Human Rheumatoid Factors with Restrictive Specificity for Rabbit Immunoglobulin G: Auto- and Multi-reactivity, Diverse V_H Gene Segment Usage and Preferential Usage of V_λIIIb

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Summary

To determine the molecular and functional properties of human rheumatoid factors (RF), we established stable hybridomas and Epstein-Barr virus–transformed B cell lines from the synovial fluid or peripheral blood of three patients with rheumatoid arthritis and one patient with systemic lupus erythematosus. 17 cell lines were obtained that produced high-titer immunoglobulin M (IgM) RF that reacted exclusively with rabbit but not human IgG or IgG of other mammalian species. Certain anti–rabbit IgG RF also had specificity for other mammalian antigens (Ag), including cytoskeletal proteins and intracellular proteins found in HeLa cells, as well as for Ag present in an extract prepared from the cell wall of group A streptococci. 13 of the 17 RF contained λ-type light (L) chains, of which 12 were classified serologically as members of the λ-L chain variable region (V_λ) subgroup, designated V_λIII. The heavy chain V region (V_H) and V_λ sequences of nine of these IgMλ RF were determined at the cDNA level. Five V_H genes in three V_H families were used by these antibodies (Ab), including V_H1 (dp21/1-4b and dp10 [51p1]/hv1051), V_H3 (dp38/3-15 and dp77/13-21), and V_H4 (dp70/4-4b). The deduced V gene–encoded amino acid sequences of the λ chains of these IgMλ RF confirmed their serological classification as λIII, and they were further classified as members of the relatively uncommon V_λIII subgroup, designated V_λIIIb. Based on cDNA analyses, nine were the product of three different V_λIIIb germline genes. Two such genes, designated hsiggll150 and hsiggll295, were cloned and sequenced from genomic DNA. Unique combinations of these V_H and V_λIIIb genes could be related to distinctive patterns of reactivity among the IgMλ RF. Although the V_H and V_λ regions of these Abs were expressed primarily as germline-encoded sequences, four of nine multireactive Abs had extensive V region mutation, indicative of an Ag-driven process. The finding that λIIIb L chains are preferentially found among anti–rabbit IgG RF, and that some of these Ab have specificity for other protein, cellular, and bacterial Ag, provides new insight into the pathogenesis of RA and related diseases.

Rheumatoid arthritis (RA)[1] is a chronic inflammatory disorder primarily affecting joints, although extra-articular organs are frequently involved during the course of the disease (1, 2). Humoral, cellular, and nonimmunological factors have all been implicated in the pathological process that results in joint destruction and other manifestations characteristic of RA (3–6). In the case of humoral immunity, a variety of autoantibodies, particularly RF, interact with Ag in synovial tissue, fluid, and cartilage. This interaction, which

This work was presented in part at the Meeting of the American Association of Immunologists and the Clinical Immunology Society, Denver, CO on 21–25 May 1993. Portions of this work have appeared in abstract form (1993. *J. Immunol.* 150:238*a*).

[1] *Abbreviations used in this paper:* FR, framework region; MAP, microtubule-associated protein; RF, rheumatoid factor.

gives rise to the formation of immune complexes, complement activation, and the recruitment of neutrophils and macrophages, is responsible for the intensive inflammatory reaction and resultant morbidity.

The biological function and pathological role of RF have been the subject of extensive investigation (for reviews see references 7 and 8). The specificity of these Ab, which are most often of the IgM class, is directed towards IgG and in particular, the Fc-related epitopes. Based on reactivity patterns, Williams and Kunkel (9) showed that IgM RF could be divided into three groups: those that recognized solely (*a*) rabbit IgG; (*b*) human IgG; or (*c*) both. RF were initially detected by the Rose-Waaler test, which employs SRBC coated with rabbit IgG Ab. A positive Rose-Waaler test has been considered more specific for RA than the bentonite or latex flocculation tests, which employ human IgG as the coating protein (7). In this regard, it is also of interest to note that RF in diseases other than RA appear to be more reactive with human IgG (10–12).

Considerable progress in identifying and characterizing the genes encoding the H and L chain V regions (V_H and V_L, respectively) of anti-IgG Ab has come through study of RF-secreting hybridomas and immortalized B cell lines (for a review see reference 13). Studies to date involving human IgM RF that have specificity for human IgG or both human and rabbit IgG have indicated that these V genes are diverse and often mutated. However, limited information is available regarding the antigenic specificity and V region gene usage by RF reactive with rabbit but not human IgG (14).

As part of a long-standing study to elucidate the molecular structure of RF, we have prepared hybridomas and EBV-transformed B cell lines from synovial cells and PBL obtained from three patients with RA and one with SLE. Many of these hybridomas and cell lines secreted IgM RF that had exclusive specificity for rabbit IgG. These RF differed from anti-human IgG RF in that they contained predominately λ-type L chains that, remarkably, were the product of a relatively uncommon V_λ gene family, designated V_λIIIb (15). Our studies have also shown that some of these anti-rabbit IgG RF were multireactive and recognized additional Ag including cytoskeletal and other intracellular proteins, as well as streptococcal cell wall components.

Materials and Methods

Generation of Human Monoclonal RF-secreting Cell Lines. EBV-transformed B cell lines were established from peripheral blood (PB) and synovial non-T cells from three patients with RA (LBR, PHB, and DGA) and one patient with SLE (KES) as described previously (16). Cell lines of interest were fused with K6H6/B5 (American Type Culture Collection, Rockville, MD) according to Carroll et al. (17). The hybridomas were repeatedly cloned by limiting dilution until all clones were positive for RF activity. KES643 was cloned twice without being fused with K6H6/B5 using irradiated human T cells as feeder cells. The cell lines were shown to be monoclonal, as evidenced by PCR amplification of unique V_H and V_λ transcripts.

ELISA. The methods used for the ELISA were previously described (16). Coating Ag used for RF determination included human and rabbit IgG isolated by NH_4SO_4 precipitation and DEAE ion exchange chromatography, IgG of horse, goat, sheep, bovine, dog, and rat (Sigma Chemical Co., St. Louis, MO), and Fc and $F(ab')_2$ fragments of rabbit IgG from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Other Ag were tetanus toxoid and diphtheria toxoid from the State Laboratory Institute (Jamaica Plain, MA), thyroglobulin, double stranded (ds)DNA, human insulin, DNP-hemocyanin, bovine type II collagen, bovine muscle myosin, porcine muscle actin, and human epidermal keratin from Sigma Chemical Co. and bovine glial fibrillary acidic protein from ICN Biomedicals Inc. (Costa Mesa, CA). Porcine brain tubulin and microtubule-associated proteins (MAPs) were purified as described previously (18).

For inhibition, human IgG at 50 mg/ml in PBS was heat aggregated at 60°C for 30 min and then centrifuged at 2,000 rpm to remove insoluble and large aggregates. The aggregated IgG was incubated at room temperature for 2 h with appropriately diluted RF-containing cell supernatants. The mixtures were used in ELISA as described above.

The V_λ-subgroup nature of the λ-type IgM anti-rabbit IgG RF was determined by ELISA using murine mAbs specific for the V_λI, V_λII, V_λIV, and V_λVI subgroups and the V_λIIIa, V_λIIIb, and V_λIIIc sub-subgroups (19). The ELISA for RF-binding to streptococcal extract was kindly performed by Dr. Madeleine W. Cunningham (University of Oklahoma, Oklahoma City, OK) (20). The affinity constants of RF to rabbit IgG and myosin were measured by ELISA as described by Friguet et al. (21).

Immunofluorescence and Immunoblots. Indirect immunofluorescence to HeLa cells (16) and SDS-PAGE and immunoblots (22) were done as previously described. Porcine MAPs (12 μg/lane) and homogenized HeLa cells (10^5 cells/lane) were electrophoresed on 5–25%-gradient SDS-PAGE gels. After transferring, the blots were washed, blocked with 5% milk for 1 h, and incubated overnight at 4°C with supernatants diluted with 0.5% BSA in PBS. The blots were then treated with reagents contained in the Vectastain ABC anti-human IgM kit (Vector Laboratories, Inc., Burlingame, CA) and analyzed by enhanced chemiluminescence reagents (Amersham Corp., Arlington Heights, IL).

RNA Isolation, cDNA Synthesis, and Anchored PCR for Amplification of V_H and V_L. Total RNA was prepared from hybridoma cell lines using either the single-step technique (23) or the RNAzol™ B method (Biotecx Laboratories, Houston, TX). 3 μg of total RNA was reverse transcribed with oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase. A poly (dG) tail was added to the 3′ end of the cDNA with terminal deoxynucleotide transferase. dG-tailed cDNA was amplified using anchored PCR (24, 25) modified by the "touchdown" method (26). Oligonucleotide primers for the amplification of V_λ were PAN (5′-CACGTCGACAGGCGGCCGCGGTA) and PANpoly C (5′-CACGTCGACAGGCGGCCGCGGTACCCCCCCCCCCCCC), both containing a SalI cloning site, and LC (5′-GTTCCTGCAGAGTGTGGCCTTGTTGGCTTG), with a PstI cloning site. The three primers for amplication of V_H were SAN (5′-CTACGAGCTCAGGCGGCCGCGGACC) and SANpoly C (5′-CTACGAGCTCAGGCGGCCGCGGACCCCCCCCCCCCCC), with an SstI cloning site, and HMC (5′-CGGCAAGCTTATTCTCACAGGAGACGAGGG), with a HindIII cloning site. In the case of KES643, because the V_H had an internal SstI site, PAN and PANpoly C primers were used.

DNA Isolation and PCR for Amplification of V_L Germline Counterparts. High molecular weight DNA was extracted from the PB granulocytes of PHB, a patient with Alzheimer's disease (MRE), and two normal donors and from the T cell fraction of LBR PBMC according to Blin and Stafford (27). Briefly, frozen cell pellets were

Table 1. *17 IgM RF Specific for Rabbit IgG Show a Predominant Usage of λ L chain (13/17) and Utilize Vλ III Subgroup (12/13)*

Cell lines	Tissue	ELISA OD_{405}		L chain‡	
		Rabbit IgG	Human IgG*	Class	V_L subgroup
LBR 150	ST	50§	Nonreactive‖	λ	λIIIa/b
271	"	40	"	"	"
295	"	80	"	"	λIIIb
962	"	160	"	"	"
1045	"	40	"	"	"
1189	"	160	"	"	"
1088	"	80	"	"	"
1104	"	20	"	"	"
328	"	20	"	κ	ND
1039	"	20	"	"	ND
1052	"	50	"	"	κIII
P255	PB	20	"	λ	λIIIb
PHB 1213	"	40	"	"	"
1540	"	20	"	κ	κI
1650	"	40	"	λ	λI
KES 643	"	640	"	"	λIIIb
DGA 127	ST	20	"	"	"

* Also nonreactive with IgG from horse, goat, sheep, bovine, dog, and rat.
‡ Vλ determination was done by ELISA and Vκ were determined by cDNA sequence analysis.
§ OD_{405} multiplied by the dilution factor i.e., OD_{405} = 1 at 1:50 dilution. The OD_{405} values were the averages of three determinations.
‖ OD_{405} <0.1 at 1:10 dilution.
ST, synovial tissue.

ground into a fine powder in liquid nitrogen and incubated at 55°C for 3 h in 0.5 M EDTA, pH 8.0, containing 0.5% sodium sarkosyl and 100 μg/ml proteinase K. After RNase treatment, the DNA was purified by phenol extraction and the concentration in the dialystates determined spectrophotometrically. When necessary for PCR reaction, the DNA was concentrated by HindIII digestion and ethanol precipitation. A 5' primer, GLUNA (5'-CAGGTCGACGGYYCAGGAGGCAGARCTC), was designed from the untranslated regions of the LBR150 and LBR295 V_λ genes. The 3' primer for the LBR150 germline gene was GLCDA (5'-CCGGAATTCACCACTGCTGTCTGCTGATTG), designed from CDR3 of the LBR150 V_λ gene. The 3' primer for the LBR295 germline gene was GLCDB (5'-GCGAATTCTGATTACCACTGTAGTCTGCTGA), designed from CDR3 of the LBR295 V_λ gene. These primers contained SalI and EcoRI cloning sites, respectively. The PCR amplification procedure was essentially that described by Sota-Gil et al. (28).

Cloning and Sequencing of the Amplified DNA. The amplified DNA was digested with the appropriate enzymes and separated by electrophoresis on 1% agarose gels (Sea Plaque; FMC Corp. BioProducts, Rockland, ME). To sequence the V region in both directions, the gel-purified DNA was directionally cloned into both M13mp18 and M13mp19. The cloned single-stranded (ss)DNA was sequenced by the dideoxynucleotide chain termination method (29) using the Sequenase Version 2.0 kit (United States Biochemical Corp., Cleveland, OH). Each given sequence represented the consensus residues of eight clones generated from two independent PCR products. The DNA sequence data were analyzed using the EuGene software program (Molecular Biology Information Resource, Baylor Medical College, Houston, TX) linked to major data banks via the University of Virginia Computer Center.

Results

Preferential Usage of VλIII Gene Segments by IgM RF Reactive with Rabbit IgG. 17 IgM anti-IgG Ab were identified by screening the supernatants of EBV-transformed B cell lines from RA patients LBR (1,351 from synovial tissue and 620 from PB), PHB (2,281, PB), and DGA (311, synovial tissue) and SLE patient KES (705, PB). These RF reacted strongly with rabbit IgG but not with human IgG or the IgG from six other mammalian species (Table 1). They were solely Fc reactive. That all 17 RF had exclusively anti-rabbit IgG reactivity was further evidenced by the demonstration that heat-aggregated, human pooled IgG up to 5 mg/ml were not inhibitory. In contrast, aggregated human IgG at 0.1 mg/ml readily inhibited our other IgM RF, reactive either with human IgG or with both human and rabbit IgG.

Serologically, 13 of the 17 RF were typed as IgMλ. This

Table 2. *IgM λ RF Specific for Rabbit IgG Are Reactive with Cytoskeletal Proteins and other Ag*

RF		Isotype	Cytoskeletal proteins (ELISA OD_{405})						Other Ag*	Strept.‡	HeLa§
			MAPs	Keratin	Myosin	GFAP	Actin	Tubulin			
LBR	295 (962)‖ (1045) (1189)	μλ	18¶	–**	2	–	–	–	–	–	++
LBR	150 (271)‖	μλ	4	19	5	7	5	29	–	++	++
LBR	1104	μλ	14	20	19	26	13	56	7 (TT), 2 (DT)	++	++
PHB	1213	μλ	2	50	100	6	4	5	–	++	++
KES	643	μλ	19	7	8	13	10	22	–	++	++
LBR	1052	μκ	–	–	–	–	–	–	–	–	–
PHB	1540	μκ	–	–	–	–	–	–	–	–	++

* dsDNA, insulin, thyroglobulin, type II collagen, DNP-hemocyanin, TT, and DT.
‡ Group A streptococcal cell wall extract: (++) OD_{405} ≥2 at 1:10 dilution; (–) OD_{405} <0.1 at 1:10 dilution.
§ Intracellular staining by indirect immunofluorescence: (++) staining; (–) nonstaining.
‖ Similar results have been obtained with LBR295, LBR962, LBR1045, and LBR1189 and only data on LBR295 is presented. Similarly, only data on LBR150 is presented.
¶ OD_{405} multiplied by the dilution factor, i.e., = 1 at 1:18 dilution. The OD_{405} values were the averages of three determinations.
** (–) Negative, OD_{405} <0.1 at 1:10 dilution.
DT, diptheria toxoid, *GFAP*, glial fibrillary acidic protein, *TT*, tetanus toxoid.

biased usage of λ-L chains was in marked contrast to the predominance of κ-L chains among anti-human IgM RF. In our collection of 63 IgM RF reactive with either human IgG or with both human and rabbit IgG, 54 were typed to be κ. Of the 13 IgMλ RF, 10 were classified serologically as V_λIIIb and one as V_λI. Two remaining RF were reactive with both anti-V_λIIIa and anti-V_λIIIb mAb.

It is of considerable interest to note that the B cells which were responsible for the secretion of these RF were markedly enriched in the synovium. This was best documented in the case of LBR whose blood and synovial tissue were available for EBV transformation and immortalization; 11 out of 1,351 synovial B cell lines secreted RF of desired specificity whereas only 1 of 620 PB B cell lines did so.

Autoreactivity of IgMλ RF. Stable hybridomas were formed with 10 of the 17 EBV-transformed B cell lines fused with K6H5/B5 cells. KES643 was cloned without fusion and proved to be a stable, high IgM secretor. The RF produced by these 11 hybridomas and B cell lines were studied further regarding their reactivities with other Ag and V region usage.

With the exception of the IgMκ RF LBR1052 and PHB1540, the other nine λ-type IgM RF listed in Table 2 reacted with one or more types of cytoskeletal proteins, and, of these, LBR1104 also reacted weakly with tetanus and diphtheria toxoids. LBR1104 reacted weakly with BSA, indicating that it was an mAb with a tendency for nonspecific binding in ELISA. The predominant reactivity of one group (LBR295, LBR962, LBR1045, and LBR1189) was with MAPs and that of a second group (LBR150 and LBR271) was with keratin and tubulin. Other patterns of reactivity were found. LBR1104 had major reactivity with tubulin but also recognized other cytoskeletal proteins, as did KES643. PHB1213 was most strongly reactive with myosin and keratin. Western blot anal-

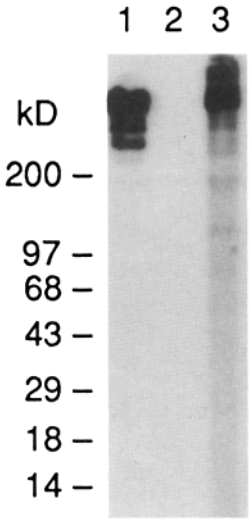

Figure 1. Immunoblot of LBR295 (lane *1*) and LBR150 (lane *3*) against MAPs. Lane *2* is an unreactive IgM antibody.

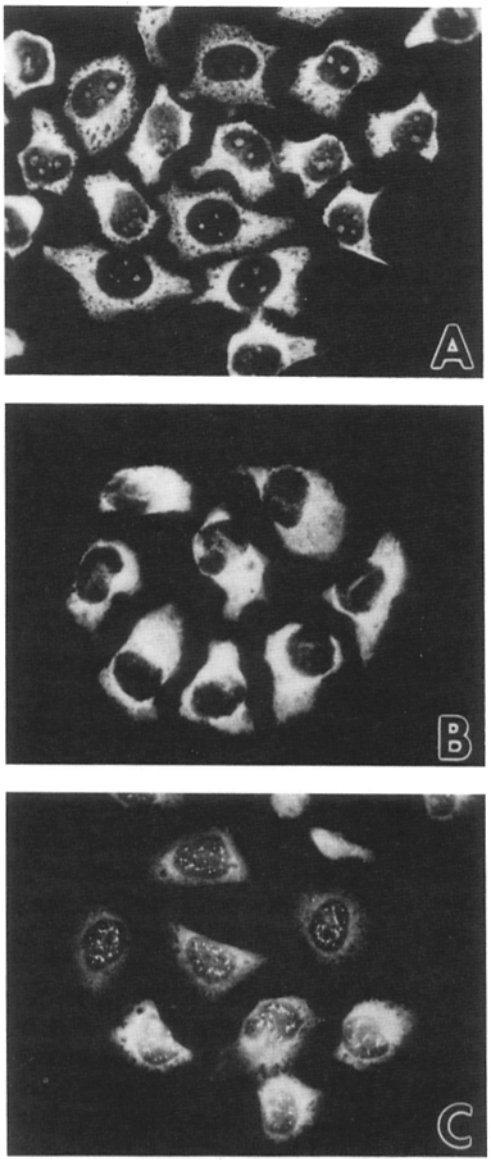

Figure 2. Immunofluorescent staining of methanol-fixed HeLa cells by IgMλ RF. LBR150 and LBR271 stained HeLa cells in an identical pattern. LBR295, LBR962, LBR1045, and LBR1189 also stained HeLa similarly. LBR1104, PHB1213, and KES643 stained HeLa with distinct patterns. Three of the RF staining are presented: (*A*) LBR150, (*B*) PHB1213, and (*C*) LBR295. Although the staining is predominantly intracellular and cytoplasmic, their nuclear staining patterns differ from each other. LBR150 stains nucleoli. Filamentous staining is apparent over the nuclei in the case of PHB1213. The nuclear staining of LBR295 looks speckled.

ysis revealed that the LBR295 and LBR150 groups were reactive with high molecular weight MAPs (Fig. 1, lanes *1* and *3*) but had different blot patterns. PHB1213 reacted weakly with the LBR150-reactive bands, although the protein concentrations in both supernatants were similar (34 μg/ml vs. 36 μg/ml). LBR1104 and KES643 did not recognize any of these MAP Ag in Western blot analysis, indicating that the MAP epitopes detected in ELISA were destroyed by SDS denaturation.

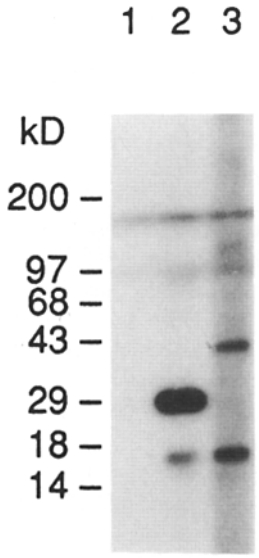

Figure 3. IgMλ RF blot different bands in Western blot analysis with HeLa cell extract as substrate. Both LBR295 (lane *2*) and LBR150 (lane *3*) stained a 17-kD band. In addition, LBR150 reacts strongly with a 40-kD component and LBR295 reacts comparably with a 29-kD band. A control supernatant with IgM of unknown specificity (lane *1*) was used as a control.

Further analyses revealed that five of the IgMλ RF were reactive with a group A streptococcal cell wall extract. All nine of the IgMλ RF and one of the two IgMκ RF stained HeLa cells intracellularly. The staining patterns of HeLa appeared to be discernable for each group of the RF (Fig. 2). By Western blotting using an extract of HeLa cells as substrate, these RF also had unique reactivity patterns. As shown in Fig. 3, LBR150 (lane *3*) and LBR295 (lane *2*) both stained a 17-kD band. However, these two RF recognized two other different cytoplasmic HeLa constituents: LBR150 reacted strongly with a 40-kD component, whereas LBR295 reacted comparably with a 29-kD band. Similar results were obtained when the HeLa extract was electrophoresed under nonreducing conditions. KES643, PHB1213, and LBR1104 blotted faintly different bands than those stained by LBR150 and LBR295.

Despite the multireactive nature of these RF, their affinity constants for rabbit IgG were relatively high, ranging from 10^{-6} to 10^{-7} M. For the LBR150 and LBR295 groups, the affinity constants were 2.8×10^{-7} M and 2.5×10^{-6} M, respectively. For PHB1213, the affinity constant for rabbit IgG was 1.6×10^{-6} M. In contrast, it had a higher binding constant for myosin (6.3×10^{-8} M).

In an attempt to demonstrate circulating RF or RF in synovial fluid with sole reactivity to rabbit IgG, the sera of the studied patients and those of six additional patients as well as six synovial fluid samples with high RF titers to both human and rabbit IgG were selected for absorption with human IgG linked to Sepharose 4B. None of these samples had demonstrable titer to rabbit IgG after extensive absorptions. These negative results may be due to in vivo absorptions and removal of the RF of interest by reactive autoantigens.

Usage of Multiple V_H Genes by IgMλ RF. The V gene utilization of the IgMλ RF was determined from sequence analyses of the cDNA encoding the V_H and V_λ portions of these Ab. One group, LBR150 and LBR271, had identical V_H and V_λ sequences. They were also grouped together in

the preceding section by virtue of similar Ag reactivity. The V_H-encoded regions of a second group, LBR295, LBR962, LBR1045, and LBR1189, were also identical, as were their $V\lambda$ regions. Thus, these latter four RF were related, and the B cells secreting them were the progeny of a single progenitor B cell. They also exhibited essentially identical binding specificities as described above.

Based on published data on human V_H genes (30, 31), the germline counterparts of the V_H segments utilized by the IgMλ RF have been identified. The cDNA sequences encoding the V_H segments of the RF have been submitted to the GenBank. Only the V_H cDNA sequence of RF LBR295, which is highly mutated, is shown.

The V_H-encoded portions of LBR150 and LBR271 were 100% homologous with that of the V_H3 germline dp38 (30). This gene is identical to the gene designated as 3-15 by Matsuda et al. (31). However, to date, the GenBank database contains no germline gene with a leader sequence identical to those of LBR150 and LBR271. The D and J_H regions of the LBR150 H chain were classified as D4 (32) and J_H4, respectively.

The V_H sequence of LBR295 was homologous to the V_H1 germline hsvi41b which was originally designated as 1-4b by Shin et al. (33). It had a leader sequence identical to that of the germline gene (Fig. 4). The V_H region of hsvi41b is most homologous to dp21 (30) with the exception of two substitutions in FR3. They are likely to be allelic genes. Thus dp21/1-4b is likely to be the germline for LBR295 V_H. The V_H region of LBR295 contained a DN4 segment (34) and a J_H region with a silent nucleotide substitution. There were 15 nucleotide substitutions that resulted in 13 amino acid substitutions (mostly in CDR1 and FR3).

The leader and V_H sequences of LBR1104 were identical to those of hswhg16g (35). This gene has been designated as 3-12 by Matsuda et al. (31). The V_H of LBR1104 was also completely identical to dp77 (30). Both DN1 (34) and D21/10 (32) segments were identified in its CDR3. It used a J_H4 segment.

The 5′ untranslated and leader sequences of PHB1213 were highly homologous to those of the germline gene humhv1051 (36). A single mutation was identified at the 5′ untranslated region. The V_H segment of PHB1213 was homologous to that encoded by the germline genes humhv1051 and hsigdp10 except for a single mutation in FR3. It appears that the V_H of PHB1213 was slightly mutated. It contained a DXP′1 region (34) and a J_H6 region. The V_H segment of this RF was homologous to the fetal V_H cDNA 51P1 (37) which is completely homologous to the coding regions of humhv1051 and dp10.

The leader sequence of the H chain of KES643 was identical to that of hsigv79 (38), a germline gene homologous with two other V_H4 germline genes, dp70 (30) and hsvh419 (39). The V_H germline gene hsigv79 has been designated as 4-4b by Shin et al. (33). It appears that hsigv79/4-4b and dp70/hsvh419 are allelic genes and that the V_H of KES643 is in germline configuration. KES643 also contained a DN1 (34) segment and a J_H4 segment.

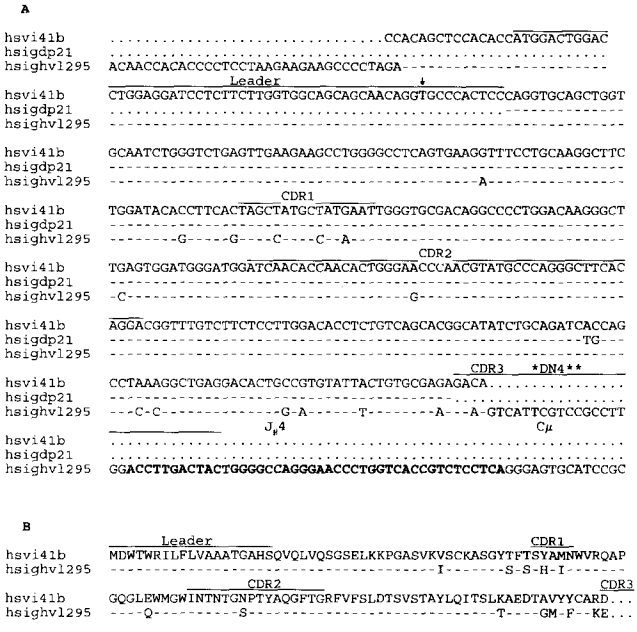

Figure 4. Identical V_H regions are utilized by LBR295 (*hsighvl295*), LBR962 (hsighvl962), LBR1045 (hsighvl1045), and LBR1189 (hsighvl1189). They are derived from the germline hsvi41b/hsigdp21 which is a member of the V_H1 family. Hsighv1295 is shown here (*A*). The leader and CDR are assigned and marked by a solid line, J_H is in bold, and D is denoted by asterisks. The intron of hsvi41b has been deleted. (↓) The position of the deleted intron of the germline. The deduced amino acid sequences are included (*B*). The identity between the leader sequences of the germline and the cDNA of these V_H indicates that this assignment is likely to be correct. There are 15 substitutions in the V_H, which result in 13 amino acid substitutions. Most substitutions are in CDR1 and FR3. The sequence data of hsighvl295 are available from EMBL/GenBank/DDBJ under accession number L29117.

The V_H, D, and J_H region usage of the nine IgMλ RF is summarized in Table 3.

Preferential Usage of VλIIIb Genes by IgMλ Anti-Rabbit IgG RF. The V regions of the λ chains of the nine IgMλ RF serologically classified as $V\lambda$IIIb were sequenced at the cDNA level (Figs. 5–8). LBR150 and LBR271 contained identical 5′ untranslated, leader, $V\lambda$, and $J\lambda1$ gene segments. PHB1213 differed only in that nine additional nucleotides (TATCCAGGG) were present at the 3′ end of the CDR3 segment (Fig. 5). With this exception, these three λ chains were most likely derived from a single $V\lambda$ germline gene. The cDNA sequences of the λ chains of LBR295, LBR962, LBR1045, and LBR1189 shared identical untranslated, leader, CDR3, and $J\lambda1$ gene segments (Fig. 6). They differed from one another by zero to three nucleotide substitutions and were also seemingly the product of the same germline gene, as was KES643, which had untranslated and leader sequences identical to those of the LBR295 group and remarkably homologous framework regions (FR) and CDR. The J segment of KES643 was $J\lambda2/3$ (Figs. 6 and 7). The $V\lambda$ cDNA sequence of LBR1104 had completely different untranslated and leader sequences from those of the LBR150 and LBR295 groups but also contained a $J\lambda2/3$ segment.

The L chain sequences of the $V\lambda$-encoded portion of all

Table 3. *Five V_H Genes Are Used by Nine IgMλ RF. Eight of Them Use J_H4*

RF	V_H designation	V_H family	Corresponding germline gene	Distance of V_H from J_H	D	J_H
				kb		
LBR 295	hsighvl295	1	dp21*/1-4b‡	155‡	DN4§	4
			Highly mutated			
LBR 962	hsighvl962	"	"	"	"	"
LBR 1045	hsighvl1045	"	"	"	"	"
LBR 1189	hsighvl1189	"	"	"	"	"
LBR 150	hsighvl150	3	dp38*/3-15‡	280‡	D4‖	4
			Unmutated			
LBR 271	hsighvl271	"	"	"	"	"
LBR 1104	hsighvl1104	3	dp77*/3-21‡	360‡	DN1§	6
			Unmutated		D21/10‖	
PHB 1213	hsighvp1213	1	dp10 (51P1)*/hv1051¶	>800‡	DXP'1§	4
			Slightly mutated			
KES 643	hsighvk643	4	dp70*/4-4b**	145‡	DN1§	4
			Mutated			

* Tomlinson et al. (30).
‡ Matsuda et al. (31).
§ Ichihara et al. (34).
‖ Buluwela et al. (32).
¶ Yang et al. (36).
** Shin et al. (33).

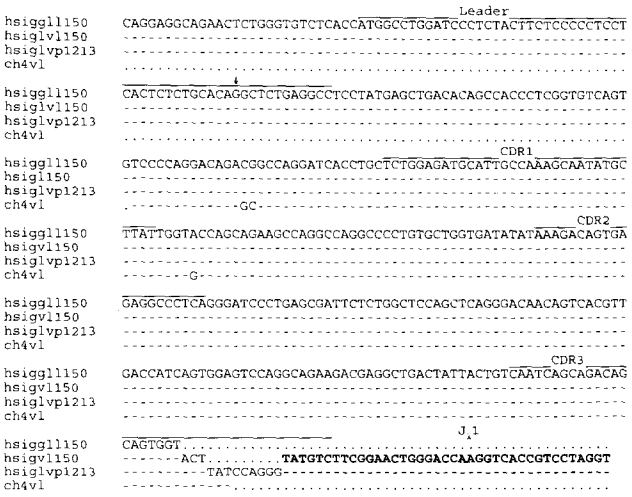

Figure 5. LBR150 (*hsiglvl150*) and LBR271 (hsiglvl271, not shown) use identical untranslated, leader, Vλ, and Jλ1 gene segments. PHB1213 (*hsig1vp1213*) differs from this sequence only in the addition of nine extra nucleotides in CDR3. These cDNA use the same unmutated germline gene hsiggll150. The leader and CDR are assigned and marked by a solid line. J_λ is in bold. (↓) The deletion of the intron. The cDNA, ch4v1 from Wong et al. (14) also uses this Vλ germline but is Jλ2/3. The differing Jλ has been deleted for our comparison. Accession numbers: L29165 for hsiggll150, L29153 for hsiglvl150, and L29163 for hsiglvp1213.

nine IgMλ RF, as deduced from cDNA analyses, were >90% homologous with the serologically and chemically defined sub-subgroup of λIII proteins designated λIIIb by Eulitz et al. (15). In contrast, these L chains were <80% homologous to proteins classified as λIIIa or λIIIc. These findings, in general agreement with the serological classification, indicated that the L chains of these anti-rabbit IgM RF were encoded by VλIIIb-related genes.

Identification of Two VλIIIb Germline Genes. The germline gene counterparts of the V_λ cDNA sequences of the anti-rabbit IgG RF were not identified in the database of the GenBank. We used the method of Sota-Gil et al. (28) to generate the germline genes encoding these λIIIb chains by PCR from the genomic DNA of patient LBR. Two of the three germline genes, designated hsiggll150 and hsiggll295, were identified. As indicated in Fig. 5, LBR150, PHB1213, and LBR271 (not shown) represented the unmutated products of the hsiggll150 gene, whereas three substitutions have been found in the FR of the L chain (ch4Vλ) in mAb H4, another anti-rabbit IgG IgMλ RF (14). In the case of hsiggll150, we have also identified an identical germline, by PCR, in the genome of two unrelated normal individuals. In contrast to the LBR150 group, the cDNAs of LBR295, LBR962, LBR1045, and LBR1189, when compared with their

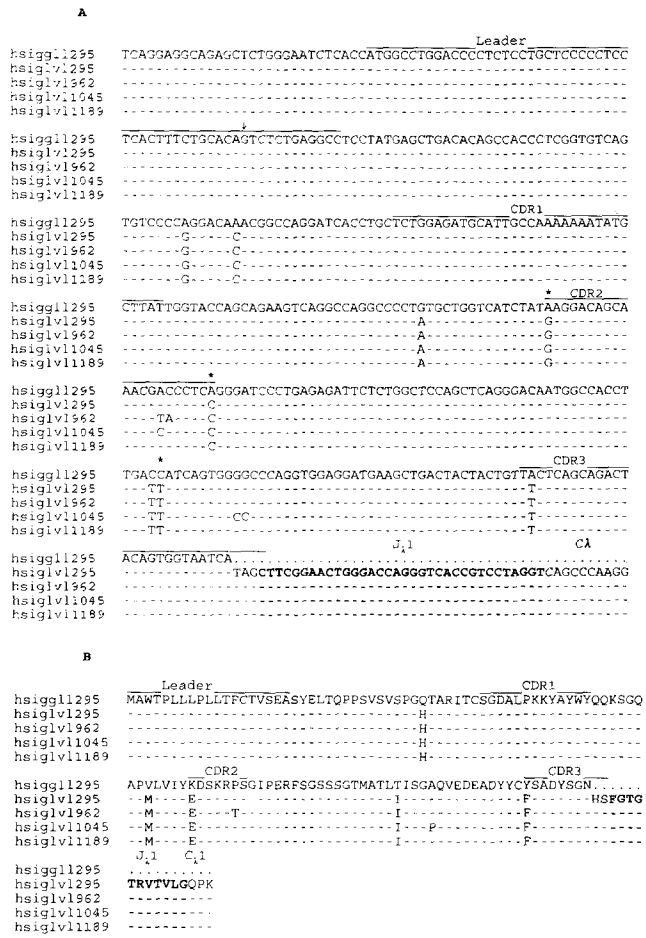

Figure 6. The Vλ cDNA sequences of LBR295 (*hsiglvl295*), LBR962 (*hsiglvl962*), LBR1045 (*hsiglvl1045*), and LBR1189 (*hsiglvl1189*) share identical untranslated, leader, CDR3, and Jλ1 gene segments (*A*). They differ from one another by zero to three substitutions. The gene, hsigg11295 is the most likely germline gene for these cDNAs. It shares an identical untranslated and leader sequence. There are 7–10 base substitutions which result in 5–6 amino acid substitutions. The leader and CDR are assigned and marked by a solid line. J_λ is in bold. (↓) The deletion of the intron from germline sequence. (*) The three substitutions that are identical to those found in KES643. The deduced amino acid sequences of the germline and cDNA more clearly show the substitutions in both FR and CDRs (*B*). Accession numbers: L29165 for hsiggll295, L29158 for hsiglvl295, L29159 for hsiglvl962, L29160 for hsiglvl1045, and L29161 for hsiglvl1189.

germline counterparts, contained a considerable number of FR and CDR substitutions (Fig. 6 *A*) that resulted in five or six amino acid changes (Fig. 6 *B*). There were fewer nucleotide substitutions in the V_λ of KES643 when compared with the germline gene hsiggll295. Moreover, three of the substitutions occurred at identical positions (Figs. 6 and 7). The deduced amino acid sequences of the two V_λIIIb germline genes hsiggll50 and hsiggll295 were most homologous with that found among $_\lambda$IIIb proteins (15). We have not as yet identified the germline gene from which LBR1104 was derived. However, as shown in Fig. 8, the V_λ portion of LBR1104 (hsiglvl1104) was more closely related to the two newly identified V_λIIIb germline genes than the V_λIII germ-

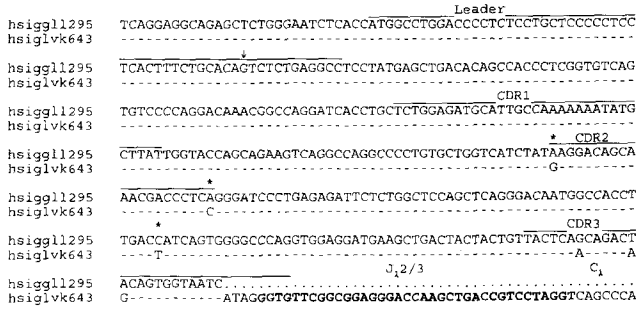

Figure 7. The Vλ cDNA sequence of KES643 (*hsiglvk643*) is homologous to germline hsiggll295 with two substitutions in CDR2, one in FR2, and three in CDR3. The leader and CDR are assigned and marked by a solid line. J_λ is in bold. (↓) The deletion of the germline intron. (*) The three substitutions that are identical to those found in the cDNAs of LBR295, LBR962, LBR1045, and LBR1189. Accession number: L29164 for hsiglvk643.

line gene hsigvlo11 (V_λIII.1) (40) which show the greatest homology with the L chain of LBR1104 among the L chain germline sequences currently available in the GenBank.

The V_λ and J_λ region usage of the nine IgMλRF is summarized in Table 4.

Discussion

A large number of IgM RF specific for the Fc region of rabbit IgG have been obtained through generation of multiple EBV-transformed B cell lines and hybridomas established from PB and synovial tissue of patients with RA and related disorders. These anti-rabbit IgG RF had relatively high binding affinity constants (10^{-6} to 10^{-7} M) and contained predominantly λ L chains. The nonreactivity of these Ab

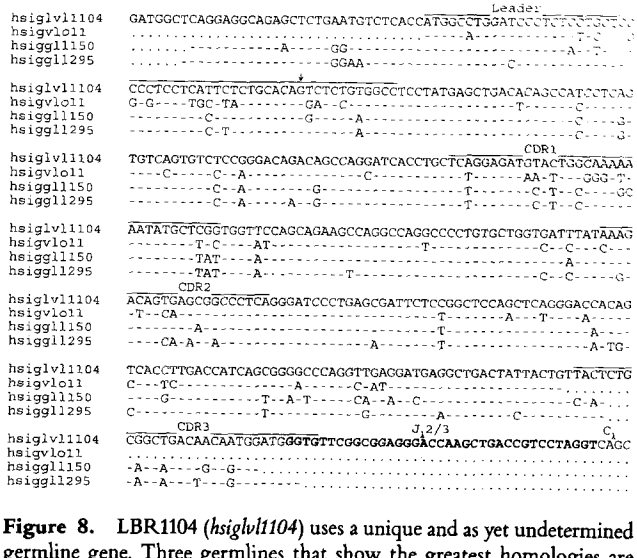

Figure 8. LBR1104 (*hsiglvl1104*) uses a unique and as yet undetermined germline gene. Three germlines that show the greatest homologies are hsiggll150, hsiggll295, and hsigvlo11 (40). It is more homologous to hsiggll150 and hsiggll295 than to hsigvl011. The leader and CDR are assigned and marked by a solid line. J_λ is in bold. (↓) The deletion of the introns from the germline.

Table 4. *Three V_λIIIb Genes Are Used by Nine IgMλ* RF. Both J_λ1 and J_λ2/3 Are Used

RF	V_λ designation	V_λ sub-family	Corresponding germline gene	J_λ
LBR 295	hsiglvl295	IIIb	hsiggll295 Highly mutated	1
LBR 962	hsiglvl962	"	"	"
LBR 1045	hsiglvl1045	"	"	"
LBR 1089	hsiglvl1189	"	"	"
LBR 150	hsiglvl150	"	hsiggll150 Unmutated	1
LBR 271	hsiglvl271	"	"	"
LBR 1104	hsiglvl1104	"	hsiggll1104 (Not yet identified)	2/3
PHB 1213	hsiglvp1213	"	hsiggll150 Unmutated	1
KES 643	hsiglvk643	"	hsiggll295 Mutated	2/3

with human IgG was established by inhibition studies using heat-aggregated IgG. Despite the lack of reactivity with other Ag often recognized by anti-human IgM RF including IgG from other mammalian species, these RF recognized epitopes associated with cytoskeletal proteins and HeLa cells (Table 2). Additionally, the reactivity with a group A streptococcal cell wall extract of certain anti-rabbit IgG RF is of considerable interest because of the role of streptococcal infections in rheumatic fever and the poststreptococcal autoimmune process related to the production of antistreptococcal Ab cross-reactive with human and mouse tissue (41). We have also found that 21 supernatants of our LBR cell lines derived from synovial tissue were highly reactive with whole streptococcal extracts (Cunningham, M. W., and S. M. Fu, unpublished results). The multireactivity of anti-rabbit IgG RF may have pathogenic importance in that these Ab, which are found in patients with RA and other related diseases, may be induced by a variety of cellular autoantigens, most notably cytoskeletal proteins, and by streptococcal infection. In this regard, it is of interest to note that a higher affinity constant to myosin was obtained in the case of PHB1213. The preferential homing of the B cells related to these RF to the synovium adds support to this thesis.

The five patterns of reactivity noted among the anti-rabbit IgG RF (Table 2) could be related to specific V_H or V_L gene usage. These five reactivity patterns are due to unique combinations of five V_H and three V_L genes. Whereas the inciting Ag and its epitopes are not known, this observation suggests that different epitopes select unique V_H and V_L combinations. This has been demonstrated in a study by Stark and Caton (42). mAb that are specific for a single amino acid interchange in a defined epitope of the influenza virus hemagglutinin use structurally distinct V regions. Thus, the use of diverse V_H and V_L gene segments by human autoantibodies (recently reviewed in 13, 43–46) is to be expected because diverse epitopes are present in most autoantigens commonly studied. With better defined epitopes, the use of V_H and V_L by autoantibodies will undoubtedly be more restricted.

Five distinct V_H gene segments, including V_H1, V_H3, and V_H4, were identified among our nine anti-rabbit IgG λ-type IgM RF (Table 3). Another RF with similar reactivity, H4 (14), contained V_H26, a V_H3 gene designated as 3-23 (31) and located ~395 kb from the J_H. Thus, these V_H genes are scattered throughout the V_H locus. As stated previously in the Results section, several of these V_H genes are encountered in fetal repertoire, and all of them have been utilized by autoantibodies of various specificities. The V_H1, V_H3, and V_H4 families have a large number of genes (30, 31), and members of these families are well presented in the repertoire of human autoantibodies and fetal repertoire (13, 43–46). Recently, we have identified a V_H2 RF (47) even though V_H2 is a small family. Thus, our data add support to the conclusions that multiple V_H genes are used by RF and other autoantibodies and that the V_H gene representation in autoantibodies reflects the normal human B cell repertoire (45).

Serologically, 10 of the 13 λ L chains of the anti-rabbit IgG RF were typed as members of the λIIIb sub-subgroup. Two others, serologically typed to be λIIIa/b, were classified to be λIIIb by amino acid sequence (deduced from their cDNA sequences) analysis. The overwhelming presence of λIIIb L chains among these Ab is in marked contrast to the

distribution of this sub-subgroup among λ-type Ig molecules in the serum of normal individuals, in whom these proteins only constitute ~10–13% of the total Igλ population (48). The preferential usage of V_λIIIb-related genes by these RF was confirmed through cDNA sequence analyses of nine such Ab. This remarkable restriction in V_λ gene utilization by anti-rabbit IgG RF extends the observation of Wong et al. (14), who found that H4, a RF monospecific for rabbit IgG, also contained $_\lambda$IIIb L chains. These data provide the first evidence that particular L chain genes, as a result of antigenic stimulus or genetic factors, are selectively utilized by certain types of RF.

Our studies have also provided new information on the human λ-L chain genome. Two heretofore undescribed V_λIIIb-related germline genes were identified that encoded the L chain V gene segments of certain anti-rabbit IgG RF. Based on cDNA sequence analyses of our nine RF, this gene family consists of at least three different members. Remarkably, two of these three were used by RF derived from two different individuals. Based on nucleotide sequence homology, the $_\lambda$IIIb L chains of the anti-rabbit IgG RF H4 (14) were most closely related to those of LBR150 and, thus, were also products of the germline hsiggll150 gene. Ermel et al. (49) have recently reported that two other synovial cell-derived anti-rabbit IgG RF from another RF patient contained $_\lambda$IIIb L chains identical to those of RF H4 (14). The predominance of $_\lambda$IIIb L chains among RF with comparable specificity, especially those that are related to the hsiggll150 V_λIIIb germline gene, implies the functional importance of this gene in certain patients with RA and related diseases.

The demonstration that the V_H or V_L portions of certain RF are derived from unmutated germline genes and that others have extensive somatic mutations (13) is of pathogenic importance. Among our anti-rabbit IgG RF, four (LBR295, LBR962, LBR1045, and LBR1189) had extensive FR and CDR mutations as compared with their germline counterparts. These RF are among the multireactive Ab with the most mutated V regions. It is also of considerable interest that the L chain of KES643 was derived from a germline similar to that of LBR 295 with identical nucleotide substitutions at three identical positions in CDR2 and FR3. These identical substitutions were unlikely to be attributable to a random event and were likely under selective pressure, reflecting an Ag-driven process. Thus, multireactive Ab should not be excluded from the repertoire generated by an Ag-driven process.

In summary, we have demonstrated that the L chains of IgMλ Ab with exclusive specificity for rabbit IgG, were predominantly products of the relatively uncommon V_λIIIb-gene family. Additionally, some of these RF reacted with a variety of proteins, cellular, and bacterial Ag. The findings of selective L chain gene usage and the multireactivity of such Ab provide new information on the molecular properties of RF and further insight into the etiology of RA and other autoimmune diseases.

We thank Dr. G. W. Litman, and R. T. Litman of the University of South Florida (St. Petersburg, FL) for their helpful advice. Dr. M. Cunningham's help in ELISA with streptococcal extract is much appreciated. The expert technical ability of T. Cook and the late Gail M. Garner is gratefully acknowledged. We thank T. Critzer for her help in preparing this manuscript.

This work was supported in part by the National Institutes of Health grants AR-39254 and AR-42207 (to S. M. Fu); AG-06348 and NS-22489 (to F. Gaskin); UVa Cancer Center Support Grant P30-CA44579; CA-10056 (to A. Solomon); and AR-20614, AR-03555, AI-18955, DK40117, AI-18745, and a VA Merit Award (to W. J. Koopman). Dr. A. Solomon is an American Cancer Society Clinical Research Professor.

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Received for publication 10 November 1993 and in revised form 27 January 1994.

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