Genetic Analysis of Self-associating Immunoglobulin G Rheumatoid Factors from Two Rheumatoid Synovia Implicates an Antigen-driven Response

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Summary

Although much has been learned about the molecular basis of immunoglobulin M (IgM) rheumatoid factors (RFs) in healthy individuals and in patients with mixed cryoglobulinemia and rheumatoid arthritis, little is known about the genetic origins of the potentially pathogenic IgG RFs in the inflamed rheumatoid synovia of patients. Recently, we generated from unmanipulated synovium B cells several hybridomas that secreted self-associating IgG RFs. To delineate the genetic origins of such potentially pathogenic RFs, we adapted the anchored polymerase chain reaction to rapidly clone and characterize the expressed Ig V genes for the L1 and the D1 IgG RFs. Then, we identified the germline counterparts of the expressed L1 IgG RF V genes. The results showed that the L1 heavy chain was encoded by a Vh gene that is expressed preferentially during early ontogenic development, and that is probably located within 240 kb upstream of the Jh locus. The overlap between this RF Vh gene and the restricted fetal antibody repertoire is reminiscent of the natural antibody-associated Vh genes, and suggests that at least part of the "potential pathogenic" IgG RFs in rheumatoid synovium may derive from the "physiological" natural antibody repertoire in a normal immune system. Indeed, the corresponding germline Vh gene for L1 encodes the heavy chain of an IgM RF found in a 19-wk-old fetal spleen. Furthermore, the comparisons of the expressed RF V genes and their germline counterparts reveal that the L1 heavy and light chain variable regions had, respectively, 16 and 7 somatic mutations, which resulted in eight and four amino acid changes. Strikingly, all eight mutations in the complementarity determining regions of the V gene–encoded regions were replacement changes, while only 6 of 11 mutations in the framework regions caused amino acid changes. Combined with L1's high binding affinity toward the Fc fragment, these results suggest strongly that the L1 IgG RF must have been driven by the Fc antigen.

Rheumatoid arthritis (RA)[2] is an extravascular immune complex disease of unknown etiology (1). The diagnostic autoantibody in most RA patients is termed rheumatoid factor (RF); this antibody bind to the Fc region of IgG molecules (2). Studies have shown that synovial fluid from the inflamed joints in RA patients contains abundant aggregates of Ig, and depressed levels of complement components. The aggregates consist of mainly IgG and RFs, which are synthesized and deposited in situ (3, 4). These findings suggest that RFs may contribute to immune complex formation, complement consumption, and chronic tissue damage in the rheumatoid synovium (5).

However, similar to other "natural" autoantibodies, RFs are also found routinely in apparently healthy individuals; such RFs generally are polyspecific, low affinity, and belong to the IgM isotype (6–10). Structural and molecular analyses of such IgM RFs from CD5$^+$ B cells of normal subjects,

[1] E. W. Lu made an equal contribution to this work and thus is considered as a co-first author.

[2] *Abbreviations used in this paper:* APCR, anchored polymerase chain reaction; FR, framework region; RA, rheumatoid arthritis; RF, rheumatoid factor.

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as well as those from patients with mixed cryoglobulinemia, have revealed that they are encoded by a restricted set of Ig variable (V) region genes with no or a few somatic mutations, i.e., up to eight amino acid substitutions per 96–98 residues in a light (L) or heavy (H) chain V region (11–15).

In contrast to the natural RFs in normal individuals, the RFs in RA patients, particularly those found in the inflamed joints, contain both IgM and IgG isotypes. Also, in addition to the low affinity and polyspecific RFs, RA patients have high affinity and monospecific RFs (2, 16, 17). Thus, to understand the role of RFs in the pathogenesis of RA, it is important to study the latter RFs, particularly the IgG RFs found mainly in rheumatoid synovium. What are the genetic bases of IgG RFs? Do they use V genes similar to those used by IgM RFs in normals and patients with mixed cryoglobulinemia? What are the inducing and/or sustaining factors for the disease-specific IgG RFs in patients? Are these antibodies driven nonspecifically by mitogens or specifically by IgG and/or unknown crossreacting antigens?

To delineate the underlying mechanisms for IgG RF synthesis in RA patients, we now report the molecular characterization of the L1 IgGγ and the D1 IgGk RFs (18). These RFs bind specifically to the human Ig Fc fragment with high affinity, as indicated by their estimated dissociation constants (K_d) of 4.1 × and 5.2 × 10^{-7} M, and, at 500 μg/ml, self-associate into homodimers. Thus, these IgG RFs have the potential to form large aggregates which are then deposited in extravascular sites, and thus trigger the inflammatory pathways leading to chronic tissue damage. In addition to defining the RF H and L chain cDNA (designated HumhalL1, HumlalL1, Humha3d1, and Humka3d1, respectively), we also isolated the L1-RF-corresponding germline V genes (designated Humhv1L1 and Humlv1L1, respectively). Interestingly, the hv1L1 germline gene is almost identical to 20P3, a cDNA found in a fetal liver, and to the V35 gene located within 240 kb upstream of the Jh locus (19–21). The data are reminiscent of the natural antibody-associated Vh genes (15), and suggest that at least part of the "potential pathogenic" IgG RFs in rheumatoid synovium may derive from the "physiological" natural antibody repertoire in a normal immune system. Indeed, hv1L1 is 99% homologous to the Vh sequence of the ML3 IgM RF found very recently in a 19-wk-old fetal spleen (22). In addition, the patterns of mutations were not random and suggested that the L1 IgG RF was highly likely to be driven by the antigen Fc fragment. Combined, these data implied that some IgG RFs in RA patients may come from natural IgM RFs of a normal immune system, possibly be escaping from the normal regulation during a transient breakdown of the immune system and/or overloading of the system with immune complexes.

Materials and Methods

DNA and RNA Isolation from the L1 and the D1 IgG RF-secreting Hybridomas and RA Patient ML. The L1 and D1 hybridomas were derived, respectively, from synovial cells of the seropositive RA patients ML and AD, who satisfied the 1987 American Rheumatism Association (American College of Rheumatology) criteria for RA (18, 23). The mAbs react strongly with the Fc fragment of human IgG, but not other six unrelated antigens, including human collagen VI, BSA, chicken OVA, KLH, tetanus toxoid, and calf thymus single-stranded DNA (18). The mRNA was isolated from the hybridoma with the Extract a Gene Kit (Oncor, Gaithersburg, MD) according to the manufacturer's instructions. Genomic DNAs were prepared from both hybridoma and PBL of patient ML.

Rapid Cloning of the RF γ H, λ and κ L chain V Regions (Vh, Vλ, and Vκ) cDNA by Anchored PCR (APCR) (24, 25). Recently, a synovium-derived RF was found to use a novel Vλ 8 gene (26). To avoid any possible problems with unknown V gene families and to clone rapidly the expressed V genes in the hybridomas, we adapted the reported APCR method (25). Briefly, the first-strand cDNA was synthesized from the hybridoma mRNA with an oligo(dT) primer and murine leukemia virus reverse transcriptase, and a poly(dG) tail was added to the 3′ end of the cDNA with terminal deoxynucleotidyl transferase. Thereafter, the tailed cDNA was amplified with two 5′ universal primers and a 3′ primer for either the γ H chain, the λ L chain, or the κ L chain. The 5′ primers were the AN (anchor) primer (5′-CACGT-CGACC-TAGGC-GGCCG-CGG) and the ANpolyC primer (5′-*AN*-CCCCC-CCCCC-CCCC, where *AN* stand for the AN primer sequence) (25); they were used at the 9:1 ratio. The 3′ primers for the γ, the λ, and the κ chain, respectively, were the GCc primer (where G, C, and c stand for the gamma chain constant region, and the complementary strand; 5′-GTAGT-CCTTG-ACCAG-GCA, complementary to nucleotides 474–457 in Fig. 1), the LCc primer (where L stands for lambda chain; 5′-AGTGT-GGCCT-TGTTG-GCTTG, complementary to nucleotides 410–391 in Fig. 2), and the KCc primer (where K stands for kappa chain; 5′-GAAGA-TGAAG-ACAGA-TGGTG-C, complementary to nucleotides 354–334 in Fig. 6). These three primers were designed to prime all γ, λ, and κ chains. Either a BamHI or a PstI site was added to the 5' end of these three primers.

To the tailed cDNA and the appropriate primers, all four deoxynucleotide triphosphates and Taq polymerase were added, and the mixture was amplified for 30 cycles. Each cycle consisted of a 45-s denaturation at 94°C, a 45-s primer annealing at 46°C, and a 1-min (except for a 7-min in the last cycle) extension at 72°C. The amplified products were visualized by running 10% (10 μl) of the reaction on a 1% agarose gel. In some cases, to improve the quantity and quality of the desired γ and κ chain gene products, the tailed cDNA was first amplified, respectively, with two additional downstream primers, GC1c and KC1c (complementary to sequences marked in Figs. 5 and 6), and the amplified product of the expected size was enriched from the agarose gel and re-amplified with GCc and KCc.

PCR-based Cloning of the Rearranged Vh and Vλ Genes (designated HumhalL1 and HumlalL1) and Their Germline Counterparts (Designated Humhv1L1 and Humlv1L1). To amplify the L1-rearranged Vh gene, 1 μg of hybridoma DNA was mixed with 100 pmol each of the appropriate primers in a buffer containing 1.5 mM Mg^{2+}. The upstream primer ha1L1U1s (where U and s denote upstream and sense strand, respectively) correspond to a sequence in the 5′ untranslated region, and the downstream primer Jh3c was complementary to a portion of Jh3 (marked in Fig. 1). For isolating the germline gene counterpart, germline DNA from the PBL of patient ML was amplified with the upstream ha1L1U1s primer and a new downstream primer, V35D1c (complementary to sequence from positions 336 to 317 in the V35 gene, marked in Fig. 3) (21). In all cases, the restriction endonuclease recognition sequences for SalI and PstI were present at the 5′ ends of the upstream and downstream primers, respectively. For amplification, the DNA was mixed with the indicated primers, all four deoxynucleotide triphosphates,

and Taq polymerase, and the mixture was incubated for 30 cycles. Each cycle consisted of 1 min melting at 94°C, 1 min annealing at 55°C, and 1.5 min (7 min during the last cycle) elongation at 72°C. After amplification, the PCR products were analyzed by gel electrophoresis.

Similarly, the rearranged L1 Vλ gene and its germline counterpart were amplified with appropriate primers. The rearranged L1 Vλ gene was amplified with the 5′ primer (la1L1U2s, corresponds to a stretch in the 5′ untranslated region) and the 3′ primer (la1L1C3c, complementary to the 3′ end of CDR3 and the first three bases of the fourth framework region (FR4) (Fig. 2). To isolate the la1L1 germline gene counterpart, la1L1U2s was paired with a series of downstream primers, corresponding to various stretches in the 3′ end of the V gene–encoding region of the la1L1 sequence and in the immediate 3′ flank of the reported Humlv117 Vλ1 gene (27). The results showed that two downstream primers yielded clean bands of the expected sizes. They were la1L1C3c1 and lv117D1c, complementary to regions marked in Fig. 4.

Cloning and Sequencing of the Amplified DNA. The amplified DNA was digested with the appropriate enzymes, and was cloned into M13mp8 (28). For the H and the L chain cDNAs, the recombinant phages were screened with the GC3s and the LC4s primers, respectively (marked in Figs. 1 and 2). The hybridization was done in 6× SSPE (20× SSPE is 3.6 M NaCl, 200 mM sodium phosphate, 20 mM EDTA, pH 7.4) containing 5× Denhart's solution (1× is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), at 55°C. Hybridized filters were washed with 6× SSPE four times at room temperature for 15 min each and the positive clones were visualized by autoradiography. Single-stranded DNA was prepared from the chosen M13 clones, and the inserted DNA was sequenced by the method of Sanger (28a) using the universal M13 sequencing primer. Because of possible base infidelity generated during PCR amplification, each of the presented sequences represents either at least two completely identical sequences from the amplified DNA or a consensus sequence from at least three 99% homologous sequences. The computer programs of the University of Wisconsin Genetics Computer Group were used to assemble, edit, and analyze all sequence data (29).

Results

Molecular Characterization of the L1 Vh and Vλ cDNA. The amplified DNA from APCR with GCc and LCc were analyzed by gel electrophoresis and were found to contain clean

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                                                                          M  D  W  T  W  R  I  L  F  L    -10
halL1     GAGCATCACCCAGCAACCACATCTGTCCTCTAGAGAATCCCCTGAGAGCTCCGTTCCTCACCATGGACTGGACCTGGAGGATCCTCTTCT    -30
       1  ---------  halL1U1s ---------+---------+---------+---------+---------+---------+---------+    90

             V  A  A  A  T  G  A  H  S  Q  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  A  S  V  K  V  S     21
halL1     TGGTGGCAGCAGCCACAGGAGCCCACTCCCAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTTAAGGTCT     61
      91  ---------+---------+---------1---------+---------+---------+---------+---------+---------+    180

                                     _____CDR1_______                                           CDR2
            C  K  G  S  G  Y  T  F  T  A  Y  Q  M  H  W  V  R  Q  A  P  G  Q  G  L  E  W  M  G  W  I      51
halL1     CCTGTAAGGGTTCTGGATACACCTTCACCGCCTACCAAATGCACTGGGTGCGACAGGCCCCTGGACAAGGACTTGAGTGGATGGGATGGA    151
     181  ---------+---------+---------+---------+---------+---------+---------+---------+---------+    270

          CDR2___________________________________________
             N  P  N  S  G  G  T  G  Y  G  Q  K  F  Q  G  R  V  T  L  T  R  D  T  S  I  S  T  A  Y  M     81
halL1     TCAACCCTAACAGTGGTGGCACAGGCTATGGACAGAAGTTTCAGGGCAGGGTCACCCTGACCAGGGACACGTCCATCAGCACAGCCTACA    241
     271  ---------+---------+---------+---------+---------+---------+---------+---------+---------+    360

                                                               _________________________________CDR3
             E  L  S  R  L  T  S  D  D  T  A  V  Y  Y  C  A  I  E  Y  F  Y  D  G  S  D  L  K  P  S  D    111
halL1     TGGAGCTGAGCAGGCTGACATCTGACGACACGGCCGTGTATTACTGTGCCATTGAGTATTTTTATGATGGCAGTGACTTAAAACCCTCTG    331
     361  ---------+---------+---------+---------+---------+---------+---------+---------+---------+    450
D21/9                                                          -----AC------A-T----.G---TT--
Dn4                                                          ------
D22/12                                                             -A-------
Dxp'1                                                                       --G---T-.---T---

          CDR3__________                                 <CH1
             V  F  D  I  W  G  Q  G  T  M  V  T  V  S  S  A  S  T  K  G  P  S  V  F  P  L  A  P  S  S    141
halL1     ATGTTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCAGCCTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCT    421
     451  ---------+---------+ Jh3c  --+---------+---------+----  GC3s  -------+---------+---------+    540
Jh3           -------------------------------------------
γ1                                                       -------------------------------------------

             K  S  T  S  G  G  T  A  A  L  G  C  L  V  K  D  Y    158
halL1     CCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTAC    474
     541  ---------+---------+---------+---------+  GCc  --+---    593
γ1        -----------------------------------------------------
```

Figure 1. The nucleotide and amino acid sequences of the L1 H chain V region, designated Humha1L1 and abbreviated ha1L1. The expressed V gene was rearranged to Jh3 and γ1 genes (33, 34); their reported germline sequences are included for comparison. The nucleotide sequence in the CDR3 of ha1L1 was compared with all reported Dh gene sequences, and the four most homologous Dh gene segments were identified (30, 31); they are given for comparison. The complete nucleotide and amino acid sequences of ha1L1 are given, while all other sequences are given only at the positions where they differ from ha1L1, in the overlapping regions. The bars and dots denote the identities and the introduced gaps, respectively. The regions of four oligomers are underlined and their names are given underneath. The CDRs are marked.

bands of expected sizes; the bands also hybridized, respectively, with the GC3s and the LC4s primers (located upstream of the GCc and the LCc primers, marked in Figs. 1 and 2). The results indicated that the bands contained the Vh and Vλ cDNAs, respectively. Accordingly, the amplified DNA was cloned into M13 and the recombinants were analyzed. The results showed that the L1 IgG RF contained Vh1 and Vλ1 genes, designated HumhalL1 and HumlalL1, respectively (Figs. 1 and 2).

The CDR3 of halL1 contained a stretch of 29 bp that was homologous to D21/9 (30), suggesting that the halL1 H chain might use this Dh gene with some mutations; alternatively, this 29-bp stretch might be encoded by three different Dh genes (30, 31), as suggested by the similarities between each of the three nonoverlapping portions in the same stretch and a particular Dh gene (32) (Fig. 1). The introduced gaps in either case may represent insertions generated by poorly characterized "gene correlation mechanisms," as proposed recently by Sanz (32). Also, the halL1 H chain used a Jh3 and a Cγ1 gene (Fig. 1); its Jh region deviated from a Jh3 sequence by one silent change (33), while its Cγ1 region was identical to the reported Cγ1 sequence over a 77-bp region (34). On the other hand, the lalL1 L chain used a Jλ2 and a Cλ2 gene (35, 36), instead of the closely related Cλ3 gene, based on a single diagnostic base (G vs. A) at nucleotide position 372 (Fig. 2). The expressed Jλ2 sequence deviated from the reported germline sequence by one replacement change and two silent changes, while its Cλ2 sequence was identical to the known Cλ2 gene over a 56-bp region.

Identification and Characterization of the Germline Vh Gene for the L1 H Chain. Since the upstream flanking and the intron regions of a rearranged Ig V gene do not encode amino acid residues, they generally have much fewer somatic mutations than the coding region. Moreover, among different gene members of a V gene family, their upstream flanks and introns are normally more heterogenous than their coding region counterparts. As such, the sequences in these regions of a rearranged V gene often provide better clues for identifying its corresponding germline V gene.

Accordingly, we first cloned the rearranged Vh and Vλ genes from L1. Using halL1U1s and Jh3c as 5′ and 3′ primers (marked in Fig. 1), the rearranged Vh gene was amplified and characterized. A comparison of the resulting halL1-rearranged gene sequence with both GenBank and EMBL databases revealed that the expressed Vh gene was most similar to V35 and 1-1 germline genes; the former is functional, while the latter is a pseudogene (21, 37). Thus, halL1 might be encoded by V35 itself or a Vh1 gene highly closely related to V35.

To differentiate between these two possibilities, we compared the 3′ flanks of both V35 and 1-1 with a large number of human Vh1 genes, and identified a stretch that was iden-

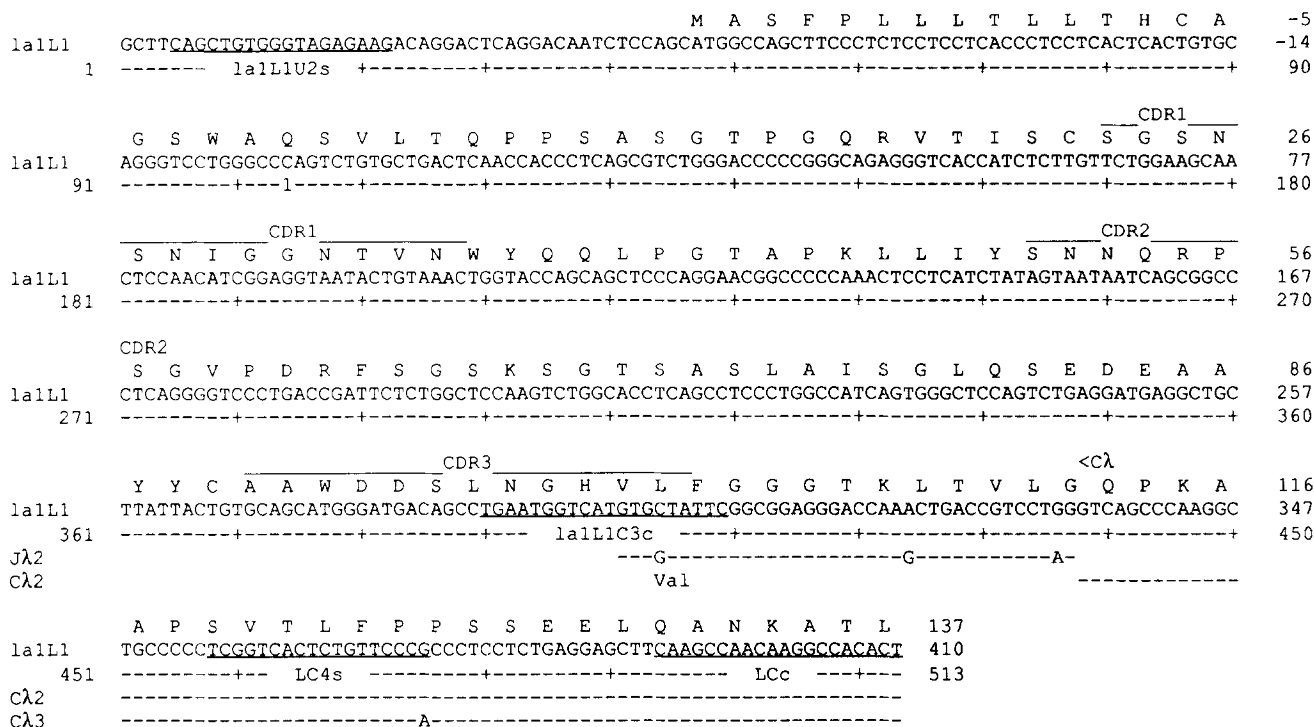

Figure 2. The nucleotide and amino acid sequences of the L1 L chain V region, designated HumlalL1 and abbreviated lalL1. The expressed V gene was rearranged to Jλ2 and Cλ2 genes (35, 36); their reported germline sequences are included for comparison. Also included is a relevant portion of the Cλ3 sequence, which differs from Cλ2 by only one nucleotide in the sequenced region. The complete nucleotide and amino acid sequences of lalL1 are given, while all other sequences are given only at the positions where they differ from lalL1, in the overlapping regions. The replacement amino acid residue is given below the replacement nucleotide change. The bars denote the identities; the regions of four oligomers are underlined and their names are given underneath. The CDRs are marked.

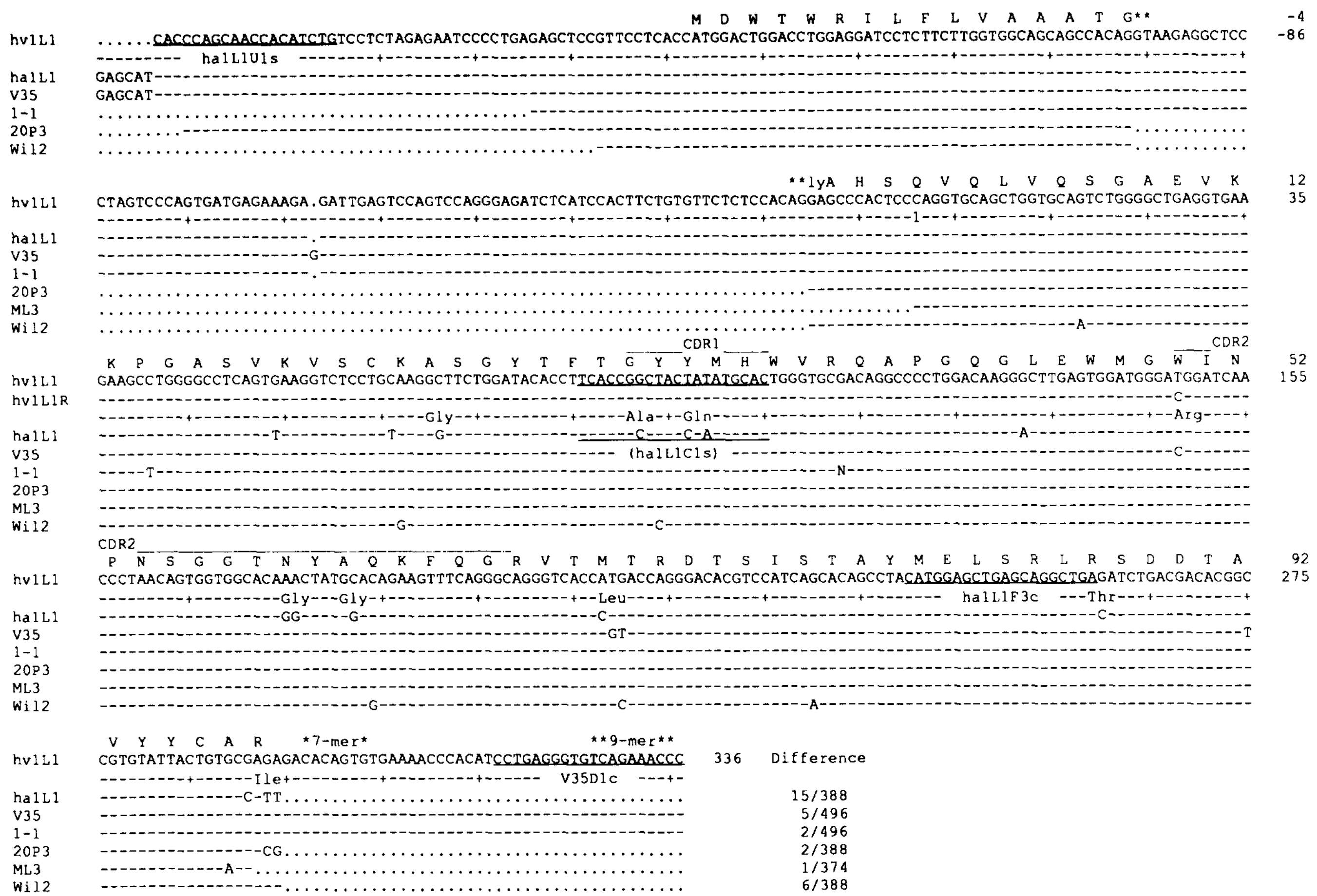

Figure 3. The genomic structures of the HumhvlL1 and the HumhvlL1R genes (abbreviated hvlL1 and hvlL1R, respectively). Also included are the sequences of the halL1 rearranged Vh gene, the V35 and 1-1 germline Vh genes, the 20P3, the ML3, and the Wil2 Vh cDNAs (19, 21, 22, 37, 79). The complete nucleotide and amino acid sequences of hvlL1 are given; all other sequences are given only at the positions where they differ from hvlL1 in the overlapping regions, except for hvlL1R, which includes only the relevant stretch. The replacement amino acid residues of halL1 and hvlL1R are given; the bars denote the identities, while the dots denote the unknown regions, the introns, or the deleted 3' flanking region. The differences between hvlL1 and each V gene are given at the end. The regions of five oligomers are underlined and their names are given. The CDRs, the splice, and recombination signal sequences are marked.

tical to both V35 and 1-1, but differed from all other remaining Vh1 genes. An oligomer complementary to this region was prepared (designated V35D1c; marked in Fig. 3), and was used with halL1U1s to amplify specifically the Ha1L1-related Vh1 germline genes from the ML germline DNA. The amplified DNA was cloned into M13, and seven recombinant clones were chosen randomly for sequencing. The results showed that all had identical sequences, except for a single base difference at nucleotide position 148; four clones had T (similar to the halL1-rearranged gene and the 1-1 pseudogene), while the remaining three clones had C (similar to the V35 functional gene) (Fig. 3). The data indicated that the gene represented by the first four clones was more likely to be the germline counterpart for halL1 and was thus designated HumlvlL1. The Vh gene represented by the latter three clones was then designated HumlvlL1R, the R stands for Arg (instead of Trp for lvlL1) at amino acid position 50.

As can be seen in Fig. 3, the hvlL1 sequence is identical to halL1 in the 5' flanking, leader, and intron regions, and differs from halL1 by 15 nucleotides in the coding regions. The data suggested strongly that hvlL1 encodes the halL1 H chain. However, could we be sure that hvlL1 was indeed the germline gene for halL1 and that the differences between them were due to somatic changes? Recently, van Es et al. (38) addressed this issue by comparative PCR, using one primer corresponding to the putatively germline sequence on one hand, while using a related primer corresponding to the putatively mutated sequence on the other hand. Based on this strategy, two oligomers were prepared; hvlL1C1s and halL1C1s (marked in Fig. 3; C1 denotes the first CDR) correspond to the putative germline hvlL1 sequence and the potentially mutated halL1 sequence, respectively. Each was paired with halL1F3c (marked in Fig. 3; F3 denotes the third FR) in PCR using genomic DNA from the L1 hybridoma or the PBL of donor ML. The results showed that amplification of hybridoma DNA with both primers yielded a clear band of expected size; in contrast, only hvlL1C1s led to a successful amplification in the PBL DNA (data not

Table 1. *Summary of Somatic Changes in the L1 V Regions, Encoded by V, D, and J Genes*

Regions	Mut	R	R/S	Conserved*	Nonconserved‡	
H chain						
Vh	15	11	2.8			
CDR	6	6	>>>§	31,	G > A	P >NP‡
				33, Y > Q		
				59, N > G		
				61,	A > G	NP > P
FR	9	5	1.8	24,	A > G	NP > P
				70, M > L		
				87,	R > T	+ > P
				98,	R > I	+ > NP
Jh	1	0	0			
L chain						
Vλ	4	3	3			
CDR	2	2	>>>	26, S > N		
				31, S > G		
FR	2	1	1	86,	D > A	− > NP
Jλ	3	1	0.5	101, L > V		

The abbreviations used are: mut, mutations; R, replacement changes; R/S, the ratio of the replacement to the silent changes.
* For each conserved replacement change, the amino acid position, the germline residue, and the mutated residue are given; > stands for "changes to".
‡ For each nonconserved change, additional information about the chemical properties (i.e. +, −, P, and NP denote + charged, − charged, polar, and nonpolar, respectively) of involved amino acid residues is given. For example, at position 31 of the H chain, the polar Gly residue changes to the nonpolar Ala residue.
§ >>>denotes infinity.

shown), suggesting that there was not a germline Vh gene in ML that contained the halL1C1s sequence. Combined with the sequence data of seven randomly picked clones, these findings demonstrated that hv1L1 was the germline gene for halL1.

Thus, the 15 nucleotides by which halL1 differs from hv1L1 represent somatic mutations; they consisted of two double-base changes and 11 single-base changes (Fig. 3 and Table 1). Of the 15 mutations, six were in the CDRs and nine in the FRs. Interestingly, all six (100%) changes in CDRs were replacement changes, while only five of nine (56%) changes in the FR were replacement changes. The replacement changes included both conserved and nonconserved changes of various natures (i.e., from polar to nonpolar or vice versa, positive charged to polar or nonpolar, etc.) (Table 1).

Identification and Characterization of the Germline Vλ Gene for the L1 L Chain. This was done similarly to the identification of the germline counterpart for the halL1 H chain. By priming with 1alL1U2s and LalL1C3c, the lalL1-rearranged gene was amplified and characterized. Comparison of the lalL1 sequence with both Genbank and EMBL databases revealed that it was equally similar to all three reported human germline Vλ1 genes (i.e., Humlv117, Vλ1.1, and IGLV1S2) (27, 39, 40). The result prevented us from rational design of the appropriate downstream primers, and thus made the identification of the lalL1-corresponding germline V gene more difficult. To circumvent the problem, we prepared a series of downstream primers corresponding to the 3′ end of the Vlλgene coding region in the lalL1 sequence and to the immediate downstream region of the reported Humlv117 germline gene sequence (27). Each of these primers was paired with lalL1U2s and was tried in the PCR amplification of the putative germline gene for lalL1; two primers led to the specific amplification of the desired Vλ gene.

When lv117D1c (complementary to the conserved nonamer region of lv117; marked in Fig. 4) was used, characterization of the clear band in the amplified DNA revealed a new Vλ1 gene, designated HumlvlL1. Importantly, amplification with the lalL1C3c1 primer (complementary to the 3′ end of the lalL1-rearranged gene sequence, marked in Fig. 4) also revealed a sequence identical to lvlL1 in the overlapped region. Together, these data showed that the lvlL1 sequence represented the authentic sequence of a new Vλ1 gene, instead of a hybrid sequence of various Vλ1 genes.

The lvlL1 gene is identical to the lalL1 gene in the 5′ untranslated, the leader, and the intron regions, while it differs

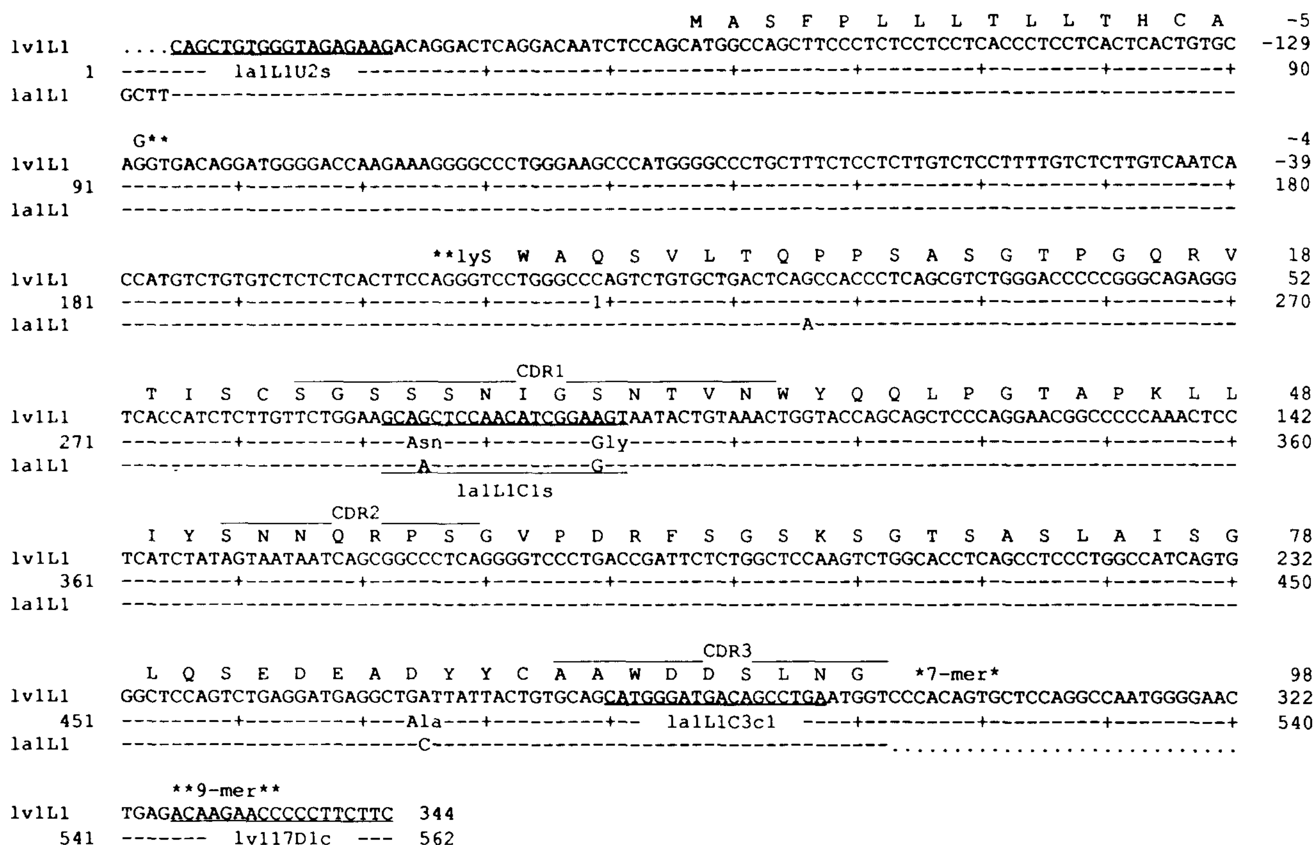

Figure 4. The genomic structures of the Humlv1L1 gene (abbreviated lv1L1). Also included is the la1L1-rearranged gene sequence. The complete nucleotide and amino acid sequences of hv1L1 are given, while the la1L1 sequence is given only at the positions where it differs from lv1L1, in the overlapping regions. The replacement amino acid residues of la1L1 are given; the bars denote the identities, while the dots denote the unknown region in the 5′ flank of lv1L1 or the deleted 3′ flanking region of la1L1. The regions of five oligomers are underlined and their names are given. The CDRs, the splice, and recombination signal sequences are marked.

from la1L1 by four nucleotides in the Vλ gene coding region (Fig. 4). The data suggested that lv1L1 encodes the la1L1 L chain. To verify this conclusion, the aforementioned comparative PCR was done. Two oligomers were prepared; lv1L1C1s and la1L1C1s (marked in Fig. 4; C1 denotes the first CDR) correspond to the putative germline lv1L1 sequence and the potentially mutated la1L1 sequence, respectively. Each was paired with la1L1C3c1 in PCR using genomic DNA from the L1 hybridoma or the PBL of donor ML. The results showed that amplification of hybridoma DNA with both primers yielded a clear band of expected size; in contrast, only lv1L1C1s led to a successful amplification in the PBL DNA (data not shown), suggesting that there was not a germline Vλ1 gene in ML that contained the la1L1C1s sequence. Combined with the sequence data of five randomly picked clones, these findings demonstrated that lv1L1 was the germline gene for la1L1, and that the four base differences in la1L1 represented mutations. Of the four nucleotide changes, two were in the CDRs and two were in the FRs (Fig. 4). Interestingly, both nucleotide changes in CDRs were replacement changes, while one-half of the changes in FRs were replacement changes (Table 1).

Molecular Characterization of the D1 Vh and Vκ cDNA. To improve the efficiency of APCR, the γ chain cDNA of the second hybridoma was amplified with one major modification, namely the tailed cDNA was first amplified with a new primer (GC1c, marked in Fig. 5), downstream of the initial GCc primer used in the first hybridoma. The amplified DNA of the expected size was enriched from the agarose gel and reamplified with the GCc primer. Similarly, two κ L chain–specific primers (i.e., KCc and KC1c, marked in Fig. 6) were designed and used to amplify the κ chain V gene cDNA. The amplified DNA was cloned into M13 and the recombinants were analyzed. The results showed that the D1 IgG RF contained Vh3 and Vk3 genes, designated Humha3d1 and Humka3d1, respectively (Figs. 5 and 6).

The CDR3 of ha3d1 contained a stretch of 10 bp that was homologous to Dk4 (31) and a nonoverlapping stretch of 5 bp identical to D21/10 (30), suggesting that the ha3d1 H chain CDR3 might be generated by a D-D fusion of these two Dh genes and with one mutation (32) (Fig. 5). Also, the ha3d1 H chain uses a Jh4 and a Cγ3 gene (Fig. 5); its Jh region is identical to a Jh4 sequence (33), and its Cγ3 region is identical to the reported Cγ3 sequence over a 77-bp

```
        E  V  Q  L  V  E  S  G  G  G  L  V  Q  P  G  G  S  L  R  L  S  C  A  A  S  G  F  T  F  S     30
ha3d1   GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGT   90
        ---------+---------+---------+---------+---------+---------+---------+---------+---------+
VH26    ------------T-------------------------------------------------------------------------T--C

        _____CDR1______                                                    __________________CDR2
        N  Y  S  M  N  W  V  R  Q  A  P  G  K  G  L  E  W  V  S  Y  I  S  S  S  S  S  T  I  Y  Y     60
ha3d1   AACTATAGCATGAACTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTTTCATACATTAGTAGTAGTAGTAGTACCATATACTAC  180
        ---------+---------+---------+---------+---------+---------+---------+---------+---------+
VH26    -G----GC-----G---------------------------------------C---GCT------G-----G--G---G--C-------

        CDR2______________
        A  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  S  L  Y  L  Q  M  N  S  L  R  A  E  D     90
ha3d1   GCAGACTCTGTGAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGAC  270
        ---------+---------+---------+---------+---------+---------+---------+---------+---------+
VH26    --------C-----------G-------------------------T--------A-G--------------------------------

                                    __________CDR3_________
        T  A  V  Y  Y  C  A  R  S  G  Y  R  G  G  D  Y  W  G  Q  G  T  L  V  T  V  S  S  A  S  T    120
ha3d1   ACGGCTGTGTATTACTGTGCGAGAAGCGGATACAGAGGGGGCGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGCTTCCACC  360
        ---------+---------+---------+---------+---------+---------+---------+---------+---------+
VH26    -----C--A-------------A-
Dk4                              -T-------
D21/10                                      -----
Jh4                                               ----------------------------------------
γ3                                                                                        --------

        K  G  P  S  V  F  P  L  A  P  C  S  R  S  T  S  G  G  T  A  A  L  G  C  L  V  K  D  Y  F    150
ha3d1   AAGGGCCCATCGGTCTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTC  450
        ---------+---------+---------+---------+---------+---------+---------+----  GCc  --------+
γ3      --------------------------------------------------------------------------------------

        P  E  P  V  T  V  S  W  N  S     160
ha3d1   CCCGAACCGGTGACGGTGTCGTGGAACTCA   480
        ---------+-------  GC1c  ----+
```

Figure 5. The nucleotide and amino acid sequences of the D1 H chain V region, designated Humha3d1 and abbreviated ha3d1. The nucleotide sequence in the Vh gene–encoded region was compared with all Vh gene sequences in the GenBank and EMBO databases, and the most homologous VH26 gene is given for comparison (45, 46). The expressed V gene was rearranged to Jh4 and γ3 genes (33, 41); their reported germline sequences are included for comparison. The nucleotide sequence in the CDR3 of ha3d1 was compared with all reported Dh gene sequences, and the 2 most homologous Dh gene segments were identified (30, 31); they are given for comparison. The complete nucleotide and amino acid sequences of ha3d1 are given, while all other sequences are given only at the positions where they differ from ha3d1, in the overlapping regions. The bars denote the identities. The regions of two oligomers are underlined and their names are given underneath. The CDRs are marked.

region (41). On the other hand, the ka3d1 L chain uses a Jk1 gene (42) (Fig. 6); its Jk and Ck regions are identical to reported germline sequences (42, 43).

Discussion

In an effort to define the mechanisms for the generation of IgG RFs in RA patients, we cloned and sequenced the expressed Ig V genes of the L1 and D1 IgG RF-secreting hybridomas, derived from the unmanipulated synovial B cells of two RA patients. Thereafter, we also identified and characterized the germline counterparts of the corresponding rearranged L1 H and L chain V genes. The results showed that the L1 H chain was generated from the Humhv1L1, the Jh3, and the Cγ1 genes; and that the L1 L chain was encoded by the Humlv1L1, the Jλ 2, and the Cλ 2 genes. In addition, the data suggest that the H chain CDR3 might derive either from the D21/9 gene only or from three separate Dh genes (i.e., Dn4, D22/12, and Dxp'1) (30, 31). The germline counterparts of the D1 H and L chain V genes are in the process of characterization. However, a computer search for homologous genes among 56,368 sequences in the GenBank and EMBO databases revealed that ka3d1 differs from the Vg gene by only one base in the third CDR (44) (Fig. 6), while ha3d1 is most similar to the VH26 gene (45, 46) (Fig. 5).

The hv1L1 germline Vh gene is almost identical to the 20P3 Vh cDNA sequence, which is a part of the restricted fetal antibody repertoire (20); 20P3 differs from hv1L1 by only one double-base at the V/D junction, suggesting that the difference might be due to an imprecise joining of an hv1L1 gene to a rearranged DJ gene. In addition, hv1L1 is highly homologous to the V35 gene, located within 240 kb of the Jh locus. Hv1L1 and V35 differ from each other by five nucleotides over a stretch of 496 bp; they are also very similar to the Humhv1L1R functional gene and the 1-1 pseudogene (Fig. 4). These four genes may represent either the allelic forms of a single Vh gene locus or different Vh genes residing at the separate gene loci. Family studies with informative probes are required to resolve this issue. Of note, similar clusters of several highly homologous germline Vh genes have been observed in a few cases (47–49).

Previously, extensive studies in mice have shown that certain Jh-proximal Vh genes are expressed preferentially during early ontogenic development, and that a few of such Vh genes frequently encode autoantibodies (50–53). Similar findings

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                                                                                 ____________________CDR1
          E  I  V  L  T  Q  S  P  A  T  L  S  L  S  P  G  E  R  A  T  L  S  C  R  A  S  Q  S  V  S      30
ka3d1     GAAATTGTGTTGACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGC    90
          ---------+---------+---------+---------+---------+---------+---------+---------+---------+
Vg        ------------------------------------------------------------------------------------------
RF-TMC1   -----------------------------------C----T-----------A------A------------------------------

          CDR1_________                                          ________CDR2________
          S  Y  L  A  W  Y  Q  Q  K  P  G  Q  A  P  R  L  L  I  Y  D  A  S  N  R  A  T  G  I  P  A      60
ka3d1     AGCTACTTAGCCTGGTACCAACAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCAGCC   180
          ---------+---------+---------+---------+---------+---------+---------+---------+---------+
Vg        ------------------------------------------------------------------------------------------
RF-TMC1   --GC----------------------------------------------------------------A--------------T--G---

                                                                                              __CDR3
          R  F  S  G  S  G  S  G  T  D  F  T  L  T  I  S  S  L  E  P  E  D  F  A  V  Y  Y  C  Q  Q      90
ka3d1     AGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGCAG   270
          ---------+---------+---------+---------+---------+---------+---------+---------+---------+
Vg        ------------------------------------------------------------------------------------------
RF-TMC1   ----------------------------------G-----T-G----------------------------C-----------------C

          CDR3_________________
          R  S  N  W  P  W  T  F  G  Q  G  T  K  V  E  I  K  R  T  V  A  A  P  S  V  F  I  F  P  P     120
ka3d1     CGTAGCAACTGGCCGTGGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGAACTGTGGCTGCACCATCTGTCTTCATCTTCCCGCCA   360
          ---------+---------+---------+---------+---------+---------+---------+  KCc  --+----  KC1c
Vg        --------------T
RF-TMC1   ----A---T-----T
Jk1                      -------------------------------------
Ck                                                            --------------------------------

          S  D    122
ka3d1     TCTGATG 367
          KC1c  -
```

Figure 6. The nucleotide and amino acid sequences of the D1 L chain V region, designated Humka3dl and abbreviated ka3dl. The nucleotide sequence of the most homologous Vg gene is given for comparison (44). Also included is the reported Vk sequence of a IgM RF from a normal individual (61). The expressed V gene was rearranged to Jk1 and Ck genes (42, 43); their reported germline sequences are included for comparison. The complete nucleotide and amino acid sequences of ka3dl are given, while all other sequences are given only at the positions where they differ from ka3dl, in the overlapping regions. The bars denote the identities; the regions of two oligomers are marked and their names are given underneath. The CDRs are marked.

have been noted in humans (15, 20, 33, 54). For example, the most Jh-proximal Vh6 gene is expressed frequently during early ontogenic development and is used by polyspecific IgM autoantibodies (20, 22, 37, 54, 55). On the other hand, structural analyses of many natural IgM autoantibodies from normal subjects have revealed that they use a restricted set of Ig V genes (12, 15, 33); and several of these V genes, such as Humhv1051/51P1 and VH26/30P1 genes, have been found to be expressed preferentially during early ontogenic development (19, 20, 22, 56, 57).

The notable overlaps of the early antibody repertoire and the natural antibody repertoire, together with other observations, have led to the "network" hypothesis (58). It contends that natural autoantibodies play an important role in the ontogenic development and the subsequent homeostasis of a normal B cell repertoire. As such, some autoreactive V genes may be evolutionarily selected for preferential expression, possible through their their proximity to the Jh locus and/or unique *cis* regulatory elements (50, 51, 59, 60). In the context of these data, the use of hv1L1 by the L1 self-associating IgG RF would suggest that L1 probably derived from a physiological natural autoantibody (with or without RF activity), but escaped from the normal mechanisms of regulation, possibility during a serious infection and/or transient dysfunction of the immune system of the host, and thus became a monospecific, high affinity (with K_d at 10^{-7} M) RF due to selection by IgG alone or IgG antibody-antigen complexes. In support of this conclusion is the recent report of the Vh gene sequence of the ML3 IgM RF, derived from the CD5$^+$ B cells of a 19-wk-old fetal spleen (22); the ML3 differs from hv1L1 by only one base over a stretch of 374 bp (Fig. 3). Similarly, the ka3d1-corresponding germline Vg gene is expressed in fetal liver and apparently encodes the L chain of a IgM RF (i.e., TMC1) from a normal individual (56, 61). Moreover, the current conclusion is consistent with extensive studies of monospecific and high affinity IgM RFs from the peripheral blood of RA patients; they were found to use certain selected Ig V genes, similar to those used by the natural autoantibodies and the fetal antibody repertoire. For example, RF SJ2 used the most frequently expressed Vh gene in fetal liver (i.e., Humhv3005f3/GL-SJ2/M72 or its homologous 56P1) (20, 49, 62); RFs TS1 and YES8c from two unrelated RA patients used Humhv1051/51P1, which also encodes the H chains of about 40% IgM RFs from unrelated patients with mixed cryoglobulinemia (14, 15, 62–64); RF KL1 used VH26/30P1; RFs TS1, SJ3, and YES8c used Humkv325/A27; RF TS2 used Humkv328h5; and RFs SJ1 and SJ2 used Humlv117 (27, 45, 46, 61–63, 65–68). However, it should be noted that RF production in RA patients may be induced by different mechanisms, and thus the afore-

mentioned hypothesis may not account for RF secretion in all RA patients.

In addition to documenting the V gene origin of an IgG RF, the current data also provide evidence for the role of antigen selection in IgG RF production. Generally, during an antigen-driven response, B cells expressing mutated antibodies with higher affinity for the antigen are progressively selected. Frequently, such affinity maturation is associated with the switch from IgM to IgG (69–75). These principles apply not only to antibody responses to foreign antigens, but also to self-antigens. For example, extensive studies of RFs and anti-DNA antibodies in normal and autoimmune mice showed that most IgG RFs from MRL/lpr autoimmune mice had an average of 4.4 somatic mutations per V region, with the most mutated V region having seven mutations; in contrast, most IgM RFs (generated by polyclonal B cell activation) had an average of only 0.5 mutation per V region, with the most mutated V region having only two mutations (76, 77). Furthermore, the former mutations occurred nonrandomly; they were located much more frequently in CDRs than in FRs, and often led to amino acid substitutions, resulting in an ratio of replacement (R) to silent (S) mutations >2.9 (76). Similar findings were obtained from analyses of IgG anti-DNA antibodies from MRL/lpr mice (78).

As summarized in Table 1, the L1 RF had 16 and 7 mutations in its H and L chain V regions, respectively. Moreover, all eight changes in the CDRs of both V genes caused amino acid substitutions, resulting in an R/S ratio of infinity (i.e., 8/0). In contrast, only 6 of 11 changes in the FRs of both V genes led to amino acid replacements, resulting in a R/S ratio of 1.2. Taken together with the high binding affinity of L1 toward the Fc fragment, these data suggest strongly that L1 RF was selected by the Fc fragment, and thus provide the first direct evidence for an Fc-driven immune response in RA synovium. As for the D1 RF, although its L chain is nearly identical to the reported germline Vg gene, its H chain differs significantly from the most closely related VH26 gene, and includes many replacement changes in the first and second CDRs. Considering the high binding affinity of D1 toward the Fc fragment, it is likely that the D1 IgG RF also derived from an Fc-driven response.

The sequence data are available from the EMBL/GenBank/DDBJ Data Libraries under the following accession numbers: X59702 (humighal1l), X59703 (humigha3dl), X59704 (humighvl1l), X59705 (humigka3dl), X59706 (humiglal1l), and X59707 (humiglvl1l).

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