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Complete sequence of the genes encoding the V_H and V_L regions of low- and high-affinity monoclonal IgM and IgA1 rheumatoid factors produced by CD5+ B cells from a rheumatoid arthritis patient

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Abstract

We have characterized the V_H and V_L genes of three low-affinity polyreactive and two high-affinlty monoreactive IgM and lgA1 rheumatoid factor (RF) mAb generated using circulating $CD5^+$ B cells from a single rheumatoid arthritis patient. We found that four and one RF mAb utilized genes of the V_HIV and V_HIII families, respectively. The V_HIV gene usage by these RF mAb differs from the preferential V_HIII, V_HI, and, to a lesser extent, V_HII gene usage by the IgM with RF activity found In patients with mixed cryoglobulinemia, Waldenstrom's macroglobulinemia, and other monoclonal gammopathies. In addition, in contrast to the preponderant χL chain usage by the RF In these patients, a λL chain was utilized by all RF mAb from our rheumatoid arthritis patient. Two RF mAbs utilized V_λI, two V_λIV, and one V_λIII L chains. The V_H genes of the two low-affinity polyreactive IgM RF mAb were in germline configuration. When compared with the deduced amino acid sequence of the putatively corresponding genomic segment, the V_H gene of the high-affinity monoreactive IgM RF mAb displayed five amino acid differences, all of which are in the complementarity determining regions (CDR), possibly the result of a process of somatic point mutation and clonal selection driven by Ag. The unavailability of the corresponding genomic V_H segment sequences made it impossible to infer whether the V_H genes utilized by the two lgA1 RF were in a germline or somatically mutated configuration. Sequencing of the genes encoding the H chain CDR3 (D segments) revealed that all three low-affinity polyreactive RF mAb displayed a much longer D segment (36–45 bases) than their high-affinity monoreactive counterparts (15–24 bases), raising the possibility that a long D segment may be one of the factors involved in antibody polyreactivity.

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Introduction

The ability of sera from patients with rheumatoid arthritis to enhance the agglutination of sheep red cells by specific rabbit antibodies was first recognized more than 40 years ago (1,2). Such a biological activity was later determined to be the property of an unusual serum component termed rheumatoid factor (RF))reviewed in 3,4). RF are autoantibodies reacting with antigenic determinants localized to the $C_\gamma 2$ and $C_\gamma 3$ domains (Fc fragment) of IgG molecules. They are mostly IgM, but they can also be IgG and IgA (3,4). Although RF are abundant in the circulation and in the synovial fluids of rheumatoid arthritis patients, they are also commonly found in other autoimmune diseases, as well as in viral and parasitic infections, chronic inflammatory diseases, some neoplasms, and various hyperglobulinemic states (4–10). Moreover, in humans, rabbits, and mice, circulating RF appear at high titer at various stages of the antibody response to foreign Ag (11–13).

In healthy humans, the precursors of RF-producing cells are mostly surface $CD5^+$ B lymphocytes and account for 10–30% of the normal B cell repertoire (14–19). In rheumatoid arthritis patients, $CD5^+$ B lymphocytes can constitute up to 60% of the circulating B cells, and are spontaneously proliferating and secreting large amounts of RF (17–20). By generating mAb-secreting cell clones using $CD5^+$ B cells from such patients, we showed that these RF are of two functionally discrete types (17,20). The first type displays a low affinity ($K_d \sim 10^{-4}$–10^{-5} M) for the human IgG Fc fragment and also binds other self and exogenous Ag. These polyreactive RF are the most numerous, and are similar to those inducible in $CD5^+$ B cells from healthy subjects (14,16,19) and to the circulating RF occurring in patients with a variety of paraproteinemic disorders (4,19,21–24). The second type of RF displays a much higher affinity ($K_d \sim 10^{-7}$ M) for the IgG Fc fragment and is monoreactive. To our knowledge, these RF have been isolated so far only from patients with rheumatoid arthritis (17,20).

To define the nature of low- and high-affinity RF, we determined the complete sequence of the genes encoding the variable heavy (V_H) and light chain (V_L regions of three polyreactive low-affinity (two IgM and one lgA1) and two monoreactive high-affinity (one IgM and one lgA1) RF mAb generated using $CD5^+$ B cells from a single patient with rheumatoid arthritis. We found that all but one of these RF mAb utilized gene members of the V_HIV family and all of them utilized a λL chain. The V_H genes of the two low-affinity polyreactive IgM RF mAb were encoded in the germline. The V_H genes of the high-affinity monoreactive IgM RF mAb possibly displayed several somatic point mutations distributed in a way consistent with a process of clonal selection driven by Ag. The analysis of the junctional V_H -diversity (D) -joining $(J)_H$ sequences suggested that the nature and length of the D segments may contribute to determine the RF poly- or mono-reactivity.

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Methods

Generation of mAb-secreting cell lines from human CD5+ B cells

Peripheral blood mononuclear cells (PBMC) were obtained at two different times from a Caucasian 58 yr old female patient with clinically active, seropositive rheumatoid arthritis (20). B lymphocytes were enriched from PBMC and $CD5^+$ B cells were purified using a FACS (FacStar Plus, Becton Dickinson & Co., Mountain View, CA) and specific mouse mAb to CD20 and CD5 (Becton Dickinson & Co.), as previously described (14,17,20,25,26). Purified $CD5^+$ B cells were infected with Epstein–Barr virus (EBV) and then immediately distributed in separate microcultures (1000 cells/well) in the presence of irradiated (2000 rad) PBMC as feeders (25–27) EBV transformed B cells from different microcultures were selected, by sequential subculturing, for the production of IgM or IgA binding to the IgG Fc fragment. These cell lines were then stabilized by fusion with F3B6 cells, a human–mouse heterohybnd, as described (16,27,28). The resulting EBV-transformed B cell hybrids were expanded in selection medium and then sequentially cloned at 0.5 cell/ well Clones were amplified and the secreted mAb were prepared as described (16,27,28). IgM and IgA RF were detected using a sensitive and specific ELISA involving purified polyclonal human IgG Fc fragment, as previously described (17,20). Competitive inhibition studies involving binding of the human mAb to the solid-phase IgG Fc fragment by the homologous soluble ligand were used to calculate K_d values (16,26,27).

Analysis of RF mAb V_H and V_λ segments

The cDNA V_H segment probes used in these studies were: 51P1 (370 bp, V_HI) (29), VCE-1 (310 bp, V_HII) (30), 56P1 (460 bp, V_HIII) (29), 58P2 (405 bp, V_HIV) (29), 83P2 (245 bp, V_HV) (29), identical with V_H251, 15P1 (350 bp, V_HVI) (31), and 20P1 (222 bp, V_HIII) (29). The $C\mu$, probe was a ~0.6 kb cDNA encompassing part of the $C\mu3$ and $C\mu4$ domains. These probes were labeled with deoxycytidine [5′-α^{32}]triphosphate (sp. act 3000 Ci/mmol, Amersham, Arlington Heights, IL) by random primer digolabeling (32). The $C_\alpha1$ probe consisted of an 18 base ^{32}P-end-labeled oligonucleotide (5′-TCACACTGAGTGGCTCCT-3′). The C_λ probe consisted of an 18 base ^{32}P-end-labeled oligonucleotide (5′-TGTGTCCTTGTTGGCTTG-3′). The C_χ DNA probe consisted of a genomic 6.5 kb *Bam*HI fragment. Cellular mRNA was isolated from EBV-transformed cell hybrids using the Fast Track kit (Invitrogen, La Jolla, CA). mRNA (2 μg) was slot blotted on Gene Screen Plus membranes (Biotechnology Systems NEN Research Products, Boston, MA) in 50% (v/v) deionized formamide and 6% (v/v) formaldehyde according to the manufacturer's protocol. Blots were hybridized with the different V_H probes and washed as previously described (33) Autoradiography was performed using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

Synthesis of cDNA, and cloning and sequencing of mAb V_H and V_L genes

Total cDNA was synthesized from 5′ poly(A+) mRNA using ohgo(dT) priming and reagents as provided by Pharmacia LKB Biotechnology, Inc. cDNA with *Eco*RI adaptors on both sides was ligated into *Eco*RI-digested, dephosphorylated λgt11 arms (Promega Biotec, Madison, Wl). Recombinant λgt11 was packaged using the Packagene (Promega Biotec) packaging extract and used to construct recombinant libraries. Replica filters (Gene Screen

membranes, Biotechnology Systems NEN Research Products) from each plate were separately hybridized with appropriate ^{32}P-labeled H and L chain gene-specific DNA or oligonucleotide probes. Genuine clones were derived from double positive viral plaques by two rounds of plating and screening. Viral DNA was purified from amplified λgt11 clones using LambdaSorb Phage Adsorbent (Promega Biotec). cDNA inserts were amplified from recombinant λgt11 phage DNA using the polymerase chain reaction (PCR) method and forward and reverse λgt11 specific primers (New England Biolabs, Beverly, MA). After amplification, full-length H chain and L chain cDNA inserts were digested with *Eco*RI, purified, and ligated into *Eco*RI-digested, dephosphorylated pUC 18 vector (Pharmacia LKB Biotechnology, Inc.,). Recombinant pUC 18 plasmids were amplified in DH5α competent *Escherichia coli* cells, purified using Qiagen-pack 100 columns (Qiagen, Inc., Studio City, CA). The V_H and V_λ gene segments were sequenced by the Sanger's dideoxy chain termination method (34), using Taq polymerase (Promega Biotec) and [α-^{35}S]dATP. The comparison of the obtained cDNA sequences and their predicted protein translations with already known DNA sequences was performed using the programs provided by the University of Wisconsin Genetics Computer Group and a VAX11/785 computer.

Isolation of the genomic RF mAb 61 V_H segment

To obtain the genomic V_H segment putatively equivalent to V_H4.18, granulocyte DNA (1–2 μg) from the rheumatoid patient under study was amplified using a 5′ primer corresponding to a part of the V2-1 leader intron (5′-GGGAATTCGTGAATGTTTCTAGGATGCAG-3′) and a primer to the 3′ end of the V2-1 and V_H4.21 segments (5′-GGGAATTCAGTAATACACAGCCGTGTCT-3′). Due to the unavalabihty of the leader intron sequence of V_H4 18, the leader intron sequence of its closest allele, V2-1 (35,36), was chosen to synthesize the putatively specific 5′ primer. Using a Perkin Elmer thermocycler (Perkin Elmer Cetus, Norwalk, CT), 35 cycles of amplification were performed, each cycle consisting of a denaturation step (94°C, 1 min), an annealing step of 2 min at 5°C below the calculated dissociation temperature, and an extension step (72°C, 3 min). After the 35th cycle, a further extension step was performed for 7 min at 72°C in order to increase the percentage of full-length molecules. The PCR product was extracted, ethanol-precipitated, dissolved, and digested with *Eco*RI. The digestion products were separated in a 1.2% agarose gel and DNA fragments of ~400 bp were isolated. These fragments were ligated into *Eco*RI-digested, dephosphorylated pUC 18 vector and used to transform DH5α cells (BRL, Life Technology, Inc.). Plasmids were prepared from colonies yielding products hybridizating with a V_HIV probe (58P2) and sequenced.

Results

Generation and immunochemical characterization of human RF mAb

Five RF mAb were studied. They were produced by monoclonal EBV-transformed B cell hybrids generated from independent microcultures using purified peripheral blood $CD5^+$ B lymphocytes from a single rheumatoid arthritis patient. The construction and immunochemical characterization of three of these RF (mAb 60, 61, and 63: an lgA1, IgM, and IgM, respectively) have been reported previously (20). At that time, RF mAb 60 and 61 had been classified as bearing a χL chain. Further studies using specific high-affinity anti-χ

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and anti-λ antibodies showed that the RF mAb actually utilized λL chains (Table 1). The two RF mAb newly generated for the purpose of this study (mAb 67 and 65, an IgM and an lgA1, respectively) also utilized λL chains (Table 1). Three RF mAb (mAb 63, 67, and 65) displayed a low affinity (K_d ~ 10^{-4}–10^{-5} M) for IgG Fc fragment (Table 1) and were polyreactive, as they bound not only to IgG Fc fragment but also to other Ag, including human thyroglobulin, human insulin, ssDNA, and tetanus toxoid, in general with a low affinity (K_d ~ 10^{-3}–10^{-6} M) (not shown). The remaining two RF mAb (mAb 61 and 60) displayed a two orders of magnitude higher affinity for IgG Fc fragment (K_d ~ 10^{-7} M) (Table 1) and were monoreactive, as they bound to IgG Fc fragment but to none of the other Ag tested (20; and results not shown).

Utilization of V_H and V_L chain genes by the RF mAb

Using slot blot analysis of the mRNA, the three polyreactive RF mAb, mAb 63, 67, and 65, and one of the two monoreactive RF mAb, mAb 61, were found to utilize a member of the V_HIV gene family (Table 1). The other monoreactive RF mAb, mAb 60, utilized a member of the V_HIII family (Table 1). Consistent with the results of the immunochemical experiments, the incubation of mRNA from the five RF mAb-producing cell clones with a [α^{32}P]dCTP-labeled genomic C_χ DNA and V_χ-specific cDNAs, and a ^{32}P-end-labeled C_λ-specific oligonucleotide resulted in strong hybridization with the latter, but not the C_χ or V_χ-specific probes.

Cloning and sequencing of the V_H genes

cDNA libraries were screened using the appropriate C_H DNA probe and the V_H DNA probe from the family assigned in the slot blot experiments. Double positive λgt11 phage clones with at least 1.5 and 1.8 kbp length inserts in the case of lgA1 and IgM, respectively, were sequenced in pUC18 vectors. All clones but one, mAb 65, proved to be full length (Fig. 1A). Their sequences were compared with published V_H gene sequences. Eighty percent similarity was used as the critenon for assignment to a V_H gene family.

The low-affinity polyreactive RF mAb 63 V_H segment displayed 97.20% similarity with the V58 genomic V_HIV sequence reported by Lee *et al.* (35). Further comparison, however, of the RF mAb 63 V_H segment sequence with a genomic allele of V58, V_H4.21, recently reported by Sanz *et al.* (36), resulted in absolute nucleotide homology (Fig. 1A). Comparison of the RF mAb 67 V_H gene segment with the (V_HIV) V79 germline sequence (35) yielded only two nucleotide differences (99.33% similarity), in positions 43 and 284, resulting in two amino acid variations (Fig. 1B). The slight departure from the germline of this expressed V_H gene was most likely due to the usage of a different V79 allele (36). In contrast, RF mAb 65, the low-affinity polyreactive lgA1 RF mAb, displayed only 87.30% similarity when compared with V71-2 (35), the closest identifiable gene among the members of the V_HIV family (Fig. 1A). Three and nine nucleotide differences were found in the complementarity determining region 1 (CDR1) and CDR2, respectively, whereas 20 differences were found in the framework regions (FR). These differences resulted in six amino acid variations in the CDR and seven in the FR when compared with the deduced protein sequence of V71-2 (Fig. 1B).

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When compared with the genomic V_H4.18 sequence (36), the V_H gene sequences of the high-affinity monoreactive IgM RF mAb 61 displayed seven nucleotide differences, resulting in 97.64% similarity (Fig. 1 A). Such nucleotide differences yielded five amino acid variations, all in the CDR1 and 2 (Fig. 1B). The sequence of the V_H gene of the second high-affinity monoreactive RF mAb, mAb 60, an lgA1, displayed 92.10% similarity to the germline V_H11 gene, a member of the V_HIII family isolated by Rechavi *et al* (37) The 23 nucleotide differences yielded five and seven ammo acid variations in the CDR and FR, respectively (Fig. 1B).

Having established that the two low-affinity polyreactive IgM RF mAb (mAb 63 and 67) V_H segments were in germline configuration, we investigated whether the V_H segment utilized by the high-affinity monoreactive IgM RF mAb 61 was the expression of a somatically point mutated gene Granulccyte DNA from the same patient used for the generation of the RF mAb was primed with a V_HIV 5′oligonucleotide [priming the V2-1 gene from the 5′ end (leader intron), as the sequence of the V_H4.18 leader is not available] and a V_HIV 3′ oligonucleotide [priming the V2-1 and the identical V_H4.18 segments from the 3′ end (nucleotides 270–290)] and amplified by PCR (see Methods). Among the V_HIV family members, the leader intron primer recognized only the V2-1 segment, due to the three unique nucleotide variations of V2-1 when compared with all the other V_HIV members (35,36). The 3′ V_HIV primer encompassed a sequence identical in both V2-1 and V_H4.18 and different in two nucleotides from any other known member of the V_HIV family (35,36). In two different experiments, six independent clones were isolated. All of them yielded nucleotide sequences identical with the published V_H4.18 (Fig. 1A: MLH4-1) genome segment (36), suggesting that the V_H gene encoding the high-affinity monoreactive IgM RF mAb 61 consists of a somatically point mutated form of the V_H4.18 gene.

The high number of still uncharacterized members of the V_HIII family (29,37,38–40) made it difficult to determine whether lgA1 RF mAb 60 utilized a mutated V_H11 gene or a different germline V_HIII member.

The D gene segments

The D segments utilized by the five RF mAb were very heterogeneous and could be accounted for only partially by previously reported germline D segments (41–48). The D gene segments of RF mAb 63, 67, and 65 were 45, 45, and 36 bp, respectively, in length. The core 31 nucleotides of the D gene encoding the RF mAb 63 were virtually identical with those of the genomic DXP4 D segment (45) (Fig. 2A). A stretch of 21 of these nucleotides was also utilized in the mAb 67 D segment (Fig. 2A). RF mAb 65 D segment displayed some homology with DxP1, a genomic D segment reported by Ichihara *et al.* (45) and identical with D21/0.5, a genomic D segment identified by Buluwela *et al.* (46). In addition, the sequence of the first half of the RF mAb 65 D gene displayed some similarity to the reverse complement of the DLR-5 gene (45). The D genes utilized by RF mAb 61 and 60 were only 24 and 15 bp in length respectively (Fig. 2A), and displayed some similarity with the genomic DLR1 sequence originally reported by Siebenlist *et al.* (41). The deduced protein sequences of the D genes of the RF mAb are reported in Fig. 2(B).

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The J_H segments

RF mAb 63 and 67 utilized germline J_H4 segments in a truncated and complete form, respectively (Fig. 2C). The only and identical variation displayed by both RF mAb, a G instead of an A, was silent at the protein level and has been recently found in several expressed J_H4 genes (29). RF mAb 65 and mAb 61 utilized truncated forms of the J_H6 segment. Assuming that the difference of a G with a C displayed by the two RF mAb was merely due to the expression of a J_H6 polymorphic allele, then mAb 61 displayed only one nucleotide difference, a G instead of T (resulting in the variation of a Tyr with an Asp), when compared with the J_H6 germline sequence (Fig. 2C and D). The RF mAb 65 J_H segment displayed six nucleotide differences when compared with the germline J_H6 sequence, resulting in four amino acid substitutions (Fig. 2C and D). Finally, RF mAb 60 utilized a full J_H5 segment displaying eight nucleotide differences when compared with the germline J_H5 sequence and resulting in two ammo acid variations (Fig. 2C and D).

Cloning and sequencing of the V_L genes

The γgt11 libraries constructed for the cloning of the H chain genes were screened with C_λ segment-specific ^{32}P-labeled oligonucleotide. The phages containing a fragment of at least 800 bp were amplified for insertion and sequencing in pUC18 vector. All the clones were full length (Fig. 3A). Both RF mAb 67 and 61 utilized a V_λI gene (Table 1 and Fig. 3A). RF mAb 61 V_λI gene displayed 11 nucleotide differences (96.26% similarity) when compared with 1B9/F2, a gene of the V_λI subgroup expressed by a Burkitt's lymphoma B cell line (49). RF mAb 67 showed six of the same 11 nucleotide differences and displayed two other differences compared with the 1B9/F2 sequence (Fig. 3A). The deduced V_λ amino acid sequences of the RF mAb 67 and 61 were identical, except for two amino acid variations, an Ala and Gly instead of a Thr and Ala in positions 91 and 98, respectively (Fig. 3B). Thus, these RF IgM mAb probably utilized the same novel allelic variant of V_λI in near germline configuration, as further suggested by the complete identity of the leader sequences of these expressed genes (Fig. 3A). The deduced protein sequences of these V_λI genes shared a high degree of similarity (92.86%) with the deduced sequence of the expressed 1B9/F2 V_λ gene and with the V_λ chain of the WAH myeloma protein (91.67–92.71%) (50) (Fig. 3B).

The low-affinity IgM RF mAb 63 and lgA1 RF mAb 65 utilized V_λ genes 95.80 and 96.85% similar, respectively, to the expressed p3C4λ5 gene (Fig. 3A), originally identified in an IgM produced by the H6-3C4 human–mouse heterohybridoma and belonging to the V_λIV subgroup (containing at least eight members) (51). The deduced protein sequence of RF mAb 63 and 65 V_λ chains were virtually identical and displayed 90.53 and 91.58% similarity, respectively, to the deduced sequence of the expressed p3C4λ5 gene (Fig. 3B). They also displayed a high degree of similarity (87.37–92.63%) to the amino acid sequence of the Sh λ type Bence–Jones protein (52) and the amyloid AL GIL protein (53) (Fig. 3B).

The V_λ segment of the high-affinity RF lgA1 mAb 60 displayed a nucleotide sequence with little similarity with any of the V_λ sequences available in the literature (Fig. 3A). In fact, the highest degree of similarity found was only 72.73%, to the V_λ chain of pC34λ5 (V_λIV subgroup) (not shown). However, the deduced protein sequence of the RF mAb 60 V_λ segment displayed a higher degree of similarity (80.65%) with the Bau λ (Bence–Jones)

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protein, a member of the V_{λ}III subgroup (54) (Fig. 3B) Compared with this, the RF mAb 60 V_{λ}III segment displayed a high number of amino acid differences, 14 out of a total of 18 ammo acids, in the CDR.

The J_{λ} segments

Three of the five RF mAb utilized $J_{\lambda}2$ segments (Table 1 and Fig. 3C) (55). RF mAb 61 displayed one nucleotide difference, resulting in variation of the second amino acid, a Val with an lieu, when compared with the translated form of the genomic $J_{\lambda}2$ (Fig. 3D). An identical variation has been reported in the $J_{\lambda}2$ segments of two different Bence–Jones λ proteins, Bau and Tro (54,56), suggesting that these RF mAb utilized an allelic variant rather than a somatically mutated form of $J_{\lambda}2$. The nucleotide differences in the RF mAb 67 $J_{\lambda}2$ resulted in the substitution of the first amino acid, a Val, with a Trp (Fig. 3C and D), whereas the two nucleotide differences of RF mAb 60 $J_{\lambda}2$ were silent (Fig. 3C and D). Finally, both RF mAb 63 and 65 utilized a $J_{\lambda}1$ segment in complete germline configuration (Fig. 3C and D).

Discussion

Rheumatoid arthritis is a systemic autoimmune disease of unknown etiology. The central clinical feature of the disease is a chronic inflammatory synovitis, an expression, in part, of immune complex pathology. Antj-IgG RF autoantibodies with high complement-fixing potential are the main constituents of such immune complexes (3–5). In rheumatoid arthritis patients, high- and low-affinity IgM, IgA, and, most likely, IgG RF autoantibodies are produced by actively proliferating $CD5^{+}$ B cells (20). High titers of RF are associated with high disease activity and extensive extra-articular (systemic) pathology (3), suggesting that RF are a major component in the pathogenesis of rheumatoid arthritis. Due to the difficulties in constructing human mAb-producing cell lines of defined specificity, the structural features of RF have been mostly inferred so far, and perhaps improperly, from the study of low-affinity IgM RF mAb isolated from individuals with monoclonal gammopathies, such as mixed cryoglobulinemia or Waldenstrom's macroglobulinemia (24). Very few of these patients had manifestations of rheumatic disease. We report here the complete nucleotide sequence of the V_H and V_L segments of not only low affinity (polyreactive) RF mAb but also of high-affinity (monoreactive) IgM and lgA1 RF mAb derived from a rheumatoid arthritis patient.

The IgG Fc fragment-binding mAb we studied are different in genetic composition from cryoglobulinemic and Waldenstrom's RF, as well as from a series of IgM RF isolated from synovial tissue and recently sequenced by Pascual *et al.* (62). The latter ones have been consistently shown to utilize V_H segments, mostly in germline configuration, of the V_HIII, V_HI, and, to a lesser extent, V_HII gene families (22,24,57–62). These are the largest in the human, containing ~30, 20, and 15 members, respectively (29,38,39). Four of the five RF mAb we generated from a single rheumatoid arthritis patient by selection for binding to IgG Fc fragment utilized four members of the relatively small (about nine members) V_HIV family (35,36). This is not likely to be due to a biased tropism of EBV for V_HIV-expressing antibody-producing cell precursors, as (i) others (63–65) and we (33,66,67; H. Ikematsu *et*

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al., in preparation) have generated by EBV-transformation a number of mAb-producing B cell lines expressing V_H genes other than those of the V_HIV family; and (ii) in these very experiments, we generated, using the same methodology, a mAb-producing cell line expressing a V_HIII family member (RF mAb 60) Expression of V_HIV family genes has been reported in at least 50% of CD5$^+$ chronic lymphocytic leukemia (CLL) B cells (63), the neoplastic equivalent of normal CD5$^+$ B lymphocytes, and, in addition, in some clonally related CLL CD5$^-$ B cells producing IgM cryoglobulins with strong anti-IgG binding (RF) activity (68). Other human mAb with defined binding activity utilizing members of the V_HIV family include three mAb reported by Sanz *et al.* (66). These, an IgM, an lgG3, and an lgA1, were generated using CD5$^+$ B cells from healthy subjects and, similar to some of the RF mAb reported here, were polyreactive and displayed a high K_d (low affinity) for IgG Fc fragment. Polyreactive 'RF' mAb from both healthy subjects and rheumatoid patients identify with the Ig that have been previously termed 'natural antibodies' (17,19,67).

The utilization of V_λ L chains by the present polyreactive and monoreactive RF mAb contrasts with the highly biased utilization of V_χ L chain, in most cases of the V_χIIIb subgroup (product of the germline V_χ 325 gene), by monoclonal RF paraproteins and Ig produced by CD5$^+$ CLL and small lymphocytic leukemia B lymphocytes (69–74). It has been speculated that, at least in some cases, the V_χIIIb chain would provide the structural basis for the binding to the IgG Fc fragment (71). The present experiments showed that RF activity can be mediated by Ag-binding sites involving V_λI, V_χIII, and V_χIV gene products. The possibility that V_χ chains underlie certain autoimmune responses would be further supported by the findings that λL chains are predominantly utilized by anti-laminin IgG autoantibodies in SLE patients (75), by the anti-TSH receptor autoantibodies produced by plasma cells infiltrating the thyroid tissue in patients with Graves' disease (76), and by the autoantibodies produced by plasma cells infiltrating the salivary gland in patients with Sjogren's syndrome (77).

Although limited to a single rheumatoid patient, our experiments showed that low-affinity polyreactive RF are encoded in the germline. In addition, although they do not rule out the possibility that selected combination of germline V_L and V_H genes may encode monoreactive high-affinity RF, they suggested that high-affinity monoreactive RF can display somatic point mutations. Absolute proof that mAb 61 V_H gene segment is somatically mutated would require differential Southern hybridization of specific oligonucleotides, encompassing the putatively mutated CDR, with the patient's genomic DNA. RF mAb 61 V_H somatic point-mutations are distributed in a way that is consistent with an Ig-receptor-dependent selection of these mutations. In the specific immune response to a defined Ag, such a selection is Ag-dnven and yields an oligoclonal B cell population producing high-affinity antibodies (78,79). Thus, the high affinity and genetic composition of the monoreactive RF mAb 61 in our rheumatoid arthritis patient suggest that this autoantibody could not result from a mere polyclonal B cell activation (80), and may mimic the affinity and the selective point mutations of the specific antibodies induced in a 'mature' Ag-driven response (78,79). Similar features apply to the RF and anti-DNA autoantibody response in autoimmune MRL/lpr mice (81,82), and contrast with the low affinity and

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germline configuration of (possibly, poly-reactive) ‘RF’ induced by LPS-activation of B cells in normal mice (83,84).

The heterogeneity of D segments expressed by the RF mAb exemplifies the high complexity of this genetic element in man. It has been suggested that the departure from the germline of the D segments expressed in human mAb often results from mechanisms of site-specific recombination or recombination following unconventional rules of gene conversion, putatively yielding D–D fusions (63,85). Along these lines, the long D segments utilized by the low-affinity polyreactive RF mAb possibly resulted from the expression of a given D segment, variously complemented by other D genes, by either deletion or inversion mechanisms. Moreover, the predominance of C and G over A and T, at both ends, suggested that, at least in part, these D genes could have been generated through N segment addition by terminal deoxytransferase (86). The difference in length between the D segments utilized by the polyreactive RF mAb (36 and 45 bases) and those utilized by their monoreactive high-affmity counterparts (15–24 bases) raises the issue of whether the configuration of the D segment may contribute to antibody poly- or monoreactivity. The possibility that a long D segment may be a prerequisite for antibody polyreactivity would be further supported by the length, 28–60 (average 45) bases, of the D segments of other polyreactive natural antibodies of various Ig classes that we have also generated from $CD5^{+}$ B cells (66). Recent three-dimensional X-ray crystallography data show that the D gene product (CDR3) plays a major role in Ag capture, particularly in antibodies binding protemic Ag (87,88). Although polyreactivity is most often associated with low affinity, as apparent from our present and previous studies (16,19,20,26,63,65), some polyreactive antibodies display a relatively high affinity for certain Ag, as best exemplified by the IgM mAb to ssDNA we generated from healthy subjects and SLE patients (16,26,27). Along these lines, it is possible that the low-affinity polyreactive (for IgG Fc fragment) RF mAb 65 arose in response to and displayed a much higher affinity for an as yet unidentified, possibly exogenous, Ag.

Finally, our findings may question the assumption that $CD5^{+}$ B lymphocytes are primordial cellular elements producing only low-affinity polyreactive antibodies, and suggest that, in some cases, these cells are capable of a ‘maturation’ process yielding high-affinity antibodies (89). The nature and complexity, unveiled here, of the V_H and V_L genes expressed by rheumatoid $CD5^{+}$ B lymphocytes may help to further our understanding of this important subset of the human B cell repertoire.

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Abbreviations

CLL	chronic lymphocytic leukemia

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CDR	complementarity determining region
D	diversity segment of Ig
EBV	Epstein–Barr virus
FR	framework region
H	heavy chain
J	joining segment of Ig
L	light chain
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
RF	rheumatoid factor
UT	untranslated nucleotide sequence
V	variable region of Ig

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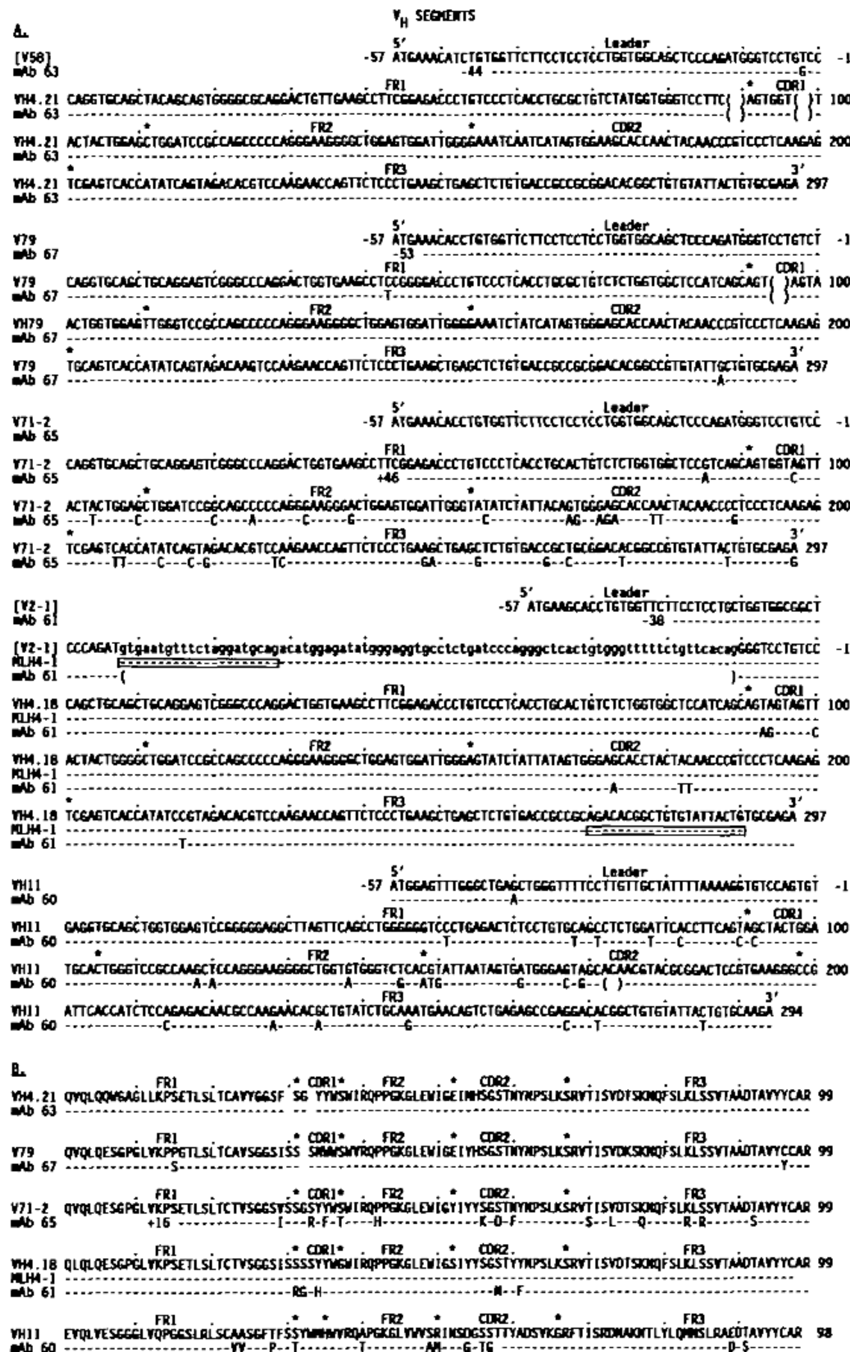

Fig. 1.

(A) Nucleotide sequence of the V_HIV and V_HIII genes utilized by the RF mAb. The top sequence in each cluster is used for germline comparison. Identities are indicated by dashes Asterisks indicate the boundaries of the CDR. The V58, VH4.21, V79, V71-2, and V_H4.18 genes are members and alleles of the V_HIV family (35,36). Parentheses in the V_H4.21 and V79 sequences denote deletions in these genes when compared with another member of the V_HIV family, V71-2 (35). Due to the unavailability of the leader sequences of V_H4.21 and V_H4.18, the leader sequences of their most similar published germline segments, [V58] and

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[V2-1], respectively, are provided. MLH4-1 is the sequence we obtained by targeted PCR amplification of the genomic DNA from the patient under study (boxes depict the sequence and the complementary sequence of the 5′ and 3′ primers, respectively, utilized in these experiments). Notice its perfect identity with the genomic V_H4.18 allele reported by Sanz *et al.* (36). Small letters denote the leader intron sequence of the V71-2 gene The V_H11 gene is a member of the V_HIII family (37). (B) Deduced amino acid sequences from the above nucleotide sequences. Identities are indicated by dashes. Blank spaces represent deletions. The new V_H nucleotide sequences presented here are available from EMBL/GenBank/DDBJ under the following accession numbers: mAb60, X54435; mAb61, X54437; mAb63, X54441; mAb65, X54443; mAb67, X54445; and MLH4-1, X54447.

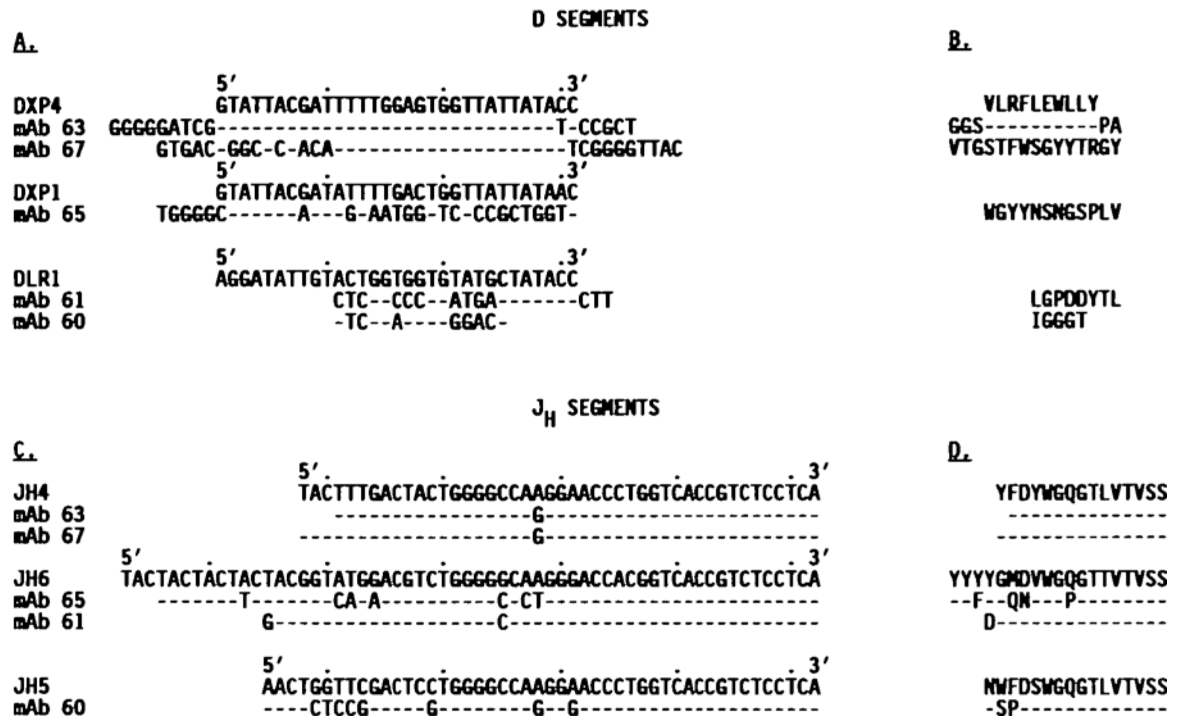

Fig. 2.

(A) Nucleotide sequences of the D segments utilized by the RF mAb. The top sequence in each cluster is used for germline comparison Identities are indicated by dashes (B) Deduced ammo acid sequences from the above nucleotide sequences Identities are indicated by dashes (C) Nucleotide sequences of the J_H segments utilized by the RF mAb The top sequence in each cluster is used for germline comparison. Identities are indicated by dashes. (D) Deduced amino acid sequences of the J_H segments Identities are indicated by dashes. The new nucleotide sequences presented here are available from the EMBL/GenBank/DDBJ under the accession numbers listed in the legend to Fig. 1.

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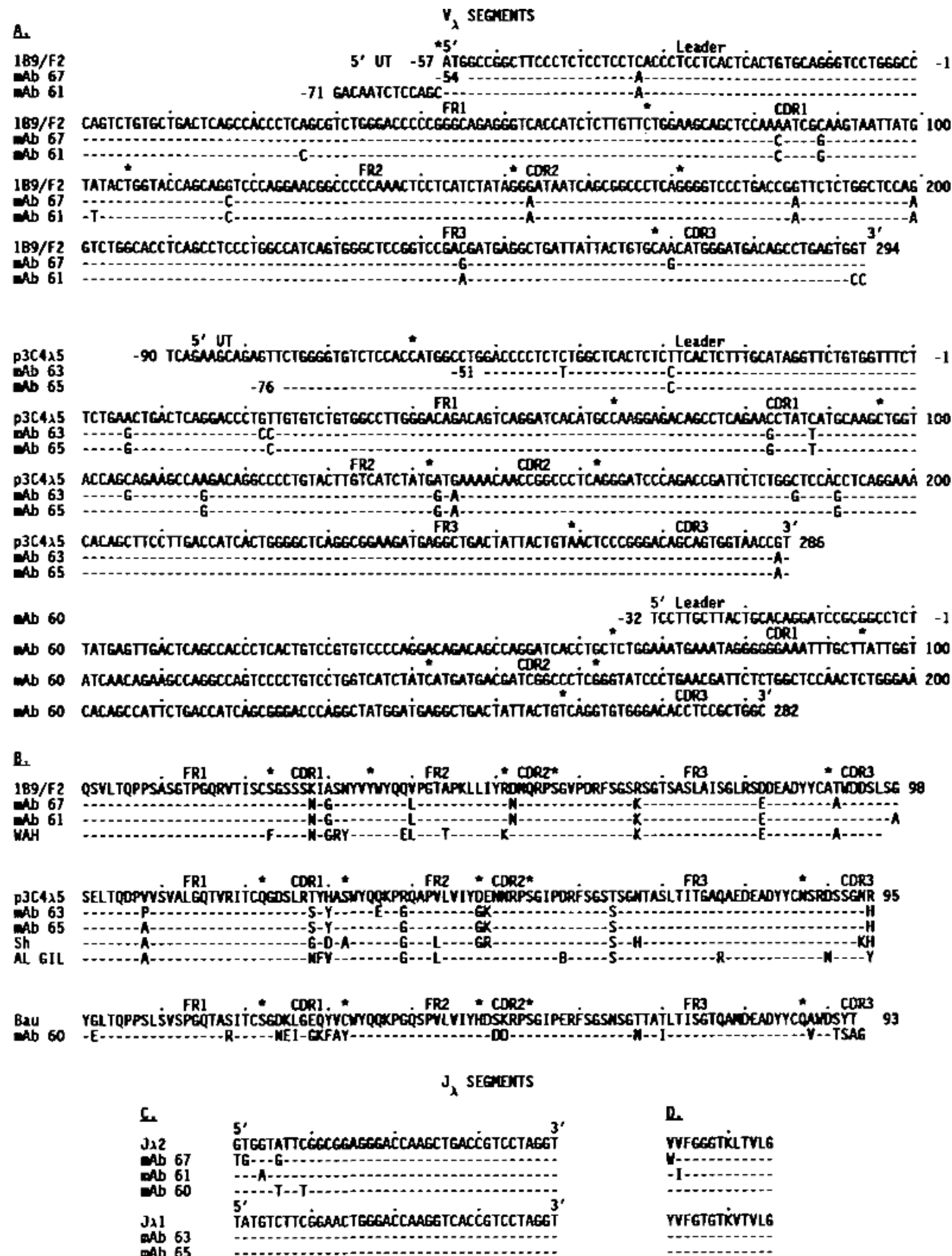

Fig. 3.

Sequences of the V_λ genes utilized by the RF mAb. The top sequence in each duster is used for comparison. Identities are indicated by dashes. Asterisks indicate the boundaries of the CDR and the 5′ untranslated regions (UT) regions. No term of comparison is given for the RF mAb 60, V_λIII gene. (B) Deduced amino acid sequences from the above nudeotide sequences. Identities are indicated by dashes. (C) Nudeotide sequences of the J_λ segments utilized by the RF mAb. The top sequence is each cluster is used for germline comparison Identities are indicated by dashes. (D) Deduced amino acid sequence of the J_λ segments.

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Identities are indicated by dashes. The new nudeotide sequences presented here are avaiable from the EMBL/GenBank/DDBJ under the following accession numbers: mAb60, X54436; mAb61, X54438; mAb63, X54442; mAb65, X54444; and mAb67, X54446.

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Table 1

H and L chains and K_d for IgG Fc fragment of RF mAb generated using discrete CD5+ B cells from a single patient with rheumatoid arthritis

RF	Bleeding[1]	H chain	V_H gene family	D gene segment	J_H gene segment	L chain	V_λ gene subgroup	J_λ gene segment	Ag reactivity	K_d (M) for IgG Fc fragment
mAb 63	A	μ	V_HIV	DXP4	J_H4	λ	V_λIV	J_λ1	Polyreactive[3]	4.0×10^{-5}
mAb 67	B	μ	V_HIV	DXP4[2]	J_H4	λ	V_λI	J_λ2	Polyreactive	4.2×10^{-5}
mAb 65	B	α1	V_HIV	DXP1[2]	J_H6	λ	V_λIV	J_λ1	Polyreactive	$1\ 2 \times 10^{-4}$
mAb 61	A	μ	V_HIV	DLR1[2]	J_H6	λ	V_λI	J_λ2	Monoreactive[3]	6.0×10^{-7}
mAb 60	A	α1	V_HIII	DLR1[2]	J_H5	λ	V_λIII[4]	J_λ2	Monoreactive[3]	20×10^{-7}

[1] Time of bleeding for the generation of monoclonal cell lines: A, 04-06-1987; B, 10-20-1988.

[2] D genes displaying only limited similarity.

[3] The Ag-binding activity and K_d values for IgG Fc fragment of these mAbs have been reported previously (20) mAb 63, 61, and 60 were previously designated as mAb 274.RA.F11, 274.RA.F4, and 274.RA.F1, respectively.

[4] V_λ group assignment based on the deduced amino acid sequence.

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