N-Region Addition in Immunoglobulin Kappa Light Chains in B Cell Subsets in Rheumatoid Arthritis: Evidence for Over-expression of TDT in B Lineage

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ABSTRACT

Background: Unusually high amounts of N region addition and CDR3 length diversity were found in immunoglobulin (Ig) light chain Vk and Jk joins in patients with rheumatoid arthritis (RA). We sought to determine whether this finding is due to excessive activity of the enzyme responsible for N region addition (terminal deoxynucleotidyl transferase [TdT]) in B lineage cells in bone marrow or from positive antigenic selection of B cells with long CDR3 lengths. Methods: We used FACS to isolate IgM⁺/IgD⁺ B cells (predominantly naive) and IgMIgD⁻ B cells (predominantly class-switched) B cells from peripheral blood of a patient with RA known to have enrichment for long Vk CDR3s and from that of two normal controls. RT-PCR of VkIII transcripts was performed, followed by sequencing of individual cDNA clones. We analyzed the CDR3 lengths and N region additions in 97 clones. Results: There was enrichment for long $\overline{CDR3}$ lengths (11 or 12 amino acids) in both IgM^+/IgD^+ and IgMIgD B cells in RA compared to B cell subsets in the normal controls. The IgM⁺/IgD^{*} B cell subset in RA was markedly enriched for N region addition and was similar to that seen in the IgM/IgD subset. Conclusion: These data suggest that enrichment for N region addition and long CDR3 lengths in RA may result from unusually high or prolonged activity of TdT in bone marrow. (Immune Network 2003;3(2):89-95)

Key Words: Rheumatoid arthritis, N region, CDR3

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 hallmark of the disease (3,4). Immunoglobulins are heteromeric proteins consisting of two heavy and two light chains, each of which is divided into a variable domain (V) that defines the antigen specificity of the molecule. V domains contain 3 hypervariable intervals of the complementary-determining regions (CDR1, CDR2, and CDR3) that are divided into 3 relatively constant domains of the framework regions (FR1, FR2, and FR3)(5-7). The V_{H} - D_{H} - J_{H} and V_{L} - J_{L} and non-germline-encoded (N) nucleotides additionally define CDR3. The CDRs are usually directly involved in antigen binding, with CDR3 at the center of antigen binding sites (6).

Previous studies showed high levels of non- germline encoded nucleotides (N regions) in VJ joins of kappa light chain from RA synovia and unsorted cells of RA peripheral blood (PB)(8,9). N-nucleotides enhance antibody diversity by encoding additional amino acid residues in CDR3. This finding may be due to positive antigenic selection or rearrangement abnormality from the bone marrow or lymph nodes.

In order to clarify these matters, we separated IgM^+ IgD⁺ B cells, which come from bone marrow without contacting antigen, and IgMIgD⁻ B cells (presumably IgG⁺ or IgA⁺) in order to compare CDR3 and N-nucleotides between RA and normal individuals. We choose the IgM⁺ IgD⁺ B cells subset as a precursor of GC B cells and the IgMIgD⁻ cells

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subset as GC derived memory B cells. To clarify whether abnormal N-nucleotides and CDR3 in RA comes from rearrangement abnormality or positive antigen selectivity, we compared N-nucleotides and CDR3 in those B cells subsets through sequencing of the V light chain.

Materials and Methods

Patient and control characteristics and cell separation of peripheral blood mononuclear cells (PBC). PBC from patient AS, a 46-year-old black female with a 10-year history of RF-positive RA, and from two normal healthy individuals, a 35-year-white female SB and a 37-year old Asian male CL, were isolated by Ficoll-Isopaque density centrifugation. PBC were incubated with anti-CD19-microbeads for 15 min at 4°C. CD19⁺ B cells were enriched by magnetic cell separation using the MiniMACS system (Miltenyi Biotec, Auburn, CA, USA). B cells were enriched 98% by a single step of magnetic sorting for analysis by immunofluorescence staining with anti-human CD19 FITC (PharMingen, San Diego, CA, USA). Cells of the CD19-enriched fraction were incubated with anti-human IgM-PE (PharMingen, San Diego, CA, USA)) and anti-human IgD-FITC for 20 min on ice, then sorted into IgM⁺IgD⁺ and IgMIgD⁻ fractions on a FACS advantage SE (Becton Dickson, Franklin lakes, NJ, USA).

Generation of cDNA and PCR amplification of $V\kappa$ -containing transcripts. Total RNA was isolated from each IgM⁺IgD⁺ subset and from IgMIgD⁻ subset B cells using Tri-zol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Oligo d(T)primed first-strand cDNA was generated from total RNA using SuperScript II for RNase H⁻ Reverse transcriptase kit (GibcoBRL, Gaithersburg, MD, USA). PCR amplifications were performed on 2-µl aliquots of first-strand cDNA, using Taq DNA polymerase. PCR conditions were: 35 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 30 sec, extension at 72°C for 1 min, with a final extension at 72°C for 7 min. PCR amplifications were designed to CK sequence as an anti-sense 3' primer (LSK-19: 5'-GCGCCGTCTAGAATTAACACTCTCCCCTGTTGA A-3') and the leader and FR1 of germline *Humkv325* as a sense 5' primer (LSK-16: 5'- CCACCGGAGAGC TCGTGTTGACGCAGTCTCCA-3'). To control for possible contamination, a mock PCR reaction mixture, lacking a template and containing products of the first strand cDNA reaction without reverse transcriptase were prepared. None of the controls contained amplified product as assessed by ethidium-stained agarose gel electrophoresis.

Cloning, transformation and sequencing of PCR products. Aliquots of PCR products were subcloned with pGEM-T vector using TA cloning kit (Promega, Madison, WI, USA). Plasmids were transformed into DH5a *E.Coli* by 37°C heat shock. For colonies that were digested with EcoR1 restriction enzyme, plasmid DNA was obtained and sequenced by dye terminator cycle sequencing using an automated sequencer (Applied Biosystems, Inc., Model ABI373, Foster, CA, USA).

Sequence analysis. Sequences were analyzed with a sequence aliment (DNAPLOT) and SAW (Sequences Analysis Workshop) software (10). Nucleotide exchanges in VK were considered for the analysis of somatic mutation. Levels of somatic hypermutation were assessed by comparing the number of nucleotides from the codon 20 of FR1 throughout the CDR3 domains (6). Non-germline encoded nucleotides at the VK-JK joint, which represent N-region insertion, and P nucleotides, which are palindromic to terminal nucleotides of the coding sequence, were not counted as somatic mutations (11).

Statistical analysis. The results are presented as the



Figure 1. Fluorescence analysis and gate for sorting of B cell subsets derived from peripheral lymphocytes of the rheumatoid arthritis (A) and normal controls (B and C).

mean±SD. Differences in the amounts of somatic mutation, N-region addition, and CDR3-length heterogeneity between RA patients and normal individuals were analyzed using the Chi-square test, Fisher exact test (two-tailed), or Student *t*-test, as appropriate.

Results

 $V\kappa$ transcripts from PBC of IgM^+IgD^+ B cells had no, or fewer, somatic mutations than those from IgMIgD⁻ B cells. Following enrichment of CD19⁺ B cell by microbeads magnetic cell separation, IgM⁺IgD⁺ and IgM IgD B cells were purified by fluorescence activated cell sorting (Fig. 1). The levels of somatic mutation, as reflected by divergence from germline, among the transcripts from the different samples are shown in Fig. 2. The transcripts from $IgM^{+}IgD^{+}$ B cells of the RA patient and normal individuals (AS, CL and SB, n=46) were significantly less mutated $(0.54\pm0.12\%)$ than their transcripts from IgMIgD B cells $(4.51\pm$ 0.42%, n=52) (P<0.0001). Somatic mutation level of the IgMIgD B cells (memory cells) in RA was also significantly higher than those in the two normal controls (P=0.04).



Figure 2. Somatic mutation in IgM^+IgD^+ and IgM^+IgD^-B cells between rheumatoid arthritis (AP, AN) and normal controls (CP, CN, SP and SN). *P=0.04: AN versus CN & SN.

Twenty-seven (59%) of 46 VK transcripts from $IgM^{+}IgD^{+}$ B cells were completely germline in the VK region, compared with none of 52 colonies from $IgMIgD^{-}$ B cells.

N-region addition. The nucleotide sequences of N regions of each clone in RA are shown in Fig. 3. The percentages of clones with at least one nucleotide of N region addition in IgM^+IgD^+ B cells were: SB 2 of 13 (15%), CL 3 of 13 (23%), and AS 10 of 19 (53%). Those of N region addition in IgMIgD B cells were: SB 4 of 14 (29%), CL 7 of 17 (41%), and AS 12 of 21 (57%)(Table I). The nucleotides of N-region addition in the RA patient $(1.3\pm0.3, n=40)$ were significantly higher than those of the two normal controls (0.4±0.1, n=58)(P=0.0003), as in previous report (9)(Fig. 3). The nucleotides of N region addition in IgM⁺IgD⁺ B cells in the RA patient (1.5 ± 0.4) were significantly higher than those of the two normal individuals (0.3 ± 0.1) (P=0.0004). But those in IgMIgD B cells did not differ significantly between RA and normal controls.

CDR3 lengths of VK transcripts. The VK repertoires of the RA patient are compared with those of the normal controls in Table I and Fig. 4, in order to demonstrate CDR3 length. Clones with unusually long CDR3s (one 11 and two 12 amino acid codons) were found in 3 of 40 (7.5%) transcripts from the RA patient, but in none of 58 transcripts from the normal controls. One 11 and one 12 amino acid codons were found in 19 transcripts of IgM⁺IgD⁺ B cells (naive cells) in RA (Fig. 5). One 12 amino acid codons was also found in 21 transcripts of IgM IgD⁻ B cells in RA. Each of 3 long CDR3s came from Humkv325, Vg, and Humkv328 VK gene segments.

Germline derivation of κ light chains amplified with Humkv325 and C κ primers. The majority of transcripts amplified with Humkv325 and κ primers were derived from Humkv325 (A27) (36%). Most of the remaining clones were derived from two other members of the κ III family, 29% from Humkv328 (L2) and 21% from Vg (L6). One clone, AN22, was

Table I. N region addition and CDR3 length in IgM⁺IgD⁺ and IgM⁻IgD⁻ cells in the rheumatoid arthritis (RA) and normal controls.

	B cell subset	CDR3>10 aa	Avg. CDR3	N addition	Avg. No. of "N"
RA	IgM^+/IgD^+	2/19 (10%)	9.3 aa	10/19 (53%)	1.0
	IgM ⁻ /IgD ⁻	1/21 (5%)	9.3 aa	12/21 (57%)	1.1
Normal 1	IgM^+/IgD^+	0/13 (0%)	9.0 aa	2/13 (15%)	0.2
	IgM ⁻ /IgD ⁻	0/14 (0%)	9.2 aa	4/14 (29%)	0.4
Normal 2	IgM^+/IgD^+	0/13 (0%)	9.2 aa	3/13 (23%)	0.3
	IgM ⁻ /IgD ⁻	0/17 (0%)	9.0 aa	7/17 (41%)	0.7

aa: amino acid.



Figure 3. N-region addition in IgM^+IgD^+ and IgM^-IgD^-B cells between rheumatoid arthritis (AP, AN) and normal controls (CP, CN, SP and SN). **P=0.0004: AP versus CP & SP, *P=not significant: AN versus CN & SN.



Figure 4. Percentage of W clones containing CDR3 length of 11 and 12 amino acids between rheumatoid arthritis (AS) and normal controls (SB, CL).

derived from the VKIII gene segment Humkv305 (A11). None of the other potentially functional members of the VK III family were represented in this analysis. Seven clones (7%) were derived from VK I gene segment Vd (DPK8), 012 (DPK9), and 018(DPK1), and six (6%) from the single member of the VK IV family DPK24 (B3). The JK4 and JK1 were frequently used in RA and normal controls. The incidence of JK in 30 sequences in RA were 11 of JK 1(36%), 11 of JK 4 (36%), 8 of JK 2 (27%), 6 of JK 5 (20%), and 4 of JK 3 (13%). The incidence of JK in 58 sequences in normal controls were 22 of JK 4 (38%), 16 of JK 1 (28%), 13 of JK 3 (22%), 4 of JK 3 (7%), and 3 of JK 5 (5%).

Analysis of clones from the IgMIgD B cells (memory B cells) in normal controls revealed two sets of 4 closely related sequences. SN 17, SN 32, CN30, and CN34 were completely identical to each other. Analysis from the IgMIgD B cells in RA revealed two sets of 5 related sequences. AN6, AN14, and AN17 were completely identical and AN2 and AN5 were completely identical to each other. But none of $IgM^{+}IgD^{+}$ B cells of RA and normal controls revealed an identical sequence.

Discussion

During B cell development, a series of highly regulated gene rearrangements generates a function antibody molecule. In the pro-B cell stage, the heavy chain locus undergoes rearrangement. Variable heavychain domains are encoded by variable (V_H), diversity (D_H), and joining (J_H) gene segments, which undergo sequential somatic rearrangement to become juxtaposed in the genomic DNA (5). Initially, one or more D_H gene segments are rearranged to a J_H gene segment, followed by rearrangement of the V_H gene segment to the D_H-J_H join. After heavy chain rearrangement, the kappa light chain segment undergoes rearrangements of V_L and J_L gene segments to generate light chain variable domains, presumably in the pre-B cell stage (7).

During the pro-B cell stage, the enzyme responsible for N-region addition, TdT is present in the nucleus of the cell. Although functional TdT has not been shown to be present at the time of kappa light chain rearrangement, N region addition is clearly present in kappa light chains from normal individuals and patients with RA (10-12,16). Therefore, TdT appears to be active throughout all stages of immunoglobulin gene rearrangements in human B cells.

Most PB B cells express IgM throughout their life and these cells are distinguished into IgM^+IgD^- and IgM^+IgD^- B cells (14,15). Sequence analysis of the V genes expressed by the B-cell subset revealed most IgM^- only cells while IgM^-IgD^- cells were mutated, but the IgM^+IgD^+ cells in PB had no, or few somatic mutations (13,14). Therefore, IgM^+IgD^+ B lymphocytes establish the naive, unmutated antibody repertoire, and if a somatically mutated subset exists among IgD-expressing PB B cells, it must be small

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AN7 AN21 AN11 AN13 AN12 AN20 Humnkv328 AN6 AN14 AN17 AN9	CAG CAG A A A A A A	CAG 	TAT -G - - C - TAT 	GGT C C - C AAT - C - C - C - C	AGC GA - T GA - T GA - - G - G - G - G - G - G	TCA	CCT T - - T - - T - - G - CCT 	cc - cc	C G TCT G G G G C	GG	G TG A G G TG	TGG TAC TTC TTC TTC TGG	ACG ACT ACT ACT ACG ACG		GGC GGC GGC GGC GGC GGC GGC	CAA CAG CCT CCT GGA CAA CAA		ACC ACC ACC ACC ACC ACC	AAG AAG AAA AAA AAG AAG AAG	GTG CTG GTG GTG GTG CTG CTG	GAA GAG GAT GAT GAA GAA GAA GAA GAA GAA	ATC G	AAA AAA AAA AAA 	J k 1 J k 1 J k 2 J J k 2 J k 2 J k 2 J J k 2 J k 2	9 9 100 10 9 9 9 9 9 9 9 9 9 9 9
AN7 AN21 AN11 AN13 AN12 AN20 Humnkv328 AN6 AN14 AN17 AN9 AN1	CAG A CAG A A A 	CAG 	TAT -G - 	GGT C C - C AAT - C - - C - - C - - C - - C	AGC GA - T GA - T GA - - G - G - G 	TCA	CCT T - - T - - T - - G - CCT 	сс - сс	C G G TCT G G G C ACG	GG GCC	G TG A G G TG A	TGG TAC TTC TTC TGG TGG	ACG ACT ACT ACT ACT ACT		GGC GGC GGC GGC GGC GGC GGC GGC	CAA CAG CCT CCT CCT CAA CAA CAA		ACC ACC ACC ACC ACC ACC ACC	AAG - G - AAG AAA AAG AAG AAG AAG 	GTG CTG GTG GTG GTG CTG CTG GTG GTG	GAA GAG GAT GAT GAA GAA GAA GAA GAA GAA	ATC G ATC ATC C ATC G G G ATC ATC	AAA AAA AAA AAA AAA AAA AAA AAA	J k 1 J k 1 J k 2 J k 2	9 9 10 10 9 9 9 9 9 9 9 9 9 9
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AN7 AN21 AN11 AN13 AN12 AN20 Humnkv328 AN6 AN14 AN17 AN9 AN1 AN9 AN1 AN24 AN8	CAG 	CAG 	TAT -G - - C - - TAT - T - 	GGT 	AGC 	TGG	CCT T - - T - - T - - T - - G - - CCT 	cc - cc	C G G TCT G G G C C	GG GCC	G TG A G G TG A G	TGG TAC TTC TC TC TGG TGG	ACT ACT ACT ACT ACT ACT ACT ACT ACT ACT		GGC GGC GGC GGC GGC GGC GGC GGC GGC	CAA CAG CCT CCT CCT CCT CAA CAA CAA CAA CCT CCT			AAG - G - AAG - AAA AAAG AAAG AAAG AAAG 	GTG CTG GTG GTG GTG CTG GTG GTG GTG GTG	GAA GAG GAT GAT GAA GAA GAA GAA GAA GAA	ATC G ATC ATC G ATC G ATC ATC ATC ATC 	AAA AAA AAA AAA AAA AAA AAA AAA AAA AA	J k 1 11 J k 1 1 J k 1 2 2 2 3 J k 2 3 4 J k 2 3 4 J k 3 3 J k 3 3 J k 4 4 J k 1 1 1 J k 2 3 J k 3 3 J k 4 4 J k 1 1 1 2 J k 2 3 J k 3 3 J k 4 4 J k 1 1 2 J k 2 3 J k 3 3 J k 4 4 J k 1 1 2 J k 2 3 J k 3 3 J k 3 3 J k 4 4 J k 1 1 2 J k 2 3 J k 3 3 J k 4 3 J k 4 3 J k 4 4 J k 1 1 2 J k 4 3 J k 4 4 J k 4 4 4 J k 4 4 4 J k 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4	9 9 10 10 9 9 9 9 9 9 9 9 9 9 9 9 9 9 9
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Figure 5. Nucleotide sequences of CDR3 regions of clones in IgM^+IgD^+ B cells (A) and those of clones in $IgMIgD^-$ B cells (B) in rheumatoid arthritis.

fraction of CD27-expressing B cells (16). The memory B-cell compartment in humans consists of somatically mutated, class-switched, which represent IgMIgD such as IgG^+ and IgA^+ PB cells, and IgM bearing B cells (17).

If B cells succeed in generating a functional,

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non-autoreactive, antigen receptor, they come into the peripheral B cell pool as naive cells. Upon encountering antigen, the antibodies expressed by a B cell are modified by class-switch recombination and somatic hypermutation (18,19). Somatic hypermutation appears to B cells proliferating within the microenvironment of germinal center (GC)(20,21). Thus somatic mutation of V-region gene is a hallmark of GC B cell and their descendants.

 $V\kappa$ transcripts from IgMIgD B cells of a patient with RA is higher somatically mutated than those from normal individuals. The V genes of human PB IgMIgD lymphocytes, which are thought to be GC-derived memory B cells, are highly diversified by somatic mutation and independent of the age of an individual (13,22). We also found high levels of somatic mutation among VK transcripts derived from the IgM IgD B cells of the two normal individuals (3.93% and 3.74%), similar to the 3.9% reported for VK gene of IgMIgD B cells of a 67-year-old female (13). But the somatic mutation level of those cells was significant higher in the RA patient than in the normal controls (5.5%, P=0.04). Differences in the proportions of different B cell subsets between RA and normal individuals may explain the increase in somatic mutation in IgMIgD B cells in the RA patient. RA may have a higher proportion of circulating memory B cells that have been exposed to antigen, which are more likely than nave cells to express mutated antigen receptor.

Abnormal CDR3 regions encoding 11 or 12 amino acids of $V\kappa$ expressed in IgMIgD B cells in RA. We found that VK transcripts of the RA patient contain distinctively long CDR3 regions (Fig. 4). Data from other investigators show that W CDR3 regions with more than 10 codons were very rare in normal individuals (6,12,13,23). Most data showed less than 5% of CDR3 domains of 11 amino acids from normal PB. Nobody has reported CDR3 domains of 12 amino acids in VK transcripts. Our data also showed no CDR3 domains of 11 or 12 amino acids among the 59 W transcripts of two normal controls. However, there were two CDR3 domains of 12 amino acids and one of 11 amino acids expressed among the 40 VK transcripts (7.5%) of the RA patient. Our previous data from unsorted peripheral B cells in the same RA patient (AS) showed two CDR3 domains of 10 sequences with 11 amino acids (9). Interestingly, two of 3 abnormally long CDR3 domains in RA were expressed in IgMIgD B cells (memory B cells).

Three factors influence the number of nucleotides at the VK-JK join and thus determine the CDR3 length: the rearrangement site of the VK gene segment, that of the JK gene segment, and the presence of N nucleotides. Since abnormally long CDR3 was expressed in $IgM^{+}IgD^{+}$ B cells (naive B) cell), the mechanism responsible for generation of CDR3 regions of unusual length in RA maybe unusual VK and K rearrangement or abnormal N region addition in bone marrow. But we couldn't exclude peripheral antigen selection because of the 2 abnormally long CDR3 in IgMIgD B cells in RA. N-region addition is extensively higher in IgM^+IgD^+ B cells in RA than those in normal individuals. Insertion of non-germline encoded nucleotides (N-regions) during Ig gene rearrangement was first described as a mechanism for the generation of junctional diversity in heavy chain CDR3 (24,25). A previous study with unsorted PBC in normal individuals and the same RA patient has shown N-region addition was present in kappa light chains with a similar proportion (9). In our present study with sorted IgM⁺IgD⁺ B cells in RA, N-region addition was extensively higher than in normal controls. There are several possible explanations for the presence of non-germline encoded nucleotides at the V-J junctions of light chains. Most likely, TdT in an amount sufficient to introduce N regions into the light chains junction is expressed from the pro-B cell to pre-B cell stage (26,27). Alternatively, light chain rearrangement can potentially precede heavy chain rearrangement in normal pro-B cells that express TdT (28). Thus, this high proportional N-nucleotides addition of IgM⁺IgD⁺ B cells in RA may result from abnormal regulation of TdT or light chain rearrangement in bone marrow level.

In conclusion, these data suggest that enrichment for N region addition and long CDR3 lengths in RA may be from unusually high or prolonged activity of TdT in bone marrow B lineage cells.

Acknowledgments

We thank Stephanie Bayer for technical assistance.

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