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J Clin Invest. 1995;96(2):831-841. <https://doi.org/10.1172/JCI118129>.

Research Article

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Somatic Mutation and CDR3 Lengths of Immunoglobulin κ Light Chains Expressed in Patients with Rheumatoid Arthritis and in Normal Individuals

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Abstract

Immunoglobulin secretion by plasma cells infiltrating synovial membranes is a prominent feature of RA. Previous analyses of a cDNA library generated from synovium of RA patient BC revealed immunoglobulin κ light chain transcripts with extensive somatic mutation, frequent N region addition, and unexpected variation in the lengths of CDR3 regions which form the center of the antigen binding site. To determine if these characteristics are present in other individuals, we performed reverse transcription–polymerase chain reaction amplification and sequenced ≥ 10 V κ -containing amplicons from nine tissue samples: synovia of three individuals with long-standing RA (including patient BC), PBLs of two of these individuals, and PBLs or splenocytes of four normal individuals. Increased levels of somatic mutation in PBLs appeared to correlate with increased age, which may reflect accumulation of circulating memory cells and/or decreased bone marrow production of naive B lymphocytes. Two of three RA synovial samples and both RA PBL samples exhibited increased proportions of clones with unusual CDR3 lengths. Enrichment for these antibody binding sites could be due to abnormal regulation of the emerging repertoire or to selection for B lymphocytes bearing antibodies of unusual specificity, and may play a role in the pathogenesis of RA. (*J. Clin. Invest.* 1995. 96:831–841.) **Key words:** synovium • B lymphocytes • aging • immunoglobulin genes • immunoglobulin variable region

Introduction

Rheumatoid arthritis (RA) is a systemic disease marked by synovial inflammation and hyperplasia that often destroys involved joints (1, 2). Infiltration of the synovium by antibody-

secreting plasma cells is a characteristic feature of the disease (3, 4). This local production of antibody is thought to participate in the pathogenesis of RA, although the exact mechanism(s) of tissue injury remains unclear (2).

Antibodies consist of two heavy chains and two light chains (either κ or λ). Each polypeptide chain contains a variable domain for antigen recognition and a constant domain, which is responsible for effector functions. The variable domain is assembled at the DNA level by splicing together variable (V),¹ diversity (D, heavy chain only), and joining (J) gene segments (5, 6). Immunoglobulin variable domains contain three intervals of sequence hypervariability (CDRs) that are separated from each other by four intervals of relatively constant sequence called framework regions (FRs) (for review see reference 7). The V gene segment encodes CDR1 and CDR2, whereas CDR3 is the product of V-(D)-J joining. In the mature protein, the heavy and light chain CDRs are juxtaposed to form the antigen binding site. Because the CDR3 intervals form the center of the antigen binding site, their sequence and structure play a major role in defining antigen specificity of antibodies.

B lymphocytes use both germline and nongermline (somatic) mechanisms to generate antibodies potentially capable of recognizing a tremendously large number of antigens. In the human kappa locus, there are ~ 28 potentially functional V κ gene segments that can be grouped into six families based on shared nucleotide sequence homology (8, 9). Random combinations of these germline V κ gene segments with one of five J κ gene segments can produce a large array of different light chain rearrangements. Somatic mechanisms, however, account for the vast majority of repertoire diversification. Initial variation is focused at the site of heavy chain gene segment splicing (10). Loss of a variable number of nucleotides at the termini of the V, D, and J gene segments in association with the addition of nongermline encoded nucleotides (N regions) generates a heavy chain CDR3 repertoire that varies tremendously in length, structure, and sequence (7, 10).

N region nucleotides are added to the exposed termini of rearranging gene segments through the activity of terminal deoxynucleotidyl transferase (TdT) (11, 12). Expression of TdT is thought to be restricted to the pro-B cell stage, when heavy chain rearrangement occurs. TdT protein is not detectable in cytoplasmic C μ^+ pre-B cells, the stage at which immunoglobulin light chain gene segments typically undergo rearrangement (12, 13). However, there is substantial evidence that N region addition occurs in V κ -J κ joins derived from B cells of normal individuals (8, 14–16) as well as from patients with RA (17, 18). Despite the presence of N region addition, rearrangement of κ light chain gene segments results in minimal V κ -J κ junctional diversity, largely because of the lack of D gene segments and similar lengths of the five known J κ gene

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Received for publication 28 October 1993 and accepted in revised form 17 May 1995.

1. *Abbreviations used in this paper:* CDR, complementarity determining region; D, diversity; FR, framework region; J, joining; RF, rheumatoid factor; TdT, terminal deoxynucleotidyl transferase; V, variable.

The Journal of Clinical Investigation, Inc.
Volume 96, August 1995, 831–841

segments. The resultant V κ -J κ joins usually encode either 9 or 10 amino acids (codons 89–97 according to Kabat [19]).

In a previous study, we cloned and sequenced randomly isolated κ light chain transcripts from a cDNA library generated from diseased synovium of a patient with long-standing, RF-positive RA (17). Unexpectedly, there was a high proportion of light chains containing N regions at the V κ -J κ junctions, resulting in uncommon variation in the lengths of CDR3 regions as compared with previous compilations of κ light chain sequences. In addition, there were clonally related transcripts in the cDNA library, suggesting that immunoglobulin production in the synovium was antigen driven (20).

With these observations in mind, we sought to determine if expression of κ light chain transcripts with unusual CDR3 lengths was specific to this particular sample of RA synovium or was reflective of a more generalized process. We chose to focus on transcripts derived from the V κ III family, particularly the gene segment *Humkv325*. *Humkv325*(A27 in Zachau nomenclature [8]) is highly conserved among different individuals in several outbred populations, it is expressed early in fetal life (21), and is a common component of CD5⁺ B cells. In addition, *Humkv325* is frequently expressed in chronic lymphocytic leukemia (for review see reference 22) and is often found in IgM κ RF paraproteins (23, 24) and RFs from patients with RA (18, 25, 26). Finally, as mentioned above, the initial RA synovial cDNA library we analyzed contained clonally expanded transcripts. These were derived from *Humkv325* and contained CDR3 regions 11 codons long (20).

We PCR amplified cDNA and performed sequence analysis of V κ transcripts expressed in synovium or PBLs of our previously described patient, from two additional individuals with long-standing RA, and from PBLs or spleen cells of four controls. The unusual length distribution of the CDR3 domains expressed in the original patient's synovium was also present in her PBLs. One of the two other individuals with RA demonstrated similar enrichment for κ light chains with unusual CDR3 lengths in both synovium and PBL. These findings suggest that V κ repertoires expressed in synovial tissue and PBLs of some patients with RA are different from normal individuals, which may have important implications regarding the pathogenesis of RA.

Methods

Patient characteristics and isolation of synovial cells and PBLs. Clinical characterization of the RA patient we initially studied (patient BC) and the methods used to process the synovial tissue and peripheral blood have been reported previously (17, 20). Two additional patients with long-standing RA were studied; synovial tissue was obtained at the time of surgery and peripheral blood was obtained during the postoperative period or at the time of the initial outpatient follow-up visit. The total amounts of IgM, IgA, and IgG from synovial cell culture supernatants were quantitated by ELISA and modifications of a previously described solid-phase RIA (27). Measurements of serum IgM, IgA, and IgG, as well as IgM rheumatoid factor (RF) and IgA-RF were performed by quantitative ELISA (28).

Patient JR, a 60-yr-old white male with a 20-yr history of RA, underwent right total knee arthroplasty in August 1993. Synovial tissue obtained at the time of surgery yielded a total of 5.8×10^7 cells. Net immunoglobulin production by synovial cells (ELISA results minus cycloheximide control) was 3,529 ng/ml IgG, 691 ng/ml IgM, and 134 ng/ml IgA. A substantial amount of IgM RF was secreted by cultured synovial cells (net 635 ng/ml). The serum IgM RF was 164 μ g/ml and IgA RF was 12.8 μ g/ml.

	<--	Leader	-->	<--	Framework 1	-->
V κ III <i>Humkv325</i> (A27)		TACCACCGGA	GAAATTGTGTTGACGCGAGTCTCCA			
LB-62		GGGG.ATTG				
LSK-16			<u>GC.C</u>			
V κ III <i>Humkv305</i> (A11)						
V κ III <i>Vg</i> (L6)						
V κ III <i>Humkv328</i> (L2)						
V κ IV (B3)		.G..TA..G	.C..C..A..C			
V κ I <i>Yd</i> (L8)		.G..GAT.T	.C..CCA..C			
V κ I <i>Ya'</i> (L18)		.G..GAT.T	.CC..CCA..C			
V κ I <i>O8</i>		.G..GAT.T	.C..CCA.A..C			

Figure 1. Nucleotide sequences of oligonucleotide primers LSK-16 and LB-62 used to preferentially amplify *Humkv325*-derived sequences. All sequences, including those of the primers are compared with germline *Humkv325*, with dots indicating identity. The germline sequences of gene segments represented among the different tissue samples are shown for purposes of comparison. The underlined sequences indicate modifications to encode SacI or EcoRI restriction sites for subcloning.

Synovial tissue from patient AS, a 42-yr-old black female with a 6-yr history of RF-positive RA, was obtained at the time of synovectomy of the right elbow. A total of 7.9×10^7 cells was obtained from this synovial tissue. The net synovial cell IgM production was 2,655 ng/ml, IgA 933 ng/ml, and IgG > 10 μ g/ml. The IgM RF was 886 ng/ml and IgA RF was 227 ng/ml. The serum IgM RF was 329 μ g/ml and IgA RF was 80 μ g/ml.

Control tissues analyzed included PBLs of a normal 60-yr-old white female (IT), who served as an age-, sex-, and race-matched control for patient BC, PBLs from a normal 21-yr-old white female (LK), PBLs from a normal 32-yr-old white male (LB), and cadaveric spleen cells from a 49-yr-old white male (SP) without history of autoimmune disease. Mononuclear cells were isolated from spleen or peripheral blood by Ficoll-Hypaque density gradient centrifugation (29).

Generation of cDNA and PCR amplification of V κ -containing transcripts. The guanidinium isothiocyanate technique was used to isolate total RNA from each sample (30). Oligo d(T) primed first strand cDNA was generated from total RNA as described previously (20), with the exception of synovial tissue from patient BC. Because all of the RNA that had bound to the oligo d(T) column had been used in previous experiments, we generated oligo d(T) primed first strand cDNA from RNA that had been passed through the oligo d(T) column but had failed to bind.

PCR amplifications were performed on 2- μ l aliquots of first strand cDNA, as described previously (20). PCR conditions were: 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, extension at 72°C for 4 min, with a final extension at 72°C for 10 min. PCR amplifications designed to enrich for *Humkv325*-derived sequences were performed with one of two sense 5' oligonucleotide primers (LSK-16 or LB-62) derived from the leader and FR1 of germline *Humkv325*. Fig. 1 shows the nucleotide sequences of LSK-16 and LB-62 compared with corresponding regions of germline gene segments. In all but one sample, the antisense 3' oligonucleotide primer used was LSK-19, derived from the C κ sequence (5'-GCGCCGTCTAGAATTAACACTCTCCCCTGTTGAA-3'). In the case of cadaveric spleen cDNA, the antisense C κ primer used was H-170 (5'-CCAGAATTCAACTGCTCATCAGATGGCGGGAAG-3') (20). To control for possible contamination, mock PCR reaction mixtures lacking template or containing products of the first strand cDNA reaction without reverse transcriptase were prepared. None of the controls contained amplified products visible on ethidium-stained agarose gels or product detected by Southern blot analysis using a C κ -specific probe.

Cloning and sequencing of PCR products from *Humkv325* amplifications. Aliquots of PCR products from amplifications with primer LSK-16 were blunt ended with large fragment DNA polymerase. PCR products from amplifications with primer LB-62 were digested with EcoRI. Ligation into pUC-19 or pBluescript® was performed using T4 DNA ligase (GIBCO-BRL, Gaithersburg, MD) by standard protocol (31). Plasmids were transformed into TB-1 *Escherichia coli* by electroporation using a Gene Pulser (Bio-Rad, Hercules, CA). At least 10 C κ

Table I. Germline Progenitor Gene Segments of V κ -containing Clones from Patients with Rheumatoid Arthritis and Controls

Tissue (total number of clones)	Number and percentage of clones originating from gene segment					
	<i>Humkv325</i> (A27)	<i>Vg</i> (L6)	<i>Humkv328</i> (L2)	<i>Humkv305</i> (A11)	V κ IV (B3)	V κ I (L8, L18, or O8)
BC Syn [62 WF RA] (<i>n</i> = 14)	7 (50%)	4 (29%)	2 (14%)	—	—	1 (7%)
BC PBL [62 WF RA] (<i>n</i> = 15)	7 (46%)	—	4 (27%)	—	4 (27%)	—
AS Syn [42 BF RA] (<i>n</i> = 11)	5 (45%)	1 (9%)	4 (36%)	—	1 (9%)	—
AS PBL [42 BF RA] (<i>n</i> = 10)	3 (30%)	3 (60%)	4 (40%)	—	—	—
JR Syn [60 WM RA] (<i>n</i> = 14)	9 (64%)	2 (14%)	—	—	1 (7%)	2 (14%)
ICT PBL [60 WF normal] (<i>n</i> = 11)	6 (55%)	2 (18%)	2 (18%)	—	1 (9%)	—
LB PBL [32 WM normal] (<i>n</i> = 12)	9 (75%)	3 (25%)	—	—	—	—
LK PBL [21 WF normal] (<i>n</i> = 11)	1 (9%)	6 (55%)	1 (9%)	—	2 (18%)	1 (9%)
Spleen [49 WM normal] (<i>n</i> = 10)	9 (90%)	—	—	1 (10%)	—	—

positive colonies from each tissue were sequenced by the dideoxy method (32). Sequencing was performed on both strands of all CDR3 regions. In most cases, double-stranded sequencing was performed on the remaining portions of each V κ domain.

Sequence analysis. The FR and CDR domains of each of the sequences were compared individually to corresponding domains of published human V κ sequences using the computer program SAW (Sequence Analysis Workshop®) (33). Sequences were assigned to germline gene segments according to highest degree of nucleotide sequence homology. Levels of somatic hypermutation were assessed by comparing the FR1 through CDR3 domains (codons 1–95 according to Kabat [19]) of each complete transcript to the appropriate germline sequence and calculating mean divergence rates for each sample. Truncated clones BCPBL2–1, BCPBL2–6, JRSyn16, and ASPBL29 were not included in the somatic mutation analysis. Nucleotide mismatches at the 3' end of the V κ gene segment or the 5' end of the J κ gene segment were assumed to represent N region addition rather than somatic mutation.

Several clones contained contiguous FR or CDR domains which appeared to be derived from one germline gene segment juxtaposed to downstream domains that were apparently derived from a different germline gene segment (see below). To avoid errors in calculation of levels of somatic hypermutation, clones containing crossover artifacts (see below) and clones that contained truncated V κ domains were not included in the somatic mutation analysis. To avoid bias that could be introduced by including several representatives of the same clone, we included only one representative from each set of identical clones in the somatic mutation, N region addition, and CDR3 length analyses. Clones JRSyn17 (identical to clone JRSyn15), JRSyn19 (two nucleotide differences from clone JRSyn13, but with an identical V κ -J κ join), JRSyn20 (identical to clone JRSyn13), JRSyn23 (identical to clone JRSyn13 except for one nucleotide in FR4), and LBPBL8 (identical to clone LBPBL7) were thus excluded from the analyses of somatic mutation, N region addition, and CDR3 length.

Statistical analysis. Differences in the amount of somatic mutation of clones from older and younger individuals and the amount of N region addition and CDR3 length heterogeneity between patients with RA and normal individuals were analyzed using the Student's *t* test, Fisher exact test, or χ^2 test, as appropriate.

Results

Germline derivation of κ light chains amplified with *Humkv325* and *C κ* primers. The majority of transcripts (52%) amplified with *Humkv325* and *C κ* primers were derived from *Humkv325* (A27) (Table I). Most of the remaining clones were derived from two other members of the V κ III family, *Vg* (L6) 19% and *Humkv328* (L2) 16%. One clone, SP9, was derived from the

V κ III gene segment *Humkv305* (A11) (34). None of the other potentially functional members of the V κ III family were represented in this analysis. These findings are consistent with the findings of other investigators, in which the overwhelming majority of expressed V κ III gene segments is derived from *Humkv325*, *Humkv328*, and *Vg* (35). Nine clones (8%) were derived from the single member V κ IV family (B3) (36), and four (4%) were derived from V κ I gene segments *Vd*(L8), *Va'*(L18), or *O8* (8, 37). The deduced amino acid sequences of all clones from each of the nine samples are shown in Fig. 2, and the nucleotide sequences of CDR3 regions (V κ -J κ joins) are shown in Fig. 3. In the three RA synovial samples, there was an increased proportion of J κ 4-containing sequences (13 of 35 nonidentical clones, 37%) compared with 9 of 68 clones (13%) from other tissues ($P = 0.01$, χ^2). This increase in J κ 4-containing clones was accompanied by a decrease in the proportion of J κ 1-containing sequences in RA synovia (7 of 39, 18%) compared with other tissues (28% of RA PBL, 44% of normal PBL, and 40% of normal spleen).

Analysis of clones from the synovial tissue of patient JR revealed the presence of a set of four closely related sequences. Clones JRSyn13, JRSyn20, and JRSyn23 are completely identical to each other. Clone JRSyn19 has two expressed differences from these three clones, resulting in a glycine at codon 25 in CDR1 and a serine at codon 62 in FR3. Although PCR artifact due to *Taq* polymerase error cannot be excluded, these may represent a set of clonally expanded sequences in this synovial tissue.

PCR amplifications were performed using an annealing temperature that was well below the calculated melting temperatures of the oligonucleotides to avoid bias toward less mutated *Humkv325* cDNAs. Because of the high degree of nucleotide sequence homology among members of the V κ III family, the finding of non-*Humkv325*-derived sequences was not surprising. We found that only four (*Humkv325*, *Humkv328*, *Vg*, and *Humkv305*) of nine potentially functional V κ III gene segments were amplified despite using an annealing temperature low enough to occasionally amplify gene segments from other families (V κ IV and V κ I). This restricted number of expressed V κ III gene segments is in agreement with other analyses of V κ repertoires (8, 15).

Comparison of PCR analysis and cDNA library analysis of the same RA synovial tissue sample. The results of the present

C. Normal PBL

Codon	Framework 1			Framework 2			Framework 3			CDR3	Jκ	107
	9	23	24	34	35	49	50	56	57			
<i>Humkv325</i>	GTLSLSPGERATLSC	RASGSV		SSSTLA	WYQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGGTDFLTISRLEPEDFAVYTC	QGYGSSP				
ITPBL3	V.....VA.R Jκ2
ITPBL7AG Jκ4
ITPBL9T Jκ4
ITPBL11S Jκ2
ITPBL12F Jκ3
ITPBL13GLT Jκ2
LBPBL2K Jκ2
LBPBL3R Jκ1
LBPBL5M Jκ2
LBPBL6Q Jκ3
LBPBL7 Jκ1
LBPBL8 Jκ1
LBPBL9H..D Jκ1
LBPBL2-1F G Jκ1
LBPBL2-2 Jκ3
LKPBL27G Jκ1
Yg (L6)	ATLSLSPGERATLSC	RASGSV		SSSTLA	WYQKPGQAPRLLIY	DASHRAT	GIPARFSGSGGTDFLTISRLEPEDFAVYTC	QGRSHMP			 Jκ1
ITPBL2C Jκ1
ITPBL14 Jκ1
LBPBL1NS Jκ1
LBPBL2-4C Jκ2
LKPBL1 Jκ2
LKPBL4R..NY Jκ4
LKPBL7L..F..GD..R Jκ5
LKPBL16D Jκ1
LKPBL18RG Jκ2
LKPBL22I Jκ3
<i>Humkv328</i>	ATLSVSPGERATLSC	RASGSV		SSHLA	WYQKPGQAPRLLIY	GASTRAT	GIPARFSGSGGTDFLTISRLEPEDFAVYTC	QGYNHMP			L Jκ4
ITPBL6H..ER Jκ1
ITPBL16D..P Jκ1
LBPBL2-3R Jκ1
LKPBL32L R Jκ1
Yδ1	DSLAVLGERATINC	KSGSVLYSSHMKDTLA		WYQKPGQAPRLLIY	MSTRRES	GVPDRFSGSGGTDFLTISRLEPEDFAVYTC	QGYTETP				P.....G..M Jκ1
ITPBL5A Jκ1
LKPBL5CC Jκ1
LKPBL19 Jκ4
Yα1 (L18)	SSLASVQDRVITTC	RASGSI		SSALA	WYQKPGQAPRLLIY	DASSLES	GVPDRFSGSGGTDFLTISRLEPEDFAVYTC	QGFNSYP			 Jκ4
LKPBL38MKNL Jκ4

D. Cadaveric Spleen

Codon	Framework 1			Framework 2			Framework 3			CDR3	Jκ	107
	9	23	24	34	35	49	50	56	57			
<i>Humkv325</i>	GTLSLSPGERATLSC	RASGSV		SSSTLA	WYQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGGTDFLTISRLEPEDFAVYTC	QGYGSSP				
SP1N...R Jκ1
SP3S..T..T Jκ1
SP4 Jκ1
SP5HSD..T Jκ2
SP6HSD..T Jκ3
SP10P Jκ1
<i>Humkv302</i>	ATLSLSPGERATLSC	RASGSV		SSSTLA	WYQKPGQAPRLLIY	DASHRAT	GIPDRFSGSGGTDFLTISRLEPEDFAVYTC	QGYGSSP			V Jκ3
SP9 Jκ1
Yg (L6)	ATLSLSPGERATLSC	RASGSV		SSSTLA	WYQKPGQAPRLLIY	DASHRAT	GIPARFSGSGGTDFLTISRLEPEDFAVYTC	QGRSHMP			 Jκ2
SP7C...Y Jκ2
SP8T..RF Jκ3
<i>Humkv328</i>	ATLSVSPGERATLSC	RASGSV		SSHLA	WYQKPGQAPRLLIY	GASTRAT	GIPARFSGSGGTDFLTISRLEPEDFAVYTC	QGYNHMP			 Jκ5
SP2R..EP.....PF Jκ5

Figure 2 (Continued).

cycles (25 cycles), 10 of the 108 clones appeared to contain PCR crossover artifacts (see Figs. 2 and 3). Clone BCSyn1 contains FR1, CDR1, and FR2 domains most homologous to *Humkv328*, whereas the sequence of CDR2 through Jκ, including N region, is identical to clone 10S2, a *Humkv325*-derived clone previously shown by cDNA library analysis to be expanded in this synovial sample (20). Clone BCSyn1, as well as BCSyn8, ASSyn13, ASSyn15, BCPBL10, LBPBL2-3, LBPBL5, SP2, SP7, and SP8 are likely the result of PCR artifact termed jumping PCR (38-40) (see Figs. 2 and 3 for details). Although the evidence for crossover is not as clear, clone BCPBL4 may also contain artifact. BCPBL4 is most homologous to *Humkv325* in each FR and CDR domain except CDR1. BCPBL4 does not encode an amino acid at codon 27A in the middle of CDR1 (Fig. 2 B), which suggests it contains a CDR1 domain from Vg and may thus represent another example of PCR artifact.

Crossovers artifacts occur when an incomplete product of one amplification cycle serves as a primer for a related sequence, generating a chimeric molecule (38-40). Our sequence analysis software enabled us to easily identify sequences containing crossover events by comparing FR and CDR domains of PCR sequences with corresponding domains from multiple germline gene segments. We cannot exclude the possibility of intragene crossovers, e.g., the presence of a clone that has the 5' end of a *Humkv325*-derived sequence and the 3' end of a different *Humkv325*-derived sequence. However, because each CDR3 domain was compared with those of all reported germline gene segments, crossover artifact did not affect analyses of

CDR3 length and N region addition. The reliability of our approach is further enhanced by the knowledge that the κ locus has been completely cloned and few sequence polymorphisms have been described.

Somatic hypermutation. The levels of somatic mutation among the transcripts from the different samples as reflected by divergence from progenitor germline genes are shown in Fig. 4 and Table II. Somatic mutation levels did not appear to be affected by disease status, as clones from RA PBL samples were not significantly more mutated than those from controls. The age of the individual, however, did appear to influence the degree of somatic mutation. The transcripts from PBLs of individuals 32 yr of age or younger (LB and LK, n = 20) were significantly less mutated (1.5±1.4%) than those from PBLs of individuals 42 yr of age or older (BC, AS, and IT, n = 30) (3.2±2.2%). This difference reached statistical significance (P = 0.002, Student's t test). Strikingly, 4 (44%) of 9 Vκ transcripts from the PBLs of normal individual LB and 2 (18%) of 11 clones from LK PBL were completely germline in the Vκ region, compared with none of 32 clones from the PBLs of individuals BC, AS, and IT. One clone from spleen contained an unmutated Vκ gene segment. Synovial clones were very mutated, with mean divergence from germline for the three RA patients ranging from 4.2 to 6.3% (Table II).

N region addition. The nucleotide sequences of N regions of each clone are shown in Fig. 3. The percentages of clones with at least one nucleotide of N region addition were: BCSyn 8 of 14 (57%), BCPBL 10 of 15 (67%), ASSyn 6 of 11 (55%), ASPBL 7 of 10 (70%), JRSyn 3 of 10 (30%), LKPBL 6 of

C. Normal PBL

Codon	CDR 3					95A 95B	96 97	98 99 100 101 102 103 104 105 106 107	Framework 4	CDR 3 Length (# aa codons)	
	89 90 91 92 93 94 95	95A 95B	96 97	98 99 100 101 102 103 104 105 106 107	95A 95B					96 97	98 99 100 101 102 103 104 105 106 107
Humkv325	CAG CAG TAT GGT AGC TCA CCT CC									Jκ2	9
ITPBL3C... ..C... ..TC ..									Jκ4	9
ITPBL7C... ..G... ..									Jκ4	9
ITPBL9C... ..C... ..	GA								Jκ4	9
ITPBL11T... ..T... ..									Jκ2	9
ITPBL12T... ..	TT								Jκ3	10
ITPBL13T... ..CT ..	GGT								Jκ2	9
LBPBL2	A								Jκ2	9
LBPBL3									Jκ1	9
LBPBL5 ‡	G A								Jκ2	10
LBPBL6	AA								Jκ3	9
LBPBL7									Jκ1	9
LBPBL8									Jκ1	9
LBPBL9A... ..									Jκ1	8
LBPBL2-1	C TTC G								Jκ1	9
LBPBL2-2	C								Jκ3	9
LKPBL27	GGA								Jκ1	9
Vκ	CAG CAG CGT AGC AAC TGG CCT CC									Jκ1	8
ITPBL2									Jκ1	8
ITPBL14									Jκ1	8
LBPBL1A... ..G... ..									Jκ1	10
LBPBL2-4									Jκ2	9
LKPBL1G... ..A... ..T... ..	C A								Jκ2	10
LKPBL4									Jκ4	9
LKPBL7A... ..A... ..C... ..G... ..									Jκ5	9
LKPBL16	A								Jκ1	9
LKPBL18	GA GG								Jκ2	11
LKPBL22C... ..C... ..									Jκ3	9
Humkv328	CAG CAG TAT AAT AAC TGG CCT CC									Jκ4	9
ITPBL6C... ..G... ..A... ..C... ..									Jκ1	10
ITPBL16C... ..T... ..									Jκ1	9
LBPBL2-3G... ..									Jκ1	9
LKPBL32	TC A								Jκ1	9
VκIV	CAG CAA TAT TAT AGT ACT CCT CC									Jκ1	9
ITPBL5G... ..G... ..									Jκ1	10
LKPBL5G... ..G... ..T... ..C... ..									Jκ1	9
LKPBL19G... ..									Jκ4	9
VκI (VκL1)	CAA CAG TTT AAT AGT TAC CCT CA									Jκ4	10
LKPBL38	AT AAA AAC CTC								Jκ4	10

D. Cadaveric Spleen

Codon	CDR 3					95A 95B	96 97	98 99 100 101 102 103 104 105 106 107	Framework 4	CDR 3 Length (# aa codons)	
	89 90 91 92 93 94 95	95A 95B	96 97	98 99 100 101 102 103 104 105 106 107	95A 95B					96 97	98 99 100 101 102 103 104 105 106 107
Humkv325	CAG CAG TAT GGT AGC TCA CCT CC									Jκ1	9
SP1TTA... ..A... ..	T								Jκ1	9
SP3C... ..C... ..A... ..									Jκ1	9
SP4C... ..C... ..A... ..G... ..	AA AGT								Jκ2	9
SP5									Jκ3	10
SP6									Jκ1	8
SP10T... ..	A								Jκ1	8
Humkv305	CAG CAG TAT GGT AGC TCA CCT CC									Jκ3	9
SP9	A AGG								Jκ3	9
Vκ	CAG CAG CGT AGC AAC TGG CCT CC									Jκ2	9
SP7T... ..									Jκ3	9
SP8C... ..	G								Jκ3	9
Humkv326AA... ..A... ..GG... ..									Jκ5	10
SP2 ‡									Jκ5	10

Figure 3 (Continued).

cal analysis regarding CDR3 lengths. Of note, however, the two RA patients whose PBLs were studied also had significant proportions of Vκ CDR3s of 11 amino acids expressed in their synovia: BCSyn, 3 of 14 (21%); and ASSyn, 3 of 11 (27%). All clones from the synovium of the remaining RA patient (JRSyn) contained CDR3s of 9 or 10 amino acids. Three of four normal individuals had Vκ CDR3 lengths of exclusively 8, 9, or 10 codons. The fourth control sample, LKPBL, contained one clone with a CDR3 length of 11 codons. In summary, the PBL repertoires of two RA patients and the synovial repertoires of two of three RA patients were enriched for κ light chains bearing CDR3 regions of unusual length.

Unusually long CDR3 regions do not seem to be limited to transcripts derived from one particular Vκ gene segment. Including three clones from our previous analysis (20), we have now isolated a total of 12 RA-derived clones containing CDR3 regions of 11 codons. Of these 12 clones, 4 (33%) are derived from Humkv325, 2 (17%) from Vg, 4 (33%) from Humkv328, 1 from a VκI gene segment, and 1 from VκIV. Finally, there was not an association between long CDR3 regions and the use of a particular Jκ gene segment, as three clones used Jκ1, four Jκ2, four Jκ3, and one Jκ4.

Discussion

Vκ transcripts from PBLs of younger individuals are less somatically mutated than those from older individuals. We found

higher levels of somatic mutation among Vκ transcripts derived from PBLs of individuals 42 yr of age or older than among those from individuals 32 yr of age or younger. Many factors such as recent infections, exposure to medications, or normal temporal variation can potentially influence the extent of somatic mutation. We have studied a limited number of individuals at one point in time, so as yet we cannot determine whether or not the finding of an age-related increase in somatic mutation will be generalizable to the entire population. However, a similar age-associated increase in the extent of immunoglobulin sequence mutation has been reported in a study of transcripts bearing VH gene segments from the VH5 family (41).

Differences in the proportions of different B cell subsets between older and younger individuals may, in part, explain the apparent age-associated increase in somatic mutation in PBL Vκs. Older persons may have higher proportions of circulating B lymphocytes that have been exposed to antigen, which are more likely than naive B cells to express mutated antigen receptors. This hypothesis is supported by several findings. Healthy aged individuals have been found to have fewer circulating B lymphocytes and higher levels of IgG and IgA, but not IgM, than younger individuals (42). In addition, Klein et al. (43) have recently analyzed somatic mutation levels of different peripheral blood CD19+ (B cell) subsets from a normal 67-yr-old man. They found that VκIII and VκIV clones from IgM+IgD+ B cells contained an average of only 0.3% diver-

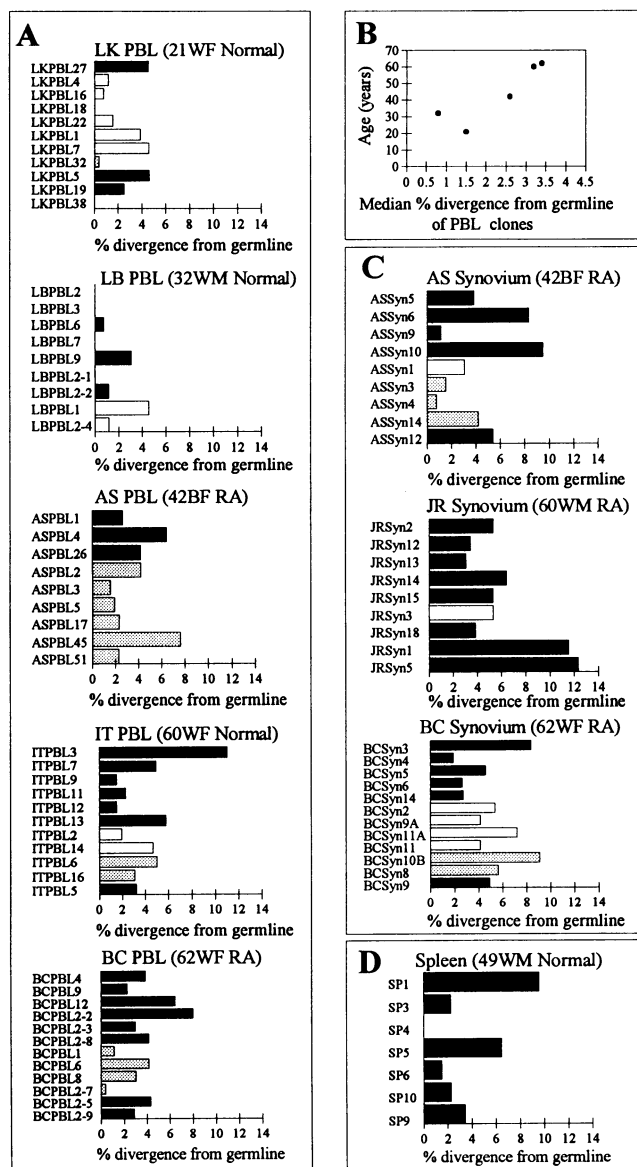


Figure 4. Nucleotide sequence divergence of V_{κ} transcripts from germline. Each bar represents an individual clone from the indicated tissue sample. Germline derivation: black bar, *Humkv325*; white bar, *Vg*; dotted bar, *Humkv328*; grey bar, other V_{κ} . (A) Percent nucleotide sequence divergence from germline of clones from PBLs, arranged by age. (B) Median percent divergence from germline of the five PBL samples in A. (C) Percent nucleotide sequence divergence from germline of rheumatoid arthritis synovial clones. (D) Percent nucleotide sequence divergence from germline of clones from cadaveric spleen.

gence from germline. In contrast, clones from $IgM^{-}IgD^{-}$ (presumably IgG^{+} or IgA^{+}) B cells from the same individual demonstrated an average of 3.9% divergence from germline (14). Analysis of somatic mutations in $VH4$ -containing rearrangements PCR amplified from tonsillar and peripheral blood B cells from a 4-yr-old child yielded similar results (43). IgD^{+} tonsillar B cells and IgM^{+} peripheral blood B cells were very homologous to germline (0.4 and 0.35% mutated, respectively). The most mutated subsets of B cells were tonsillar IgG^{+} germinal center cells and $IgG^{+}CD5^{-}$ peripheral blood B cells, with mean mutation levels of 3.3 and 3.2%, respectively.

Table II. Somatic Mutation of Clones from RA Patients and Controls of Different Ages

Tissue sample	No. of clones analyzed	Divergence from germline sequence		
		Mean \pm SD	Median	Range
		%	%	%
LK PBL (21 WF normal)	11	2.2 \pm 1.9*	1.5	0.0–4.6
LB PBL (32 WM normal)	9	1.2 \pm 1.6*	0.5	0.0–4.6
AS PBL (42 BF RA)	9	3.7 \pm 2.1	2.6	1.5–7.6
IT PBL (60 WF normal)	11	4.1 \pm 2.8	3.2	1.5–11.0
PB PBL (62 WF RA)	12	3.6 \pm 2.1	3.4	0.4–8.0
AS Syn (42 BF RA)	9	4.2 \pm 3.1	3.8	0.8–9.5
JR Syn (60 WM RA)	9	6.3 \pm 3.4	5.3	3.0–12.3
BC Syn (62 WF RA)	12	5.1 \pm 2.2	4.7	1.9–9.1
Spleen (49 WM normal)	7	3.6 \pm 3.3	2.3	0.0–9.5

Incomplete sequences or those containing PCR crossover artifacts were excluded from analysis. * The V_{κ} clones from PBLs of younger individuals LB and LK are less mutated than those from older individuals (RA patients BC and AS, and normal control IT) ($P = 0.002$, Student's t test).

Quantitative differences in immunoglobulin mRNA expression among B cells at different stages of differentiation may also be a factor in observed differences in levels of somatic mutation among individuals of different ages. Activated mature B cells have been shown to express approximately twice as much immunoglobulin mRNA as immature B cells (44). Because cDNA analyses reflect mRNA abundance, transcripts from activated cells are more likely to be isolated than transcripts from quiescent B cells. Thus, a decline in de novo B cell production by bone marrow coupled with an increase in the relative proportion of circulating memory and class-switched B cells may account for a higher amount of somatic mutation among the older individuals in this study.

Increased levels of somatic mutation in normal aging individuals may have important ramifications with regard to immune surveillance. Accumulation of memory B cells may enable mature individuals to respond more rapidly to previously encountered common pathogens in their environment. However, such an accumulation could possibly lead to a proportional decrease in the naive repertoire, resulting in decreased ability to respond to novel antigens. This loss of plasticity may theoretically contribute to increased susceptibility of aged individuals to infections.

κ light chain repertoires in some patients with autoimmune diseases are enriched for CDR3 regions encoding 11 amino acids. We found that V_{κ} transcripts in two of three RA patients analyzed contain a distinctively high proportion of long CDR3 regions. Data from other investigators corroborate our findings that V_{κ} CDR3 regions > 10 codons are rare in normal individuals (Fig. 5). The overwhelming majority ($\sim 96\%$) of V_{κ} CDR3s reported in the Kabat compilation (19) contain 8, 9, or 10 amino acid codons (Fig. 5A). Strikingly, none of these 130 V_{κ} sequences contain a CDR3 region of 11 amino acid codons. In a study of V_{κ} transcripts PCR amplified from the peripheral blood lymphocytes of a normal 67-yr-old patient, none of 36 clones contained CDR3 lengths of 11 codons (14). Analysis

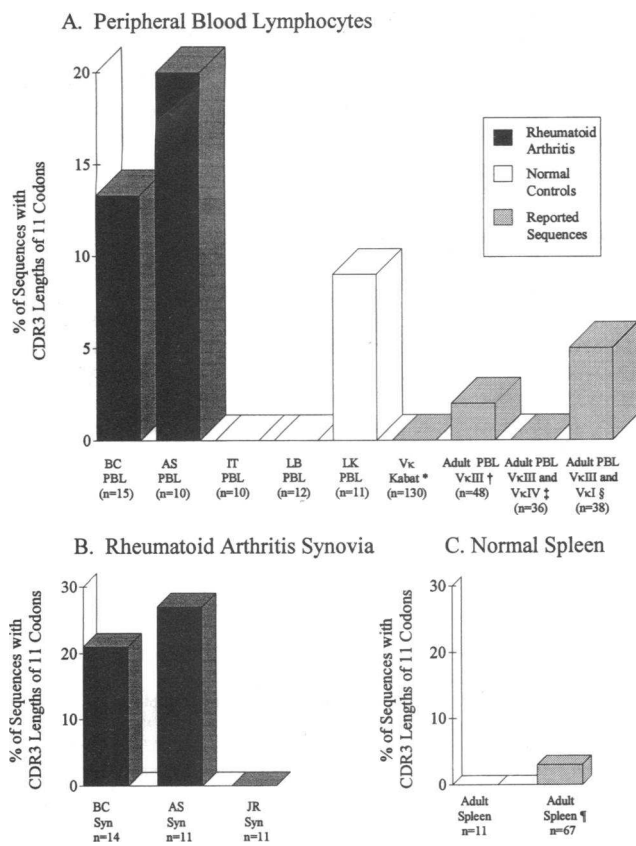


Figure 5. Percentage of Vκ clones containing CDR3 lengths of 11 amino acids. *Reported Vκ sequences in Kabat et al. (19). †Clones from a PCR-amplified cDNA library from PBLs of a normal adult (reference 15). ‡Clones from IgM⁺/IgD⁺, IgM⁺/IgD⁻, and IgM⁻/IgD⁻ PBLs from a normal 67-yr-old man (reference 14). §Clones amplified from the PBLs of two normal adult individuals (reference 45). ¶Clones from a cDNA library from the spleen of a normal 86-yr-old man (reference 8).

of PCR-amplified cDNA libraries from PBLs of a normal adult demonstrated that only 1 (2%) of 48 VκIII clones and 1 (11%) of 9 clonal rearrangements from CD5⁺ B cells had CDR3 regions of 11 codons (15). Victor et al. (45) amplified VκIII and VκI transcripts from PBLs of 2 normal individuals and noted that only 2 of 38 sequences (5%) had CDR3 domains of 11 codons (45). Although strict comparisons between PBL and spleen B lymphocytes cannot be made, we found no CDR3 domains of 11 amino acid codons from 10 transcripts amplified from cadaveric spleen, and Zachau and colleagues found that only 2 of 66 (3%) clones from a spleen cDNA library had CDR3 regions of 11 codons (8). In summary, κ light chains with CDR3 domains of 11 amino acids are found in B lymphocytes from normal individuals, but some patients with RA appear to have repertoires enriched for these long Vκ CDR3s.

Our results in RA are in agreement with findings of other investigators who have noted the presence of long κ chain CDR3s in some patients with autoimmune diseases. Of 20 VκIII-Jκ joins of polyclonal RFs derived from PBLs and synovial fluid B lymphocytes of a patient with RA, 2 (10%) had CDR3 regions of 11 codons (18). Similarly, 2 (40%) of 5 VκIII gene segments (1 from SLE PBLs and 1 from RA synovium) contained 11-codon CDR3s (26, 46). However, despite the

apparent enrichment for 11-codon Vκ CDR3s in some patients with autoimmune disease, the majority of autoantibodies do not exhibit unusual κ light chain CDR3 length. Most CDR3 domains of κ light chains of RFs from patients with RA contain 8, 9, or 10 codons (25, 47–50). Although 11-amino acid CDR3s are therefore not required for RF activity, the frequent presence of κ chains with 11-amino acid CDR3s in some patients with autoimmune diseases may be a marker for an antibody repertoire that has been abnormally regulated or abnormally activated.

Three factors influence the number of nucleotides at the Vκ-Jκ join and thus determine the CDR3 length: the site of rearrangement of the Vκ gene segment, the site of rearrangement of the Jκ gene segment, and the presence of N nucleotides. To delineate the mechanism(s) responsible for generation of CDR3 regions of unusual length in RA, we analyzed patterns of gene segment rearrangement. One of the clones in the present report, BCSyn14 (Fig. 3) has evidence of marked exonuclease activity of the Vκ gene segment (seven nucleotides removed), resulting in a distinctly unusual CDR3 region of only seven codons. Only two VκIII sequences reported in Kabat et al. (19) contain CDR3 regions of less than eight amino acids—one from a multiple myeloma that produced Bence Jones protein (51) and one from an individual with non-Hodgkin's lymphoma (52). Little is known about regulatory control of exonucleolytic loss during gene rearrangement (53).

Variation in the sites of gene rearrangement of the Vκ to Jκ gene segments also influences CDR3 length. The lengths of the five known germline Jκ gene segments are very similar to one another, with Jκ2 containing 38 nucleotides (8 in the CDR3 region and 30 in FR4) and Jκ1, Jκ3, Jκ4, and Jκ5 containing 37 nucleotides each (7 in the CDR3 region and 30 in FR4). Similarly, most Vκ gene segments contain 23 nucleotides in the CDR3 region. Analysis of the sites of Vκ-Jκ joining revealed patterns of rearrangement that were consistent throughout all tissues analyzed. Inclusion of 7 nucleotides from the Jκ gene segment in conjunction with exonucleolytic loss of 3 nucleotides from the Vκ gene segment (leaving 20 nucleotides) was the most common pattern (22 of 104 rearrangements). In the absence of N region addition, this rearrangement yields a CDR3 region of nine codons. The second most common rearrangement, seen in 15 joins, was a contribution of 7 nucleotides from the Jκ gene segment and no loss of the Vκ sequence (resulting in the inclusion of 23 nucleotides and a CDR3 of 10 codons if there is no N region addition). 13 joins demonstrated the presence of 5 nucleotides from the Jκ and 1 nucleotide lost from the Vκ (leaving 22). This analysis demonstrates that there is more variability in the number of nucleotides removed from the Vκ than in the number of nucleotides removed from the Jκ gene segment. Similar consistency in the sites of gene segment rearrangement have been observed in mouse κ light chains (54).

Recently, N region addition has been noted to contribute extensively to the diversity generated at sites of Vκ→Jκ joining. In the present study, normal individuals and RA patients were found to contain a similar proportion of transcripts with N region addition (Fig. 3). However, despite the frequent presence of N region addition, the lengths of the vast majority of CDR3s from normal individuals were relatively constant, with > 95% of κ transcripts containing either 9 or 10 amino acids codons. In contrast, the consequences of N region addition appear to be quite different in some patients with RA, as Vκ transcripts containing 11 amino acids in their CDR3s were commonly

found in synovia and PBLs of two of the three RA patients. These long CDR3s can only be generated through N region addition, as the germline V κ gene segments and the longest J κ gene segment, J κ 2, provide only 31 of the 33 nucleotides required for an 11-amino acid CDR3.

There are several plausible explanations for the presence of nongermline-encoded nucleotides at the V-J junctions of these light chains. Most likely, there is a point during the transition from the pro-B cell to pre-B cell stage at which there is TdT in an amount sufficient to introduce N regions into the light chain junction. The level of TdT expression in some pre-B cells could be insufficient for detection by staining, but sufficient for addition of N regions, as has been shown in T lymphocytes from human fetal thymus (55). Studies using fluorescence-activated cell sorting have identified a small subpopulation of pre-B cells expressing both nuclear TdT and cytoplasmic μ heavy chains (56). Alternatively, light chain rearrangement can potentially precede heavy chain rearrangement in normal pro-B cells that express TdT. Kubagawa et al. (57) first documented the existence of pro-B cell lines that had undergone κ rearrangements at one or both alleles but contained heavy chain loci in either germline configuration or with DH-JH rearrangement. These κ -only cell lines appear to represent an alternative pathway of B lineage development in which light chain rearrangement precedes heavy chain rearrangement, presumably in the presence of TdT. Recently, it has been shown that the light chain rearrangements in these κ -only cell lines may indeed contain N regions (58). Thus, sequences that contain N regions in light chains could represent the progeny of this alternative B cell pathway.

Enrichment of the repertoire for B lymphocytes expressing κ light chains with unusual CDR3 lengths can potentially expand the diversity of antigens recognized. The CDR3 region is very important with regard to function, as alteration of the V κ -J κ junctional sequence can abrogate the ability of antibody to bind antigen (59). In the absence of N region addition, only eight different amino acids can be generated at the Humkv325-J κ splice site, residue 96 according to Kabat (19). However, if nongermline-encoded nucleotides and variation of CDR3 length are allowed, the potential junctional diversity of the κ repertoire increases 1,000-fold. Unusually long CDR3 regions may lead to conformational changes that influence heavy chain and light chain pairing (20). We speculate that individuals capable of generating variant CDR3 intervals, such as patients with RA, may be more likely to generate novel antigen binding sites than individuals constrained toward a more conventional light chain repertoire.

V κ transcripts with unusual CDR3 lengths may be the result of abnormal gene rearrangement or antigenic selection. There are at least two possible explanations for the enrichment of V κ domains with unusually long CDR3 domains in some RA patients. There may be intrinsic differences in the mechanisms of gene rearrangement in these patients, such as alterations in regulation of TdT or exonuclease, that result in abnormal development of the preimmune repertoire in bone marrow. If so, family members may also express altered repertoires, representing a potential risk factor for the development of RA. An alternative possibility is that in normal individuals there may be selection against normal B cells that by chance contain κ chains with unusual CDR3s, with positive selection by antigen and clonal outgrowth of such B cells in some patients with RA. Because of the mounting evidence that RA is an antigen-driven

disease (20, 60) and the observation that κ light chains with CDR3 regions of 11 codons can encode autoantibodies, we favor the hypothesis that enrichment for κ light chains with unusual CDR3 intervals is the product of antigenic selection. In either case, it is apparent that the antibody repertoires of some RA patients are different from those of normal individuals.

Acknowledgments

We thank Jennifer Collins for excellent technical assistance.

This work was supported, in part, by a Veterans Administration Merit Award (W. J. Koopman) and National Institutes of Health grants AR-01867 (S. L. Bridges, Jr.), HL-07553 (M. L. Johnson), AR-03555 (H. W. Schroeder, Jr. and W. J. Koopman), AI-18958 (W. J. Koopman), AI-18745 (W. J. Koopman), AR-20614 (W. J. Koopman), DK-40117 (W. J. Koopman), and AI-33621 (H. W. Schroeder, Jr.). S. K. Lee received support from the Faculty Research Fund and the Myung-Sun Kim Memorial Foundation of Yonsei University College of Medicine.

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