearranged genes are translated into a protein product that may be expressed as part of a functional antigen receptor. B cells expressing surface immunoglobulin can be influenced by a variety of selective forces resulting either in their preferential expansion or deletion from the repertoire (11). Assuming that productive and nonproductive Vκ genes within the same B cell arise by a similar process of rearrangement of random gene segments, comparison of the two repertoires should provide an indication of the influence of selective pressures in shaping the repertoire.

To assess the dimensions of the human Vκ repertoire and deduce some of the influences that shape it, a previously described single cell PCR technique was used (10) to examine productive and nonproductive VκJκ rearrangements from individual IgM^+ B cells. By amplifying genomic DNA of resting B cells, > 350 productively rearranged and 250 nonproductively rearranged Vκ genes have been analyzed. Nearly every functional Vκ gene was found to be rearranged, with a small number of Vκ genes predominating. No difference in kappa gene usage was found between $CD5^+$ and $CD5^-$ B cells. Comparison of the productive and nonproductive repertoires indicated that some Vκ genes were overrepresented because of recombinational bias whereas others were positively selected. Genes that have been reported to be overexpressed in autoimmune disorders were also found to be overrepresented in the normal repertoire. The molecular mechanisms that account for CDR3 length and junctional diversity were similar in both productive and nonproductive VκJκ rearrangements. Finally, a significant number of IgM^+ B cells were found to have undergone somatic hypermutation. These results defining the normally expressed human kappa chain repertoire provide the basis for a more comprehensive understanding of the molecular and subsequent selective influences that shape it.

Methods

Cell preparation. Mononuclear cells were isolated from peripheral blood of two healthy donors by centrifugation on a ficoll-hypaque gradient as previously described (12). B cells were enriched using a CEPRATE LC kit (Cellpro, Inc., Bothwell, WA) that permits separation of $CD19^+$ cells by affinity column chromatography. The enriched B cell population was stained with PE-labeled anti–human CD19 monoclonal antibody (Sigma Chemical Co., St. Louis, MO), FITC-labeled anti–human IgM monoclonal antibody (PharMingen, San Diego, CA), a biotinylated anti–human CD5 monoclonal antibody (Becton-Dickinson, Mountain View, CA), and RED613-labeled streptavidin (GIBCO BRL, Gaithersburg, MD).

Cell sorting. Single $CD19^+IgM^+CD5^+$ and $CD19^+IgM^+CD5^-$ cells were sorted into 96-well PCR plates (Robins Scientific, Sunnyvale, CA) using a FACStar^{Plus} outfitted with an automated cell deposition unit (Becton-Dickinson). Each well contained 5 μl of an alkaline lysing solution (200 mM KOH/50 mM DTT). Four wells on each plate received no cell and served as negative controls.

Primer extension preamplification. After sorting single cells directly into lysing solution and incubating for 10 min at 65°C, the lysing solution was neutralized (5 μl of 900 mM Tris-HCl, pH 9.0, 300 mM KCl, 200 mM HCl). Afterward, 20 μl of a lower reagent mix (7.5 mM $MgCl_2$, 300 μM each dATP, dCTP, dGTP, and dTTP) was added to each well followed by a wax pellet (AmpliWax PCR Gem 100; Perkin Elmer Corp., Norwalk, CT). After sealing the wax, 30 μl of an upper reagent mix (20 mM Tris-HCl, pH 9.0, 0.2% [vol/vol] Triton X-100, 66.7 μM 15-base random oligonucleotides [Operon Technologies, Alamed, CA], 5 U Taq DNA Polymerase [Promega Corp., Madison, WI]) were added to each well. Amplification for 60 cycles was performed in a PTC-100 programmable thermal controller (MJ Research, Watertown, MA) according to the following program: initial denaturation for 10 min at 95°C, annealing for 2 min at 37°C, programmed ramping of 10 s/degree to 55°C, and extension for 4 min at 55°C. The denaturation step for all subsequent cycles was performed for 1 min at 94°C. All plates were stored at 4°C for further analysis by specific amplification.

Amplification of rearranged Vκ genes. The sequences of all family specific PCR primers are shown on Table I. All external primers bind in the leader sequence and all nested primers bind at the 5′ end of FR 1. For the external amplification, 25 μl of a lower reagent mix (100 μM each dNTP, 1.0 μM each primer, $MgCl_2$ according to an optimum determined for each primer set) were added to each well of a 96-well PCR plate (Robbins Scientific, Sunnyvale, CA). A wax pellet was used to seal each well. Next, 50 μl of an upper reagent mix (10 × PCR buffer, 2 U Taq DNA Polymerase (Promega Corp.) was added followed by 5 μl of template generated in the preamplification step. The amplification program consisted of one cycle of 95°C for 7 min, 56°C for 1 min, 72°C for 1 min 30 s, followed by 30 cycles of 94°C for 1 min, 56°C for 30 s, 72°C for 1 min 30 s. After cycle 30 all products received a final extension of 5 min at 72°C. The nested PCR was performed in an identical manner, using appropriate primers and 5 μl of each reaction product from the external PCR as template. The only difference in the nested program was an annealing temperature of 65°C.

Sequence analysis. All PCR products were separated by electrophoresis through 1.5% SeaKem LE agarose (FMC Bioproducts, Rockland, ME) and visualized with ethidium bromide. Positive bands were cut from the agarose and purified using GenElute Agarose Spin Columns (Supelco, Bellefonte, PA). The purified products were directly sequenced using the DyeDeoxy Termination Cycle Sequencing Kit (Applied BioSystems, Inc., Foster City, CA) and analyzed with an ABI 377 automated DNA sequencer (Applied BioSystems). The primers used for the second round of PCR amplification were used for sequencing. Vκ gene sequences were analyzed using GeneWorks software (IntelliGenetics, Inc., Mountain View, CA) and the Genebank and EMBL databases. The nomenclature of Zachau (6) has

*Table I. Sequences of Oligonucleotide Primers for Amplification of VκJκ Rearrangements**

External 5′ primers	
VκI/IIE	5′-GCTCAGCTCCTGGGGCT-3′
VκIIIE	5′-GGAA(AG)CCCCAGC(AGT)CAGC-3′
VκIV/VE	5′-CT(CG)TT(GC)CT(CT)TGGATCTCTG-3′
VκVI/VIIE	5′-CT(GC)CTGCTCTGGG(CT)TCC-3′
External 3′ primers	
Jκ2E	5′-ACGTTTGATCTCCAGCTTG-3′
Jκ5E	5′-CTTACGTTTAATCTCCAGTC-3′
Internal 5′ primers	
VκI-I	5′-CATCCAG(AT)TGACCCAGTCTCC-3′
VκII-I	5′-TCCAGTGGGGATATTGTGATGAC-3′
VκIII-I	5′-GTCT(GT)TGTCTCCAGGGGAAAGAG-3′
VκIV-I	5′-GACATCGTGATGACCCAGTCTC-3′
VκV-I	5′-GGGCAGAAACGACACTCACGCA-3′
VκVI-I	5′-TCCAGGGGTGAAATTGTG(AC)TGAC-3′
VκVII-I	5′-GCTGCAATGGGGACATTGTGCT-3′
Internal 3′ primers	
Jκ2I	5′-CAGCTTGGTCCCCTGGCCAAA-3′
Jκ5I	5′-CCAGTCGTGTCCCTTGGCCG-3′

*External 5′ primers hybridize to the leader sequence and internal primers hybridize to the 5′ end of framework region 1. Nucleotides in parentheses indicate mixed bases at that position.

been used throughout this paper. Designation of functional and nonfunctional Vκ gene segments is according to the analysis of Zachau (6) as modified by Tomlinson (13).

Estimation of Taq polymerase error. To estimate the fidelity of the amplification (14, 15) and sequencing with Taq polymerase, V_H and Vκ genes from the genomic DNA of individual B cells from a 4-yr-old child with X-linked hyper IgM syndrome were analyzed. Patients with hyper IgM syndrome do not form germinal centers and subsequently their B cells do not undergo somatic hypermutation (16). Of 9,429 nucleotides sequenced from kappa chains, 11 were mutated and of 13,696 nucleotides sequenced from heavy chains, 23 were mutated. This suggests maximal Taq error rates of 1.2 to 1.7 x 10^{-3} per base pair, respectively. This error rate corresponds to approximately 0.5 mutations per variable region, and is in close agreement with published Taq error rates calculated from sequences of B cell hybridomas and murine PALS-associated foci (17). Based on a conservative interpretation of the above calculations, kappa sequences with one or two mutations were considered to be germline.

Statistics. Chi square analysis was used for comparison of productive and nonproductive rearrangements, as well as for comparison of $CD5^+$ and $CD5^-$ populations. The chi square goodness of fit test was used to compare observed with expected frequencies in all cases in which the expected frequencies were known.

Anticipated frequency of Vκ genes. Of the 76 Vκ genes, 32 are potentially functional, 25 are pseudogenes, 16 have minor defects, and 3 have been found to have both potentially functional and slightly defective alleles (6). A reanalysis of the Vκ genes has found 11 genes with minor defects that may be sufficient to render them nonfunctional (13). This suggests that there is a total of 40 functional Vκ genes. Four genes with germline-encoded stop codons were also found in nonproductive rearrangements. Therefore the expected number of Vκ genes in the non-productive repertoire is 44, whereas the anticipated number in the productive Vκ repertoire is 40.

Results

Amplification of kappa light chain genes from individual B cells. The majority of individual $CD19^+IgM^+CD5^+$ or $CD5^-$ B cells contained either one or two rearranged kappa light chain genes (Table II). A rearrangement was considered productive if the VκJκ junction maintained the reading frame into the J segment. Rearrangements that involved pseudogenes were considered nonproductive, as well as those that introduced stop codons. Of the 736 individual B cells that were sorted, 450 yielded at least one rearranged kappa sequence, for an overall efficiency of 61%. Similar numbers of sequences were obtained for the $CD5^-$ and $CD5^+$ B cells from each donor, although the efficiency was higher for donor No. 2 (67% vs 45% for donor No. 1). It is unlikely that the efficiency of the method affected the distribution of VκJκ rearrangements detected. The major determinant of efficiency appeared to be the effectiveness of the cell sorter in depositing a single B cell per well. Analysis of β-actin DNA indicated that the cell sorter deposited at least one cell in 60–80% of the wells. This implies an efficiency of amplification of VκJκ rearrangements of $> 75\%$. Moreover, the method detected 89% of all Vκ gene segments and all Jκ genes.

The majority of B cells from which kappa gene PCR products were obtained, yielded one productively rearranged kappa sequence only (233/450, 52%), whereas just one nonproductively rearranged sequence was obtained from 118 cells (26%). 51 cells (11%) yielded one productive and one nonproductive rearrangment, and 26 cells (6%) yielded two nonproductive rearrangements. 16 cells (3%) yielded two productively rearranged kappa genes. In five cases (1%), two nonproductive rearrangements and one productive rearrangement were detected. In one of these cases, two cells must have been deposited in the well. In the others, however, three kappa chain rearrangements could have occurred within one cell. In those cells, two rearrangements could have occurred on one chromosome, and the third on the other chromosome (see below). In one case, the FACS recorded depositing six cells in a single well. Four sequences were obtained from that well; three productive and one nonproductive rearrangement.

Table II. Summary of Kappa Genes Amplified from Individual IgM^+ B Cells

	Donor No. 1		Donor No. 2	
	$CD5^-$	$CD5^+$	$CD5^-$	$CD5^+$
Total number of B cells	92	92	276	276
Wells with one sequence:	33	30	156	132
wells with 1 P	24	17	103	89
wells with 1 NP	9	13	53	43
Wells with two sequences:	7	10	38	38
wells with 1 P + 1 NP	3	5	24	19
wells with 2 NP	1	5	8	12
wells with 2 P	3	0	6	7
Wells with three sequences:	1	0	1	3
wells with 1 P + 2 NP	1	0	1	3
Efficiency (%)	46	43	71	62

P, productive; NP, nonproductive.

All six functional Vκ families are represented. Kappa light chain genes are classified into seven families based on sequence similarity (6). Six of these families contain functional genes. The VκVII family is made up of a single member that is thought to be a pseudogene (18). Table III compares the distribution of Vκ sequences obtained to the known distribution of Vκ families in the genome (6, 13). Of the 232 nonproductive sequences detected, 67 (29%) were members of the VκII family. This is a significantly higher frequency than expected from the presence of VκII family members in the genome ($P \leq 0.025$). VκIV family members were also found in the set of nonproductive rearrangements at a significantly higher fre-

Table III. Distribution of Vκ Families in IgM^+ B Cells

Known functional genes/Vκ family			Nonproductive (n = 232)		Productive (n = 321)	
VκI	19	(47.5%)	109	(47%)	142	(44%)
VκII	9	(22.5%)	67	(29%)*	61	(19%)
VκIII	7	(17.5%)	30	(13%)	94	(29%)*§
VκIV	1	(2.5%)	14	(6%)*	17	(5%)*
VκV	1	(2.5%)	10	(4%)	6	(2%)
VκVI	3	(7.5%)	2	(1%)‡	1	(< 1%)‡

*Significantly more frequent than predicted from its presence in the genome; $P \leq 0.025$ for Vκ2, $P \leq 0.005$ for Vκ4 productive, $P \leq 0.001$ for Vκ4 nonproductive. ‡Significantly less frequent than predicted from its presence in the genome, $P \leq 0.001$. §Significantly more frequent in the productive repertoire than in the nonproductive repertoire, $P \leq 0.001$.

quency than expected ($P \leq 0.001$), whereas VκVI family members were found significantly less frequently than expected ($P \leq 0.001$). The frequencies of nonproductively rearranged VκI, VκIII, and VκV families were not significantly different than expected from their presence in the genome.

Within the group of productively rearranged Vκ genes, the VκIII family was found more often than predicted from its presence in the genome ($P \leq 0.001$). The VκIV family was also found significantly more often than expected ($P \leq 0.005$), whereas VκVI was found significantly less often than expected ($P \leq 0.001$). The distribution of productively rearranged VκI, VκII, and VκV families was not significantly different than expected. Comparison of the distribution of Vκ family members between productive and nonproductive populations demonstrated a significant overrepresentation of VκIII family members among the productively rearranged genes ($P \leq 0.001$).

Table IV. 43 Rearranged Vκ Genes Are Detected in IgM$^+$ B Cells

Vκ gene*	Vκ family	Nonproductive‡‡ (n = 232)		Productive‡‡ (n = 321)	
B3 (HSIGK18)	VκIV	14§	(6%)	17§	(5%)
B2 (EV15)	VκV	10§	(4%)	6	(2%)
L12a	VκI	6	(3%)	26‖§	(8%)
L11 (HUMIGKL11a)	VκI	3	(1%)	5	(2%)
L10 or L25	VκIII	2‡	(< 1%)	0	
L9 (HSIGK15)	VκI	8	(3%)	7	(2%)
L8 (HSIGK14)	VκI	1	(< 1%)	6	(2%)
L6 (Vg;HSIGKLC1)	VκIII	9	(4%)	20§	(6%)
L5/L19a (HSIGK9)	VκI	2**	(< 1%)	9	(3%)
L4 (ψ)	VκI	2	(< 1%)	0	
L2 (humkv328)	VκIII	6	(3%)	23‖§	(7%)
L1 (HUMIGKVE)	VκI	8	(3%)	3	(1%)
A30	VκI	9	(4%)	13	(4%)
A27 (humkv325)	VκIII	12§	(5%)	46‖§	(14%)
A26/A10	VκVI	2**	(< 1%)	1**	(< 1%)
A23	VκII	4	(2%)	4	(1%)
A21 (ψ)	VκII	6	(3%)	0	
A20	VκI	9	(4%)	7	(2%)
A19/A3	VκII	13	(6%)	28§	(9%)
A18 (ψ)	VκII	14§	(6%)	0	
A17	VκII	16§	(7%)	22§	(7%)
O18/08	VκI	18§	(8%)	20	(6%)
O14/04	VκI	8	(3%)	6	(2%)
O12/02	VκI	20§	(9%)	34¶§	(11%)
O11/O1	VκII	4**	(2%)	1**	(< 1%)
A1	VκII	4	(2%)	1**	(< 1%)
A2 (HUMIGKVAE)	VκII	2	(< 1%)	1**	(< 1%)
A4 (ψ)	VκI	1	(< 1%)	0	
A7	VκII	4	(2%)	0	
A11	VκIII	0		2**	(< 1%)
L15 (HUMIGKVG)	VκI	2	(< 1%)	0	
L16	VκIII	1	(< 1%)	1**	(< 1%)
L19 (HSVK1B)	VκI	2	(< 1%)	3	(1%)
L20 (HSVKIII)	VκIII	2	(< 1%)	1**	(< 1%)
L23	VκI	4	(2%)	3	(1%)
L24	VκI	4	(2%)	3	(1%)
L25	VκIII	0		1**	(< 1%)

*Vκ genes are arranged in order from J-proximal to J-distal. Number of productive Vκ = 40; nonproductive Vκ = 44. ‡In this case L25 cannot be distinguished from L10. §Found significantly more often than predicted from its presence in the genome. ‖Significant difference between productive and nonproductive rearrangements; L12a, $P \leq 0.01$; L2, $P \leq 0.02$; A27, $P \leq 0.001$. ¶Significant difference between productive and nonproductive rearrangements in donor No. 1, $P \leq 0.02$. **Found significantly less often than predicted from its presence in the genome. ‡‡All sequences have been submitted to the EMBL database; accession numbers Z85397 to Z85948.

Distribution of individual Vκ genes in IgM$^+$ B cells. 39 genes that could be considered potentially functional or mildly defective were found in both productive and nonproductive rearrangements (Table IV). Genes from each cassette were noted, with J-proximal genes being found more frequently. Within the regions sequenced, several genes from the J-distal cassette, including O1, O2, O4, L8, A3, and A10, are identical to the genes in the J-proximal cassette and, therefore, members of the pairs cannot be distinguished. In the nonproductive group, 143 Vκ genes were found from the proximal cassette, 26 from the distal cassette, and 63 that could not be distinguished as to cassette. In the productive group, 214 Vκ genes were found from the J-proximal cassette, and 16 genes were found from the J-distal cassette, with 90 genes that could not be distinguished. Of note, four pseudogenes, L4, A21, A18, and A4 were also found to be rearranged by IgM$^+$ B cells. Three potentially functional genes, all from the distal cassette, A14, L14, and L18, as well as two mildly defective genes, also from the distal cassette, A29 and L22, were not detected.

Several genes were found significantly more often than would be expected from random usage based on their distribution in the genome. One pseudogene, A18, along with O18/O8 and B2 were found at a significantly higher frequency than expected ($P \leq 0.001$, 0.025, and 0.05, respectively), and represent instances of increased frequency in the nonproductive repertoire only. Additional Vκ genes found at significantly increased frequency in nonproductive rearrangements were B3, A27, A17, and O2/O12 ($P \leq 0.001$, 0.01, 0.001, and 0.025, respectively). Those genes that were found at significantly higher frequencies in the productive repertoire than predicted from their distribution in the genome were B3, L12, L6, L2, A27, A19/A3, A17, and O12/O2 ($P \leq 0.005$, 0.001, 0.001, 0.001, 0.001, 0.005, 0.001, and 0.001, respectively).

Several genes were found significantly less often than would be predicted from random usage (Table IV). Vκ genes that were found in the set of nonproductive rearrangements significantly less frequently than expected from their distribution in the genome were L5/L19a, A26/A10, O1/O11, A11, and L25 ($P \leq 0.01$, 0.005, 0.025, 0.025, and 0.025, respectively). Those Vκ genes that were found in productive rearrangements significantly less frequently than predicted were A26/A10, O4/O14, O1/O11, A1, A2, A11, L16, L20, and L25 ($P \leq 0.001$, 0.025, 0.025, 0.025, 0.025, 0.025, 0.05, 0.025, and 0.025, respectively).

Each Vκ gene was also analyzed for differences in its distribution between the productive and nonproductive repertoires. No functional genes were found at a significantly lower frequency in the productive than in the nonproductive repertoire. However, three genes, L12, A27, and L2, were found at significantly higher frequencies in productive than in nonproductive rearrangements ($P \leq 0.006$, 0.001, and 0.017, respectively). One gene (pair), O12/O2 was found at a significantly higher frequency in the productive repertoire of donor No. 1, but not

Table V. Distribution of Jκ Genes in IgM⁺ B Cells

Joining segments	Nonproductive (n = 232)		Productive (n = 321)	
Jκ1	50*	(21%)	97*‡	(30%)
Jκ2	92‡	(40%)	115‡	(36%)
Jκ3	9§	(4%)	18§	(6%)
Jκ4	46	(20%)	56	(17%)
Jκ5	35§	(15%)	35§	(11%)

*Significant difference in CD5⁺ population between productive and nonproductive rearrangements, $P \leq 0.04$. ‡Found at significantly higher frequency than expected. §Found at significantly lower frequency than expected.

in that of donor No. 2. Of note, six (or seven) Vκ genes, L12, L6, L2, A27, A17, and O12/O2 were found to account for 53% of the productively rearranged repertoire. The remaining 47% of the productive repertoire was made up of sequences from 32 other Vκ genes.

Vκ genes rearrange to all Jκ gene segments. All five Jκ gene segments were found in both productive and nonproductive rearrangements (Table V). The most frequently used Jκ segment was Jκ2, followed in order by Jκ1, Jκ4, Jκ5, and Jκ3. In nonproductive rearrangements, Jκ2 was found at a significantly higher frequency than expected from its presence in the genome ($P \leq 0.001$), and Jκ3 was found at a lower frequency than expected ($P \leq 0.001$), whereas Jκ1, Jκ4, and Jκ5 were all found at their expected frequencies. By contrast, only Jκ4 was found at the expected frequency in productive rearrangements, whereas Jκ1 and Jκ2 were found at significantly higher frequencies than expected ($P \leq 0.001$), and Jκ3 and Jκ5 were found at significantly lower frequencies than expected ($P \leq 0.001$). No significant differences between productive and nonproductive repertoires or between donors were noted.

Table VI. CDR3 Lengths of VκJκ Genes

Length	Nonproductive (n = 232)		Productive (n = 321)	
bp				
8	2	(1%)		
10	2	(1%)		
12	2	(1%)*		
13	3	(1%)		
14	1	(< 1%)		
15			2	(< 1%)
17	2	(1%)		
18			3	(1%)
19	3	(1%)		
20	4	(2%)		
21	2	(1%)*	7	(2%)
22	7	(3%)		
23	12	(5%)		
24	21	(9%)	29	(9%)
25	25	(11%)		
26				
27	6	(3%)*	211	(66%)
28	45	(19%)		
29	61	(26%)		
30	2	(1%)*	60	(19%)
31	15	(6%)		
32	11	(5%)		
33	1	(< 1%)*	7	(2%)
34	3	(1%)		
35	2	(1%)		
36			2	(< 1%)

*Pseudogenes.

CDR3 length. 66% of the productively rearranged kappa genes contained a CDR3 of 27 nucleotides in length (from codons 89 to 98) and 19% of the in-frame rearrangements had a CDR3 length of 30 nucleotides (Table VI). In the nonproductive repertoire, 68% of the kappa genes had a CDR3 length of 25–29 nucleotides. Shorter and longer CDR3 regions were found, but at much lower frequencies. The mean CDR3 length of productive rearrangements was 27.2±2.5 nucleotides (mean±SEM) and the mean CDR3 length of nonproductive rearrangements was 26.7±4.5 nucleotides. No significant differences between the two donors were noted.

Sequence diversity in the CDR3 is provided by exonuclease activity, terminal deoxyribonucleotide transferase activity, and p-nucleotide formation. Despite the nearly uniform CDR3 length of VκJκ rearrangements, no two CDR3 sequences were identical. No sequences appeared to be clonally related and no common sequence motifs were evident at the VκJκ junction. Table VII shows the CDR3 sequences of VκJκ rearrangements utilizing A27, the most commonly rearranged Vκ gene. Evidence of noncoded nucleotide (n-nucleotide)[1] addition, palindromic nucleotide (p-nucleotide) formation, and exonuclease trimming can all be observed. Of note, the degree of enzyme activity as well as p-nucleotide formation appeared to be independent of the Vκ or Jκ gene utilized for rearrangement. As shown in Table VIII, the majority of rearrangements contained n-nucleotide additions, although the frequency was different in the productive and nonproductive repertoires (57 and 69%, respectively, $P \leq 0.004$). The most frequent occurrence was the addition of one n-nucleotide, followed by two, three, and four. The greatest number of n-nucleotides detected was 15. No significant differences between the two donors were noted.

Evidence of exonuclease activity was detected on both the Vκ (5′) and Jκ (3′) sides of the coding join (Table IX). More than 75% of all VκJκ rearrangements exhibited evidence of exonuclease activity on either the 5′ or the 3′ side of the junction and ~60% of all rearrangements had exonuclease activity on both sides. As shown in Table IX, the most frequent occurrence on the Vκ (5′) side was the removal of three nucleotides; the two germline nucleotides adjacent to the heptamer RSS and the last nucleotide of codon 95 (Table VII). On the Jκ (3′) side of the join, the most frequent occurrence was removal of one nucleotide by exonuclease. Removal of one nucleotide eliminates the single germline nucleotide of Jκ1, Jκ3, Jκ4, and Jκ5, and one of the two germline nucleotides of Jκ2; removal of more than one nucleotide generally extends the trimming into codon 96 of Jκ or beyond.

Multiple VκJκ rearrangements may occur on the same chromosome. 98 of 450 B cells (22%) yielded more than one kappa light chain gene rearrangement. 34 of the 98 B cells re-

1. *Abbreviations used in this paper:* n-nucleotide, noncoded nucleotide; p-nucleotide, palindromic nucleotide; TdT, terminal deoxyribonucleotide transferase.

arranged Vκ gene segments to the same Jκ segment and therefore must have rearranged both loci. Of the remaining 64 B cells, 31 contained a nonproductive gene that rearranged by a deletional mechanism. In these cells, the particular Vκ or Jκ segment utilized in the productive rearrangement is located between the nonproductively rearranged V and J segments and therefore would have been deleted. These cells must also have rearranged a kappa gene on each chromosome. However, in 33 B cells (34%), it is possible that both rearrangements occurred sequentially on the same chromosome.

Table VII. CDR3 Regions of A27 Genes

	Productive rearrangements with CDR3 lengths of 27 nucleotides												
Sample	89	90	91	92	93	94	95	GL	N or P	GL	96	97	Jκ
A27 germline gene	CAG	CAG	TAT	GGT	AGC	TCA	CCT	CC					
BF1N-A09	...	...	...	...	...	...	.--	--*	GAC	--	-AC	ACT	2
BF1N-D01	...	...	...	...	...	...	...	C-		-	-GG	ACG	1
BF1N-D04	...	...	...	...	...	...	...	C-	GA	-	---	ACG	1
BF1N-E03	...	...	...	...	...	...	..-	--		G	TGG	ACG	1
BF1P-B04	...	...	...	...	...	...	.--	--	G	-G	TAC	ACT	2
BF1P-F02	...	...	...	...	...	...	..-	--	CCA	-	--G	ACG	1
BF1P-H08	...	...	...	...	...	...	..-	--	CC	-	-TC	ACC	5
BF2N1-A01	...	...	...	.A.	...	...	..-	--		-G	TAC	ACT	2
BF2N1-A05	...	...	...	...	...	...	..-	--		G	CTC	ACT	4
BF2N1-D06	...	...	...	...	...	..C	...	..	G	-	---	ACT	3
BF2N1-B07	...	..C	...	...	..T	...	..-	--	GG	-	-GG	ACG	1
BF2N2-B01	...	...	...	...	...	...	...	.-	TA	-	---	ACT	5
BF2N2-H03	...	..A	...	...	..T	...	..-	--		-G	TAC	ACT	2
BF2N2-E04	...	...	.G.	...	...	...	..-	--	AA	-	-GG	ACG	1
BF2N2-B05	...	...	...	...	...	...	---	--	TT	G	CTC	ACT	4
BF2N2-G05	...	...	...	...	...	...	..-	--		A	TTC	ACT	3
BF2N2-F06	...	A..	...	...	..T	...	---	--	ACGG	-	-GG	ACG	1
BF2N3-A09	...	...	...	...	...	...	..-	--	GGAA	-	---	ACG	1
BF2N3-G10	...	...	...	...	...	...	...	..		-	--C	ACT	2
BF2P1-A01	...	...	...	A..	GA.	...	---	--	TC	-G	TAC	ACT	2
BF2P1-F05	...	...	...	...	..T	..-	---	--	GGT	G	TGG	ACG	1
BF2P1-G11	...	...	...	...	...	...	...	.-		-	-GG	ACG	1
BF2P3-E11	...	...	...	.A.	.AG	...	..-	--		G	CTC	ACT	4
BF2P3-E02	...	...	...	...	...	.--	---	--	TTAC	G	TGG	ACG	1
BF2P3-B08	..A	...	...	...	...	...	...	--	GC	--	--C	ACT	2

	Productive rearrangements with CDR3 lengths other than 27 nucleotides												
Sample	89	90	91	92	93	94	95	GL	N or P	GL	96	97	Jκ
A27 germline gene	CAG	CAG	TAT	GGT	AGC	TCA	CCT	CC					
BF1N-A05	...	...	...	...	...	...	---	--	A	-	-TC	ACT	4 (24)
BF1N-F01	...	...	...	...	...	...	---	--		-	CTC	ACT	4 (24)
BF1N-G01	...	...	...	...	...	G..	---	--		-	TTC	ACT	3 (24)
BF2N1-A06	...	...	...	...	...	..-	---	--	T	-	TGG	ACG	1 (24)
BF2N1-B04	..A	...	...	A..	..T	...	.--	--	GA	--	---	ACT	2 (24)
BF2N1-D07	...	...	...	...	...	...	..-	--		-	--G	ACG	1 (24)
BF2N1-D09	...	...	...	...	...	...	..-	--	GGA	A	TTC	ACT	3 (30)
BF2N1-D10	...	...	...	...	...	...	..-	--	CCTGT	-	-TC	ACC	5 (30)
BF2N1-G11	...	...	...	...	...	T..	---	--		-	CTC	ACT	4 (24)
BF2N1-H05	...	...	...	...	...	...	..-	--	CCA	A	TTC	ACT	3 (30)
BF2N2-G03	...	...	...	...	...	.--	---	--	T	G	CTC	ACT	4 (24)
BF2N2-H09	...	.T.	C..	C..	GAT	...	...	..	GGGG	-	TTC	ACT	3 (33)
BF2N3-B07	...	...	...	...	...	...	.--	--	T	-	---	--T	4 (21)
BF2P1-C10	...	...	...	...	...	...	...	..	A	--	TAC	ACT	2 (30)
BF2P2-C09	...	...	...	...	...	...	...	..	CC	TG	TAC	ACT	2 (33)
BF2P2-C10	...	...	...	...	...	...	...	..		G	TGG	ACG	1 (30)
BF2P2-E01	...	...	...	...	...	...	...	--		--	---	ACT	2 (24)
BF2P2-F05	...	...	...	...	...	..-	---	--	GCG	--	--C	ACT	2 (24)
BF2P3-B10	..C	..T	...	.A.	.A.	A..	.--	--		-	-GG	ACG	1 (24)

Table VII. (*continued*)

Sample	Nonproductive rearrangements												
	89	90	91	92	93	94	95	GL	N or P	GL	96	97	Jκ
A27 germline gene	CAG	CAG	TAT	GGT	AGC	TCA	CCT	CC					
BF1P-G02-np	...	...	...	...	...	...	..-	--	CTC	-	TTC	ACT	3 (29)
BF1P-A10-np	...	...	...	...	...	...	...	--	GGGTGCAAAA	-	---	--G	1 (32)
BF1P-C10-np	...	...	...	...	...	...	.--	--	ATGTGG	--	--C	ACT	2 (29)
BF2N1-F01-np	...	...	...	...	...	...	..-	--		-	---	ACC	5 (23)
BF2N2-A03-np	...	...	...	.--	---	---	---	--	CCCCGT	--	--C	ACT	2 (20)
BF2N2-B03-np	...	...	...	...	...	...	..-	--	A	G	TGG	ACG	1 (28)
BF2N3-E06-np	...	...	...	...	...	...	...	--	GTG	--	--C	ACT	2 (28)
BF2P1-C11-np	...	..A	...	...	.T.	---	---	--	ACGG	--	---	---	2 (19)
BF2P1-B12-np	...	...	...	...	...	...	...	..	GCCTT	-	---	--T	3 (29)
BF2P3-C10-np	...	...	---	---	---	---	---	--	GCCC	--	---	---	2 (10)
BF2P3-E09-np	...	...	...	...	.A.	---	---	--	GGAAA	-	---	ACT	4 (23)

*Dots indicate identity with germline nucleotides. Dashes indicate nucleotides removed by exonuclease.

The order of Vκ family rearrangements is not random. 51 cells contained one productive and one nonproductive kappa gene rearrangment. Assuming that nonproductive rearrangements precede productive rearrangements, it is possible to determine the Vκ genes rearranged initially or subsequently. 40% of these B cells rearranged a VκI family member first (nonproductively), 38% rearranged a VκII family member first, and only 6% rearranged a VκIII family member first. In contrast, a VκIII family member was found to be productively rearranged in 47% of these B cells (Table X). Productive rearrangements involving a VκI or VκII family member occurred less frequently; 37% for VκI and 10% for VκII. Second rearrangements could occur on the opposite chromosome or could involve residual family members on the originally rearranged chromosome. In the few cases in which a VκIII family member rearranged first, a second rearrangement on the same chromosome was not possible because the Jκ gene used for the second rearrangement would have been deleted. In those cells in which a VκI family member rearranged first, a second rearrangement on the same chromosome would have been possible in 45% of B cells. In the cases in which a VκII family member rearranged first, a second rearrangement on the same chromosome would have been possible in 47%. Despite this, second rearrangments to another member of the same family occurred rarely. The smaller Vκ families were found in cells with both a productive and a nonproductive rearrangement too infrequently for a more detailed analysis.

In contrast to the apparent nonrandom use of Vκ segments for first and second rearrangements, Jκ segments appeared to be used comparably in first and second rearrangements. Moreover, the same B cell frequently used the identical Jκ for both rearrangements (Table X). The only exception to this was Jκ3, which was rarely used during the first rearrangements but was found somewhat more frequently in the productive rearrangements, presumably because of positive selection of the VκJκ rearrangements using this Jκ segment.

A subset of IgM$^+$B cells undergoes somatic hypermutation. Approximately 90% of the rearranged kappa genes from the first donor were found to be in germline configuration, whereas ~ 10% were found to be mutated (Table XI). No differences were noted in the mean frequencies of mutations between productively and nonproductively rearranged genes. When the B cells from a somewhat older donor (45 vs 25 yr) were analyzed, ~ 30% of the expressed repertoire was found to be mutated. In the older donor, the mean frequency of mutations was significantly higher in the productive repertoire when compared with the non-productive repertoire ($P \leq 0.001$). The number of mutations per rearrangement was also compared between the two donors. Kappa genes from the 25-yr-old donor that were most mutated were still 95% homologous to germline (10–11 mutations in a sequence of ~ 230–280 nucleotides). In contrast, highly mutated kappa genes from the 45-yr-old donor could be as little as 85% homologous to germline (as many as 37 mutations in ~ 250 nucleotides). In the mutated populations from either donor, genes that were 97–98% homologous to germline (5–7 mutations) were found most often (Table XII).

Of note, the distribution of Vκ and Jκ gene segments was not different between the mutated population and the unmutated population (data not shown). The six most commonly used genes were found at the same frequencies in the unmutated population as they were in either the mutated population or the total population. Similarly, the distribution of J segments rearranging to Vκ genes that were not subsequently mutated was not different from the distribution of J segments rearranging to all Vκ genes. Finally, when the distribution of J segments rearranging to the six most commonly utilized Vκ genes was analyzed no differences were found between mutated and unmutated genes or the total population.

In a small number of cells, mutations to stop codons were found outside of the CDR3. Eight of these were in Vκ genes that were nonproductive because of an out of frame rearrangement. In five cells, the mutation to a stop codon occurred in an otherwise productively rearranged gene.

Comparison of CD5$^+$ and CD5$^-$ B cell populations. Very few differences were found between the CD5$^-$ and CD5$^+$ B cell populations. No differences were seen in the particular Vκ or Jκ segments undergoing rearrangement. No differences were found in either the distribution of CDR3 lengths or in the enzyme activity apparent on the CDR3. One difference between the populations was a small overrepresentation of JκI family members in the nonproductive repertoire when compared

Table VIII. TdT Activity on Rearranged VκJκ Genes from IgM⁺ B Cells

Number of N-nucleotides	Nonproductive (n = 232)		Productive (n = 321)	
0	72	(31%)	138	(43%)
1	42	(18%)	61	(19%)
2	48	(20%)	50	(16%)
3	29	(13%)	38	(12%)
4	17	(7%)	16	(5%)
5	12	(5%)	9	(3%)
6	6	(3%)	3	(1%)
7	2	(1%)	2	(1%)
8	1	(< 1%)	2	(1%)
9	0		1	(< 1%)
10	2	(1%)	1	(< 1%)
15	1	(< 1%)	0	

with the productive repertoire of CD5⁺ but not CD5⁻ B cells (33% in nonproductive vs 22% in productive, $P \leq 0.036$). In addition, the VκIV family was significantly overrepresented in the nonproductive repertoire in CD5⁻ B cells compared with the nonproductive repertoire of CD5⁺ cells (10 vs 2%, $P \leq 0.05$). The most notable difference between CD5⁻ and CD5⁺ B cells was found in the frequency of mutated genes in the productively rearranged repertoires (Tables XI and XII). 34% of the productively rearranged genes in the CD5⁻ population of donor No. 2 were mutated, whereas only 18% were mutated in the CD5⁺ population ($P \leq 0.004$). In contrast, there was no difference in the frequency of nonproductively rearranged Vκ genes that were mutated between CD5⁻ and CD5⁺ cells of this donor (7 and 5%, respectively). A similar, although less marked, trend was seen in donor No. 1.

Table IX. Exonuclease Activity on VκJκ Genes from IgM⁺ B Cells

Nucleotides removed	Vκ CDR3 Nonproductive (n = 232)		Vκ CDR3 Productive (n = 321)		Jκ CDR3 Nonproductive (n = 232)		Jκ CDR3 Productive (n = 321)	
0	43	(18%)	49	(15%)	61	(26%)	81	(25%)
1	32	(14%)	52	(16%)	53	(23%)	88	(27%)
2	33	(14%)	36	(11%)	19	(8%)	45	(14%)
3	50	(22%)	118	(37%)	27	(12%)	32	(10%)
4	26	(11%)	22	(7%)	30	(13%)	33	(10%)
5	15	(6%)	25	(8%)	13	(7%)	26	(8%)
6	8	(3%)	5	(2%)	6	(3%)	2	(1%)
7	3	(1%)	6	(2%)	2	(1%)	9	(3%)
8	9	(4%)	6	(2%)	8	(3%)	2	(1%)
9	1	(< 1%)			2	(1%)	2	(1%)
10	1	(< 1%)			4	(2%)	1	(< 1%)
11	1	(< 1%)			2	(1%)		
13	1	(< 1%)			1	(< 1%)		
14			1	(< 1%)	1	(< 1%)		
15	1	(< 1%)						
16	1	(< 1%)			1	(< 1%)		
17	2	(1%)	1	(< 1%)				
18	2	(1%)						
20	2	(1%)						
22	1	(< 1%)			1	(< 1%)		
26					1	(< 1%)		

Table X. Order of VκJκ Rearrangements in IgM⁺ B Cells

Vκ family*	Rearrangement 1st	Rearrangement 2nd	Number of sequences (n = 102)	Jκ gene	Rearrangement 1st	Rearrangement 2nd	Number of sequences (n = 102)
VκI	20	19	39	Jκ1	14	13	27
VκII	19	5	24	Jκ2	18	13	31
VκIII	3	24	27	Jκ3	0	6	6
VκIV	4	1	5	Jκ4	9	11	20
VκV	4	2	6	Jκ5	10	10	20
VκVI	1	0	1				

Individual B cells‡	Vκ family 1st	Vκ family 2nd	Number of B cells (n = 51)	Individual B cells	Jκ gene 1st	Jκ gene 2nd	Number of B cells (n = 51)
VκI/II	I	II	3	Jκ1/1	1	1	7
VκI/III	I	III	16	Jκ1/2	1	2	3
VκI/V	I	V	1	Jκ1/3	2	3	2
				Jκ1/5	1	5	2
VκII/I	II	I	13				
VκII/II	II	II	1	Jκ2/1	2	1	1
VκII/III	II	III	5	Jκ2/2	2	2	5
				Jκ2/3	2	3	1
VκIII/I	III	I	3	Jκ2/4	2	4	7
				Jκ2/5	2	5	4
VκIV/III	IV	III	3				
VκIV/V	IV	V	1	Jκ4/1	4	1	3
				Jκ4/2	4	2	3
VκV/I	V	I	2	Jκ4/3	4	3	1
VκV/II	V	II	1	Jκ4/4	4	4	2
VκV/IV	V	IV	1				
				Jκ5/1	5	1	2
VκVI/I	VI	I	1	Jκ5/2	5	2	2
				Jκ5/3	5	3	2
				Jκ5/4	5	4	2
				Jκ5/5	5	5	2

*The distribution of Vκ and Jκ genes used by 51 B cells containing both a productive and nonproductive VκJκ rearrangement is shown. By convention, the nonproductive rearrangement was considered to have occurred first. ‡The usage of Vκ and Jκ genes by 51 individual B cells containing both a productive and a nonproductive VκJκ rearrangement is shown. By convention, the nonproductive rearrangement was considered to have occurred first.

Discussion

VκJκ rearrangement is not random. The distribution of Vκ genes found in VκJκ rearrangements was not representative of their presence in the genome. Two general levels of bias in Vκ use were detected. First, recombinational bias was detected when nonproductive rearrangements were analyzed. Secondly, bias dependent on the expression of a kappa chain protein was revealed when productive and nonproductive VκJκ rearrangements were compared.

Table XI. Frequency of Kappa Chain Mutations in Two Donors of Different Ages

		Percentage of rearrangements with homology to germline genes of ≤ 98%		
			Donor No. 1 (26 yr)	Donor No. 2 (45 yr)
	Productive	5%	18%	$P \leq 0.01$
$CB5^+$ B cells	Nonproductive	7%	5%	NS
	Productive	17%	34%	$P \leq 0.004$
$CD5^-$ B cells	Nonproductive	12%	7%	NS

Analysis of nonproductive VκJκ rearrangements provided evidence that recombinational bias based on a variety of putative molecular mechanisms may have accounted for the underrepresentation of specific Vκ genes. The underrepresented Vκ genes included nine from the J-distal cassette and three pair that have one member in each cassette. One of the underrepresented gene pairs having a member in the J-proximal cassette (A26/A10) has previously been reported to have defects that could alter the efficiency of rearrangement (13). Although these defects may explain underutilization of these Vκ genes, not all putatively defective genes, including A20, are rearranged infrequently. For example, A20, has a defective recombination signal sequence as a result of a single base pair substitution at the fourth position of the heptamer (19). Although this defect would be predicted to reduce efficiency of recombination (20), A20 was found at the expected frequency in VκJκ rearrangements. One underrepresented J-proximal gene, O11, is known to have a structural defects (13). It is unlikely, however, that a structural alteration would account for a decrease in the frequency of appearance of a Vκ gene in nonproductive VκJκ rearrangements. In this regard, both members of the gene pair O14/O4 are structurally defective in that they encode Gly in place of an invariant Cys at position 88 (21) that would disrupt a disulfide bridge and compromise the integrity of the expressed protein (13). This major structural abnormality would not be expected to alter the use of these Vκ genes in nonproductive rearrangements. As expected, these Vκ genes were found at the expected frequency in nonproductive rearrangements, but surprisingly they were also found at the expected frequency in productive rearrangements. Therefore, it is unlikely that putative structural defects can account for the underrepresentation of specific Vκ genes in VκJκ rearrangements, and may not even preclude their appearance from the expressed repertoire.

The frequency of Vκ genes from the J-distal cassette in VκJκ rearrangements was significantly less than the frequency of J-proximal Vκ genes. Eight individual J-distal genes were significantly underrepresented, as were the J-distal genes in aggregate. At least two molecular mechanisms could contribute to the underutilization of the J-distal genes. All J-distal genes rearrange by inversion rather than by deletion. Inversion may be a significantly less efficient process, as suggested by studies using artificial recombination substrates (22). It is unlikely, however, that the requirement for inversion versus deletion entirely accounts for the underutilization of J-distal Vκ genes, since two J-proximal genes, B2 and B3, also rearrange by inversion (18), but are found at an increased frequency in either the nonproductive repertoire (B2) or in both nonproductive and productive repertoires (B3). A second molecular mechanism that could contribute to the infrequent use of Vκ genes from the J-distal cassette is the requirement for recombination over a long expanse of DNA. The J-distal cassette is ~ 1,500–2,000 kb from the nearest Jκ gene. It is possible that the recombination machinery is less efficient over distances > 1,000 kb. In this regard, the most J-distal Vκ gene, L25, was rarely found to be used in rearrangements. However, the penultimate J-distal gene, L24, that must rearrange over nearly the same expanse of DNA, was found at the expected frequency. This makes it unlikely that distance from Jκ is the sole determinant of the Vκ genes used in rearrangements, although it appears to play a role. Whether subtle differences in transcription rates or gene availability also contribute to the underrepresentation of J-distal genes in VκJκ rearrangements is not currently known. Regardless of the precise mechanism, the underutilization of the J-distal Vκ gene segments could limit the heterogeneity of the expressed kappa repertoire, although the similarity of the J-distal genes to the J-proximal genes would tend to mitigate the impact of this bias.

When the nonproductive repertoire was analyzed in detail, seven (or nine) genes, B2, B3, A27, A18, A17, and the gene pairs O18/O8 and O12/O2, were found to be rearranged at greater frequencies than expected. Included among the overrepresented Vκ genes was A18, a germline pseudogene. Of note, three other pseudogenes, L4, A21, and A4, were also found in the nonproductive repertoire. As the recombinational signal sequences of these four pseudogenes are intact (6), they can be used in VκJκ rearrangement, but never appear in the expressed repertoire. The functional Vκ genes that are overrepresented in the nonproductive repertoire, B2, B3, A27, A17, and the gene pairs O18/O8 and O12/O2 are between 50 (B3) and 1,500 (O2) kb from the J segments and belong to five different Vκ families. Four rearrange by inversion (B2, B3, O8, O2) and four by deletion (A27, A17, O18, O12). A common molecular mechanism responsible for the overrepresentation of these Vκ genes in the nonproductive repertoire was not,

*Table XII. Vκ Mutational Frequencies**

	$CD5^-$ B cells		$CD5^+$ B cells	
Homology to germline gene	Nonproductive (n = 112)	Productive (n = 177)	Nonproductive (n = 120)	Productive (n = 144)
100%	96 (85%)	113 (64%)	106 (88%)	110 (76%)
99%	7 (6%)	11 (6%)	7 (6%)	11 (8%)
98%	2 (2%)	13 (7%)	5 (4%)	12 (8%)
97%	3 (3%)	14 (8%)	–	4 (3%)
96%	–	8 (5%)	1 (1%)	4 (3%)
95%	1 (1%)	5 (3%)	–	1 (< 1%)
94%	1 (1%)	5 (3%)	–	1 (< 1%)
93%	1 (1%)	5 (3%)	1 (1%)	–
92%	–	2 (1%)	–	–
91%	–	1 (< 1%)	–	–
90%	–	–	–	1 (< 1%)
85%	1 (1%)	–	–	–

*Data from both donors have been combined since differences in the number of mutations did not affect the distribution of B cells with specific numbers of mutations.

therefore, immediately obvious. It is possible that subtle differences in transcription rates may play a role in the overutilization of these genes. In this regard, it has been proposed that germline transcription of an unrearranged gene may be an initial step in recombination or indicate that the DNA of that region is available to the recombinase enzymes, Rag1 and Rag2 (23). Germline transcripts of B3 have been found in a human pre–B cell line and in fetal bone marrow cells (24), implying that this frequently used Vκ gene may be a more readily available recombination substrate. Whether germline transcription of the other frequently used Vκ genes occurs or can explain their frequent usage is currently unknown. In summary, at least four molecular mechanisms may contribute to the differential frequency of rearrangement of individual Vκ genes in VκJκ rearrangements. These are likely to include distance from the J segment, transcriptional orientation, efficiency of transcription, and integrity of the recombination signal sequences. The subtle interplay of these four elements may determine the individual Vκ genes utilized in VκJκ rearrangements.

8 (to 10) Vκ genes were overrepresented in productive VκJκ rearrangements. The frequencies of three (or four) of these (B3, A17, O12/O2) were also significantly greater than expected in the nonproductive repertoire. Moreover, the frequencies of B3 and A17 in both donors (Table IV), and the frequency of O12/O2 in one of the two donors (data not shown), were not significantly different in the productive and nonproductive repertoires. Consequently, the frequent appearance of these three (or four) Vκ genes in the productive repertoire appears to be related to their overutilization in the rearrangement process and not to selection dependent upon expression of a kappa chain protein. Four (or five) of the remaining five (or six) genes A19/A3, L12a, L6, and L2, were overrepresented in the productive, but not the nonproductive, repertoire. This finding implies that their biased appearance in the expressed repertoire is strictly related to events after VκJκ rearrangement, and, therefore, is dependent on the expression of a kappa chain protein. In contrast, the remaining Vκ gene, A27, was found to be overrepresented in both the nonproductive and productive repertoires, and its frequency was significantly greater in the productive compared to the non-productive repertoire. Therefore, its frequent occurrence is related both to its overutilization in the recombination process and subsequent selection based upon its expression as part of a kappa chain protein. Recombinational bias as well as subsequent selection of kappa light chains containing the A27 gene product together resulted in A27 being the most frequently used Vκ gene segment in the expressed repertoire. This was found both in the current analysis as well as in previous reports (7–9, 13, 25). Of note, A27 has frequently been found as a component of autoantibodies (26), although this may merely reflect its overall frequency in the repertoire.

Overrepresentation of 8 (to 10) Vκ genes in the expressed repertoire appeared to relate to features of the Vκ gene segments themselves and not other components of the kappa chain, including the Jκ segment or the CDR3 region. This would be expected for the three (or four) Vκ genes that were overrepresented because of recombinational bias; however, it was also the case for the five (or six) Vκ genes whose overrepresentation was dependent on the expression of a kappa protein. The mechanism of overrepresentation of these latter Vκ gene products is uncertain. It is possible that the proteins encoded by these Vκ segments pair more effectively with rearranged heavy chain gene products. Although possible, there are no data to suppport this conclusion as almost all functional Vκ segments have been found as part of immunoglobulin molecules. Moreover, preliminary analysis of productive V_H and Vκ rearrangements in the same B cell has not shown preferential pairing of V_H and Vκ genes (data not shown). It is more likely that overrepresentation of specific Vκ gene segments in the expressed repertoire reflects positive selection of B cells expressing kappa chains containing V regions encoded by these Vκ genes. Selection may not be by a conventional antigenic mechanism, as it results in the overrepresentation of specific Vκ segments, but not the major antigen binding CDR3 regions. It is possible, however, that low avidity antigenic selection, mediated by interaction with the CDR1 and/or CDR2 regions of the Vκ gene products, plays a role in the overrepresentation noted. Alternatively, selection of B cells expressing kappa chains containing V regions encoded by specific Vκ gene segments may result from superantigen mediated selection. A number of B cell superantigens, including staphylococcal protein A, staphylococcal entertoxin D, and HIV gp120 have been reported (27, reviewed in reference 28). However, each of these has been shown to have the capacity to influence the repertoire in a V_H and not a Vκ-specific manner (27–29), as might be expected since immunoglobulin heavy chain is analogous to the β chain of the T cell receptor, a structure known to bind conventional superantigens (30). It is possible that a similar superantigenic mechanism may account for Vκ-specific biases. In this regard, protein L, a cell wall component of *Peptostreptococcus magnus* (31, 32), has been shown to bind specifically to VκI, VκIII, and VκIV gene products by interacting with framework residues (33). Whether a related or comparable binding event may bias the immunoglobulin repertoire in a Vκ-specific manner remains to be determined.

The various mechanisms shaping the human light chain repertoire, including combinational biases and selection events, serve to limit the extent of the expressed repertoire, as has previously been shown for the V_H repertoire (10). As a result, only 6 of the 40 potentially functional Vκ gene segments are used by $>$ 50% of circulating IgM^+B cells. Although most other functional Vκ genes are used by small numbers of B cells, the various biases appear to limit the expressed light chain repertoire markedly. One potential implication of this restriction in Vκ usage is a much greater reliance on combinatorial diversity to generate a broad array of antibody specificities.

CDR3 length and sequence diversity. Molecular mechanisms appear to impose strict limits on the length of the CDR3 segment of VκJκ rearrangements. The majority (66%) of the productive rearrangements were 27 nucleotides in length (from codons 89 to 98). Several previous studies have noted that the CDR3 regions of expressed human kappa chains are usually 27 nucleotides in length (13, 25). A new observation from the current study is that the restriction in CDR3 length is also noted in the nonproductive rearrangements as no statistical difference was noted in the CDR3 lengths of nonproductive and productive repertoires (26.7±4.5 and 27.2±2.5 nucleotides, respectively). These data are similar to findings of restricted CDR3 lengths in murine productive and nonproductive kappa rearrangements (34). Therefore, the restricted length of the CDR3 region of kappa chains must relate to tight regulation of the mechanisms governing junctional diversity.

Even though the total CDR3 length was restricted, there was still considerable sequence diversity in both nonproductive and productive kappa rearrangements. Downregulation of terminal deoxyribonucleotide transferase (TdT) at the time of kappa gene rearrangement has been reported, and as a result n-nucleotides have seldom been noted in murine light chains (35). However, n-nucleotides have been reported in some human kappa chains, especially those expressed by B cells accumulating at sites of inflammation (26), suggesting that TdT is not downregulated as completely in human pre–B cells. In the current analysis, 57% of the productive and 69% of the nonproductive rearrangements contained n-nucleotides, supporting the conclusion that TdT is not completely downregulated at the time of human VκJκ rearrangement. However, it appears that the number of n-nucleotides introduced in VκJκ rearrangements is limited in that fewer than four nontemplated nucleotides were usually found. In contrast to the limited number of n-nucleotides in VκJκ rearrangements, a mean total of 15 n-nucleotides was found for V-to-D and D-to-J heavy chain junctions from the same B cells (Brezinschek, H.-P., S.J. Foster, R.I. Brezinschek, T. Dörner, R. Domiati-Saad, and P.E. Lipsky, manuscript submitted for publication). In addition to the differences in average number of n-nucleotides, fewer kappa sequences contained n-nucleotides than did heavy chain sequences. This would indicate that the downregulation of TdT at the time of light chain rearrangement results in fewer n-nucleotides per kappa gene as well as fewer VκJκ genes with n-nucleotides, but not an absence of n-nucleotide additions in VκJκ rearrangements.

Palindromic sequences (p-nucleotides), produced as a result of resolution of the hairpin structure generated by the action of Rag1 and Rag2 (36), were rarely observed. This contrasts with murine kappa chains in which p-nucleotide formation is seen more often (35). Whether this difference in human rearrangements relates to a more precise resolution of hairpins to the site of the initial strand break, or their rapid removal by exonuclease activity is uncertain.

Generation of restricted CDR3 lengths requires precise regulation of a number of diverse molecular mechanisms, including the activity of TdT, the degree of exonuclease digestion, and the method of hairpin resolution. The method by which these various diverse processes can be regulated to yield a mean CDR3 length of 27 nucleotides in nonproductive rearrangements is not known. However, it is clearly different than the mechanism generating a larger range of CDR3 lengths in V_H rearrangements (Brezinschek, H.-P., S.J. Foster, R.I. Brezinschek, T. Dörner, R. Domiati-Saad, and P.E. Lipsky, manuscript submitted for publication).

Jκ gene usage is not random. As with Vκ genes, the frequency of Jκ gene segments found in VκJκ rearrangements is not representative of their frequency in the genome. Jκ1 was found significantly more often in the productive repertoire than in the nonproductive repertoire of $CD5^+$ B cells. This observation suggests that there may be a biased usage of Jκ1 based on the expression of an immunoglobulin protein in $CD5^+$ B cells. In $CD5^-$ B cells, Jκ2 was overrepresented in both productive and nonproductive rearrangements, suggesting a recombinational bias for Jκ2 in $CD5^-$ B cells. In a previous study of expressed VκIII genes obtained from adult and neonatal cDNA libraries prepared from unstimulated B cells, preferential usage of Jκ1 and Jκ2 was also reported (7). This bias was attributed to the preferential usage of the two most 5′ J segments for rearrangement to the VκIII gene L2, although a mechanism was not provided. Of note, L2-Jκ1 rearrangements were often found in the newborn and L2-Jκ2 rearrangements were overrepresented in the adult library (7). As newborn B cells are largely $CD5^+$ (37), the biased use of Jκ1 previously reported is consistent with the current finding that Jκ1 was overrepresented in adult $CD5^+$ B cells. Similarly, adult B cells are largely $CD5^-$ and, therefore, the current finding of Jκ2 prevalence in $CD5^-$ B cells is also consistent with the previous observation. As noted above, the mechanisms underlying the biased utilization of Jκ segments in $CD5^+$ and $CD5^-$ B cells appear to differ. Moreover, no preferential association of Jκ1 or Jκ2 with the L2 gene or with the VκIII family was noted. Therefore, the relevance of the different Jκ biases noted in $CD5^+$ and $CD5^-$ B cells and their precise mechanisms remains unexplained.

Multiple VκJκ rearrangements on the same chromosome. The current data suggest that two sequential VκJκ rearrangements on the same chromosome may occur frequently. This is possible because of the organization of the kappa locus, that contains only Vκ and Jκ segments and Vκ segments in both transcription orientations (6). Because many inversional and deletional rearrangements leave functional Vκ and Jκ segments undisturbed in the locus, secondary rearrangements on the same chromosome are theoretically possible. A number of previous findings indicate that sequential kappa chain rearrangements can occur on the same chromosome, but do not permit an estimate of their frequency (38–41). In the current study, 33 of 98 B cells containing more than one rearrangement were candidates to have undergone two sequential rearrangements on the same chromosome. Three patterns were found with approximately equal frequency; an initial inversion followed by a deletion, two inversions, and an order that could not be established unambiguously. There are a number of implications of these findings. The first is that the percentage of B cells that must have rearranged the kappa locus on both chromosomes is likely to be smaller than originally estimated and may be as low as 15% (65/450). This is consistent with the previous conclusion that only a minority of B cells rearrange both V_H loci (10). A second implication of these results is that rearrangement by inversion may occur more frequently than would be predicted from the location of the Vκ gene on the chromosome. When a VκJκ rearrangement occurs by inversion the intervening DNA is retained on the chromosome in the opposite orientation. Therefore, if an intervening Vκ gene that normally rearranged by deletion were involved in a subsequent rearrangement, it would do so by inversion. This may have happened in as many as 22 of 34 B cells in which two sequential rearrangements could have occurred on the same chromosome.

The order of Vκ rearrangements is not random. Preferential usage of particular Vκ families for initial as well as subsequent rearrangements became apparent when B cells containing both a productive and a nonproductive VκJκ rearrangement were analyzed. 51 B cells containing an initial nonproductive rearrangement and a subsequent productive rearrangement were identified. If there were an equal likelihood that a second rearrangement would use the same Vκ family as was used for the initial VκJκ rearrangement, between 9 and 11 of these B cells should be VκI–VκI doubles and between four and eight should be VκII–VκII doubles. However, in only one of these 51 B cells was the same Vκ family (VκII) used for both rear-

rangements. No VκI–VκI doubles were found. Not only was a different Vκ family used for each rearrangement, but the distribution of Vκ families used for the second rearrangement was not representative of their occurrence in the genome. VκI family members were utilized in roughly equal frequencies for first and second rearrangements, but not by the same B cells, whereas VκII family members were used more frequently in first rearrangements and VκIII members were used more often in second rearrangements. Of note, no similar bias in the distribution of Jκ segments was noted in first and second rearrangements. It is possible that selection effected the frequency of VκIII family members found in the productive repertoire of B cells expressing both a productive and nonproductive rearrangement, as VκIII members appeared to be positively selected in the entire productive repertoire. This appears to be unlikely, however, as there was a significant overrepresentation of VκIII family members in the productive repertoire of B cells expressing both a productive and a nonproductive rearrangement compared to B cells containing only a single productive rearrangement (47% vs 25%, $P \leq 0.002$). This makes it more likely that molecular events in B cells containing two rearrangements influenced the distribution of VκIII in the productive repertoire beyond the influence of subsequent selective pressures. In addition, selection is unlikely to account for the diminished representation of VκII family members in the second rearrangements, as no overall evidence of negative selection of VκII family members was found. Finally, the biased presence of Vκ family members is also unlikely to reflect the use of pseudogenes in the nonproductive rearrangements, as the same distribution was noted when pseudogenes were removed from the analysis. These results, therefore, are most consistent with the conclusion that molecular events bias the utilization of Vκ, but not Jκ, segments during second rearrangements.

One explanation for the biased use of Vκ segments during first and second rearrangements could relate to availability or transcription of Vκ families during different times in B cell ontogeny. Vκ families that were available for initial rearrangement, such as VκII, may undergo changes in the rate of transcription or become less available subsequently when the B cell progenitor may attempt to rearrange the second Vκ locus. The VκIII family, in contrast, appears to be less available during initial rearrangement but more able to be involved in subsequent rearrangements. VκI appears to be used comparably for first and second rearrangements. The molecular explanation for this observation is uncertain, but could relate to developmentally controlled alterations in the amount or availability of specific regulatory factors that drive transcription or recombination of members of specific Vκ families differentially. This would be consistent with a conservation of regulatory elements by frequently used members of individual Vκ families. It is also consistent with the likely origin of family members from a common primordial gene (6). Regulation of Vκ utilization by availability of specific transcription or recombination factors might also explain the lack of utilization of members of the same Vκ family for sequential rearrangement by individual B cells. This is particularly noteworthy for the VκI family that can be used for both initial and subsequent rearrangements, but not by the same individual B cell. The initial nonproductive rearrangement of a VκI family member might consume specific regulatory factors and thereby sharply limit the likelihood of a second VκI rearrangement by the same cell. Other B cells that had previously rearranged another Vκ family member nonproductively would have sufficient specific factors available to facilitate rearrangement of a VκI family member. Although the precise mechanism is uncertain it is noteworthy that a similar mechanism appears to apply to V_HDJ_H rearrangements in that V_H3 family members are rarely used for sequential rearrangements by the same B cell (10).

One practical implication of the apparent overutilization of VκIII for second rearrangements is the potential influence of this bias on the conclusion that the VκIII family was positively selected. This conclusion was based on a comparison of the distribution of Vκ genes in productive and nonproductive repertoires and assumed that the rearrangement process was comparable in each. However, it is now clear that this assumption is not completely accurate, as the frequency of the usage of Vκ families is influenced by the order of rearrangement. As the number of B cells that rearrange two kappa loci is small, this unexpected bias might not influence the interpretation of the data. To examine this issue more completely, the distribution of Vκ families in 233 B cells containing only a productive VκJκ rearrangement was analyzed. The VκIII family was found at significantly increased frequency (25%) and was comparable to that found in the entire set of rearrangements (29%), and significantly greather than that found in the nonproductive repertoire (13%, $P \leq 0.001$). These findings confirm that positive selection is a major factor influencing the overrepresentation of the VκIII family in the expressed repertoire, although the increased availability of this family for second VκJκ rearrangement may also contribute to the increased frequency of VκIII family members found.

Two productively rearranged Vκ genes in the same B cell. In 15 of the B cells analyzed (3.5%), two productive VκJκ rearrangements were found. Although dual expression of kappa and lambda light chains has been reported for a small population of fetal B cells (42), it is unlikely that both productively rearranged kappa genes from adult B cells would be expressed. There are several potential explanations for the occurrence of two productive Vκ rearrangements in the same B cell. It is possible that one of the VκJκ rearrangements encoded for a kappa chain that was unable to pair with a heavy chain. In this regard, two cells were identified in which a VκJκ rearrangement encoded an unusually short CDR3 (18 nucleotides) or an unusually long CDR3 (36 nucleotides), along with a VκJκ rearrangement encoding a CDR3 of 27 nucleotides. In these B cells, failure of the kappa chain with an abnormal CDR3 length to pair appropriately with the heavy chain during ontogeny may have prevented the rearrangement mechanism from being downregulated, permiting a second VκJκ rearrangement to occur. In other B cells a second productive VκJκ rearrangement may have occurred much later in development after immunoglobulin expression, antigen stimulation, and somatic hypermutation. Support for this possiblilty comes from analysis of B cells with two productive rearrangements with appropriate CDR3 lengths. In one such cell one VκJκ rearrangement contained a number of mutations including one that introduced a stop codon at position 88 in FR 3, whereas the other contained no stop codons. In two other B cells, highly mutated kappa genes, (20 and 14 mutations) were found along with less mutated genes (8 and 1 mutations, respectively). In the Vκ gene with 20 mutations, two were in invariant nucleotides in the donor splice site at the 3′ end of FR 4. In all of these B cells, loss of a functional kappa chain as a result

of somatic hypermutation may have caused deletion of the B cell unless an additional light chain rearrangement occurred. Findings from murine studies indicate that the recombinase activating genes, Rag-1 and Rag-2, are upregulated in the germinal center (43). This suggests the possibility that a second wave of VκJκ rearrangement may occur in the germinal center to rescue mature B cells whose functional light chains have been disabled by somatic hypermutation. Support for this possibility derives from the analysis of human B cell clones containing a productively rearranged lambda as well as a productively rearranged kappa gene in which mutation has generated a stop codon (44). Presumably reactivation of the recombination machinery in the germinal center may rescue a mature B cell from deletion after somatic hypermutation has introduced stop codons in a functional light chain gene.

IgM^+ memory cells. Since somatic hypermutation and heavy chain isotype switching are both thought to occur in the germinal center (45–47), and since the current study analyzed IgM^+ B cells, few mutated kappa sequences were expected. However, 24% of the productive and 7% of nonproductive VκJκ rearrangements were found to be mutated, suggesting that IgM^+ memory B cells are commonly encountered in the peripheral blood. The observation that IgM^+ memory cells appeared to be more frequent in the older donor suggests that more prolonged exposure to diverse antigens may favor the development of the IgM^+ memory population. A recent study of IgM^+ B cells from a 67-yr-old donor reported somewhat disperate results. Few mutations were noted in the IgD^+ subset, but more frequent mutations were found in the IgD^- population (9). The apparent discrepency may relate to the current analysis of IgM^+ cells not being separated into IgD^+ and IgD^- subsets. It is possible that the current results reflect the contribution of highly mutated sequences in the IgM^+IgD^- cells. Alternatively, the analysis of only a small number of clones in the previous study may have influenced the interpretation of the results.

The finding of IgM^+ memory B cells suggests that the mutational process may have been activated in these cells in the absence of a signal for heavy chain isotype switching. In the germinal center the mutational machinery is thought to be activated before heavy chain isotype switching (48). Therefore, these somatically mutated B cells may represent cells at an early phase of germinal center cell maturation (45–47). It is noteworthy that IgM^+ B cells with mutated kappa chain genes were found to comprise nearly one-fourth of peripheral IgM^+ B cells, suggesting that B cells may exit germinal centers at this early stage of maturation frequently and persist in the circulating pool. It is also noteworthy that the frequency of somatic mutations in the productive rearrangements was greater than in the nonproductive rearrangements suggesting that these B cells may have been influenced by antigen mediated selection (45–47). Evidence for antigen selection of somatically mutated VκJκ rearrangements suggests that B cells expressing these gene products had passed through the light zone of the germinal center, where antigen selection occurs (45–47, 49), before reentering the circulation. The exact stage and pathway of maturation of these cells is not certain, but the data imply that antigen-selected IgM^+ memory cells occur frequently in human peripheral blood. It is important to note that the distribution of Vκ and Jκ genes did not differ between the somatically mutated and unmutated productive rearrangements, implying that the biases in Vκ usage noted in the expressed repertoire could not be explained by antigen exposures. Rather these biases are more likely to result from selective events after kappa chain expression but before antigen stimulation and clonal expansion.

$CD5^-$ vs $CD5^+$ B cells. The current analysis indicates that human $CD5^+$ and $CD5^-$ B cells contain a similar distribution of Vκ genes, and, therefore, may be somewhat different from murine B-1 B cells that use a limited set of genetic elements for immunoglobulin rearrangements (37). Moreover, other features of VκJκ rearrangements, including CDR3 length, were comparable in the human $CD5^+$ and $CD5^-$ B cells. The most striking difference between the human $CD5^+$ and $CD5^-$ population was the number of Vκ genes that exhibited evidence of somatic hypermutation. Both donors were found to have significantly fewer mutated Vκ genes in the productive repertoire of $CD5^+$ cells compared to $CD5^-$ B cells. These findings suggest that human $CD5^+$ B cells may be comparible to those in the mouse in that they are less likely to undergo somatic mutation (37, 50). These results are somewhat different from a previous report of an analysis of human V_H5 and V_H6 families in which minimal differences in mutational frequencies of $CD5^+$ and $CD5^-$ B cells were noted (51). In contrast, the current results obtained from analyzing a large number of kappa gene sequences from two separate donors clearly indicate that $CD5^-$ B cells accrue mutations in VκJκ rearrangements at a significantly greater frequency than do $CD5^+$ B cells. These findings are consistent with the conclusion that human $CD5^+$ and $CD5^-$ B subpopulations may differ in their ability to generate IgM memory B cells; perhaps relating to a limited capacity of $CD5^+$ B cells to enter germinal centers (52).

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