

V_H GENE EXPRESSION IS RESTRICTED IN ANTI-IgG ANTIBODIES FROM MRL AUTOIMMUNE MICE

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Rheumatoid factors (RF),¹ autoantibodies that bind to the Fc fragment of IgG, were first identified in the sera of patients with rheumatoid arthritis (RA; 1, 2). The presence of RF has since been shown in the sera of patients with other rheumatic diseases (3) and with a variety of other chronic or infectious diseases such as subacute bacterial endocarditis, tuberculosis, and sarcoidosis (4–6). RF associated with RA characteristically exhibit a broad range of binding specificities for homologous and heterologous IgGs, whereas RF associated with nonrheumatic diseases bind preferentially to human IgG (4).

RF production, a hallmark of RA, may contribute to tissue injury in this disease. RF-containing immune complexes have been seen (7–9) in sera and synovial fluids and within phagocytes of patients with RA. The capacity of IgG and IgM RF to activate complement under experimental conditions (10), the demonstration of complement cleavage products in synovial fluids of RA patients (11), and evidence of local RF production at sites of tissue inflammation in RA imply an active role for RF in tissue injury (12, 13).

Paradoxically, some evidence indicates that RF may represent normal components of the immune system (14). The spontaneous appearance of anti-IgG antibodies in the sera of unmanipulated normal mouse strains has been documented (15, 16). Moreover, RF have been identified during the course of anamnestic responses to soluble antigens in mice (17–19) and man (20). Other evidence suggests that RF may subserve a biologically significant role(s) in some immune responses by amplifying the function of specific antibodies (21–23).

Little is known of the molecular events underlying the expression of RF in either the pathologic or physiologic situation. Clones producing RF in the setting of autoimmune disease may use a unique set of germline genes that are under concerted regulation, or they may derive from the same germline pool as antibodies directed against exogenous antigens. Since somatic mutational and combinatorial events play an important role in determining antigen-binding specificity, these mechanisms could permit the synthesis of pathologic RF from the same germline repertoire that encodes responses to exogenous antigens. To

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¹Abbreviations used in this paper: C μ , constant region of IgM; RA, rheumatoid arthritis; RF, rheumatoid factors.

explore the genetic basis of autoantibody expression, we have taken advantage of the autoimmune mouse strain MRL/Mp *lpr/lpr*. This strain spontaneously produces high titers of anti-IgG antibodies, with detectable amounts in the serum as early as 6 wk of age (24). They also exhibit many clinical manifestations typical of human RA, including synovial cell proliferation, pannus formation, and eventual joint destruction (25, 26). Another advantage of using a mouse model system is that the genes encoding the Ig V_H regions of antibodies have been studied most extensively in mice, and they have been shown to be clustered together into at least eight homologous family groups ranging in size from 2 to 50 or 60 members tandemly linked on chromosome 12. Genes within a family share at least 75% homology, whereas genes of different families are usually <70% homologous (27, 28).

We have generated a panel of anti-IgG mAbs from young MRL mice raised under pathogen-free conditions by fusing unstimulated spleen cells with a non-secreting myeloma. Our major goals were to determine the binding specificities of each clone and to determine which V_H family encoded the antigen-binding portion of antibodies produced by each clone. We found that the monoclonals bound to different proteolytic fragments of IgG, to several IgG subclasses, and to IgG from other mammalian species just as do anti-IgG antibodies from patients with RA. We hybridized probes representing gene segments from seven V_H families with RNA from our anti-IgG clones, and we have found that one small V_H family preferentially contributes the codes for these anti-IgG antibodies regardless of the binding specificity.

These data indicate that the genetic codes for anti-IgG antibodies in young MRL mice are restricted in their heterogeneity even though the antigen-binding specificity is already heterogeneous. In addition, our finding that a single small V_H family contributes the codes for most of our anti-IgG antibodies raises questions concerning V_H gene expression and the regulation of that expression in autoimmunity.

Materials and Methods

Mice. Breeder pairs of MRL/Mp *lpr/lpr* mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were bred and maintained in Trexler type isolators (Germ-Free Supply Div., Standard Safety Equipment Co., Palatine, IL) until they were used in experiments. All food, water, bedding, and cages were autoclaved and entered aseptically into the isolators. Original breeders were later killed and screened for all common viral, mycoplasmal, bacterial, and parasitic pathogens. We performed gross and histopathologic examinations of all major organs to check for lesions. Serologic tests were performed for the following viruses: mouse hepatitis, Sendai, Theiler's poliomyelitis, minute virus of mice, pneumonia virus of mice, reovirus 3, K virus, polyoma, mouse adenovirus, mouse pox, mouse rotavirus, and lymphocytic choriomeningitis virus. Thereafter, representatives from several age groups in each isolator were killed and screened twice annually to insure pathogen-free status of the colony.

Hybridomas. Untreated spleen cells from 4–15-wk-old female MRL/Mp *lpr/lpr* mice and a 32-wk-old MRL/Mp +/+ mouse were fused with X63-Ag8.653 nonsecreting myeloma cells (29). The hybridomas were initially cultured in RPMI 1640 (Flow Laboratories, Inc., McLean, VA) supplemented with 12% FCS (Flow Laboratories, Inc.), glutamine (2.0 mM), MEM nonessential amino acids (0.01 mM), 2-ME (5×10^{-5} M), and penicillin (100 U/ml), streptomycin (100 μ g/ml), and HAT (50X stock; Flow Laboratories, Inc.). We screened culture supernatants from the resulting hybridomas for the presence

of IgM or IgA antibodies reactive with purified mouse IgG (Cappel Laboratories, Cochranville, PA) using an ELISA adaptation of an RIA for RF (30). Plates (Costar, Cambridge, MA) were coated with murine IgG at a concentration of 25 $\mu\text{g/ml}$ overnight at 4°C and then blocked with 0.15 M NaCl (pH 7.5) buffered with 0.02 M sodium phosphate (PBS) containing 1% BSA (Sigma Chemical Co., St. Louis, MO). Wells coated with PBS/1% BSA served as a control. Undiluted culture supernatants (100 μl) were added to wells and incubated overnight at 4°C. After four rinses with PBS, we added 100 μl of alkaline phosphatase-labeled goat antibody directed against IgM or IgA (Southern Biotechnology Associates, Birmingham, AL; the specificity of each goat antisera was checked by ELISA against IgG-, IgM-, and IgA-coated plates), incubated this for 6 h at room temperature, and rinsed it five times with PBS. The assay was developed with 1.0 mg/ml *p*-nitrophenyl phosphate (Sigma Chemical Co.) in 1 M diethanolamine buffer (pH 9.8) containing 0.5 mM MgCl₂; after a 30 min development, the absorbance was read at 405 nm in a Titer Tek Multiskan MC ELISA reader (Flow Laboratories, Inc.). Hybrids that exhibited strong reactivity (OD >0.8) with mouse IgG were cloned by limiting dilution in the same medium used for fusions (without HAT) and subcloned a minimum of four more times until each was stable.

Binding Assays. Binding of antibodies from selected cell hybrids to murine IgG subclasses, heterologous IgG, and proteolytic fragments of mouse IgG were analyzed as follows. Monoclonal IgG subclass proteins used were IgG1 (MOPC 21), IgG2a (UPC 10, HOPC-1), IgG2b (MOPC 195) and IgG3 (J606) (Litton Bionetics, Charleston, SC). We verified by ELISA that concentrations of all IgG subclasses coated onto the wells were comparable. Fragments of polyclonal mouse IgG [Fc, Fab, (Fab')₂] were obtained from Jackson ImmunoResearch Laboratories (Avondale, PA) and were analyzed for purity by immunodiffusion and RIA before use. Cat, horse, pig, rat, goat, rabbit, cow, and guinea pig IgGs were purchased from Pel-Freeze Biologicals (Rogers, AR) and human IgG was isolated from a pooled Cohn II fraction, as previously described (30). Polystyrene Immulon 2 Removawell strips (Dynatech Laboratories, Inc., Alexandria, VA) were coated overnight at 4°C with the IgG preparations described above at 25 $\mu\text{g/ml}$ or with goat anti-mouse IgM at 2.5 $\mu\text{g/ml}$, and were subsequently blocked with PBS containing 1% BSA for 1 h at room temperature. IgM concentrations for each hybridoma culture supernatant were determined by solid-phase RIA using the anti-IgM-coated plates and MOPC 104E as the IgM standard. Binding studies with heterologous IgGs and murine IgG fragments were done with each supernatant at concentrations ranging from 1 to 250 ng/ml of IgM. Samples were incubated overnight at 4°C, rinsed four times with PBS, then incubated overnight at 4°C with ¹²⁵I-labeled goat anti-mouse IgM (31). After extensive washing with PBS, wells were counted in a gamma counter (Beckman Instruments, Inc., Fullerton, CA).

All clones were also assayed for reactivity with double-stranded B-DNA (calf thymus DNA; Worthington Biochemicals Corp., Freehold, NJ) and Z-DNA [brominated poly(dG-dC)-poly(dG-dC)], a gift from Drs. Robert Bergen and Robert Wells (University of Alabama at Birmingham). Single-stranded DNA was prepared by heating the double-stranded calf thymus DNA in a boiling water bath for 10 min and plunging it immediately into an ice-water bath to prevent reannealing. Polystyrene wells were precoated with 150 μl (10 $\mu\text{g/ml}$) poly-L-lysine (Sigma Chemical Co.) for 1 h at room temperature, then were coated with 150 μl of the appropriate DNA at a concentration of 2.5 $\mu\text{g/ml}$ for 4 h at room temperature and then blocked with PBS containing 1% BSA for 1 h. Plates precoated with poly-L-lysine were blocked with poly-L-glutamate (50 $\mu\text{g/ml}$) and used as controls. Culture supernatants were added at IgM concentrations ranging from 10 to 1,000 ng/ml and binding was determined by RIA.

RNA Extraction and Dot Blots. Viable hybridoma cells were adjusted to 2×10^5 cells/ml. 10 ml were washed in ice-cold PBS, centrifuged, and the cell pellet was resuspended in 45 μl Tris/EDTA buffer (10 mM Tris, 1 mM EDTA [pH 7.6]) containing 2,000 U/ml of Ribonuclease Inhibitor (Sigma Chemical Co.). Samples were brought to 0.5% NP-40 with gentle mixing on ice for 5 min and were centrifuged in Eppendorf tubes for 3 min to

pellet nuclei and mitochondria. Supernatants (50 μ l) were transferred to fresh tubes containing 30 μ l 20X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate) and 20 μ l 37% formaldehyde, and they were incubated at 65°C for 15 min. Samples were initially diluted 1:10 in 15X SSC and serially diluted with a final dilution of 1:320. Aliquots (100 μ l) of each dilution were applied to BA85 nitrocellulose filter paper in a 96-well filtration manifold (Schleicher & Schuell, Keene, NH). Filters were air-dried, baked for 2 h at 80°C in vacuo, and prehybridized for 4 h at 42°C in buffer containing 50% formamide, 5X SSC, 50 mM Na₂HPO₄, 0.2% SDS, 100 μ g/ml boiled salmon sperm DNA, 100 μ g/ml heparin, and 5X Denhardt's solution (1X = 0.05% each of ficoll, polyvinylpyrrolidone, and BSA). The hybridization buffer was the same but with a 1X Denhardt's concentration and 2×10^6 cpm/ml ³²P-labeled probe. Hybridization of dot blots was done at 42°C for 16 h, followed by three low-stringency washes (2X SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 55°C) and three high-stringency washes (0.2X SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 60°C). Filters were then blotted with paper towels, wrapped in plastic wrap, and exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY) at -70°C using a Cronex Lightning-plus intensifying screen (DuPont Co., Wilmington, DE). Films were developed after 18 or 24 h and filters not displaying hybridizations after this time were reexposed for 48 h to allow low-homology hybridization to be detected.

Probes. Representatives from seven of the eight identified murine V_H families were tested for hybridization to filters containing RNA from each clone. The probes used were pSPV_H 81X (0.28 kb) from the 7183 family (32), pBS107V_H (0.3 kb) from the S107 family (33), pUV_HQ52 (0.3 kb) from the Q52 family (27), pBV_HJ558 (1.0 kb) from the J558 family (27), pBV_HJ606 (0.6 kb) from the J606 family, pB6.3R1 (2.3 kb) from the 36-60 family (34), 460-42 (0.22 kb) from the 36-60 family (34), and V_H 23-9 (0.4 kb) from the 3609 family (28). Since all clones were producing IgM, we used a cDNA probe representing the C μ constant region, pB μ ₁₂ (1.0 kb), as a positive control (35).

All probe inserts were isolated from vectors by enzyme digestion, size fractionation by agarose gel electrophoresis, electroelution into a 7.5 M ammonium acetate bridge (International Biotechnologies Inc., New Haven, CT), and ethanol precipitation. Purified inserts (50–100 ng) were nick-translated with ³²P-deoxynucleotides (36) to a specific activity of $>10^8$ cpm/ μ g, and they were added to the hybridization buffer at a concentration of 2×10^6 cpm/ml.

Densitometric Analyses. Autoradiograms of RNA dot blots were scanned with a 2202 Ultrascan laser densitometer and curves integrated with GELSCAN software (LKB Instruments, Inc., Gaithersburg, MD) in an Apple IIe computer. Lanes showing hybridization with V_H probes were scanned and their hybridization intensities were compared with the C μ (constant region of the IgM) probe hybridization on the same filter. Filters were stripped of each V_H probe in 80% formamide in distilled water for 30 min at 60°C, were exposed to film overnight to insure that the stripping was complete, and were rehybridized with the C μ probe. All comparisons were performed at the same absorbance on the same intensity scale at a scan speed of 100 mm/min. The actual intensities are arbitrary units such that a full scale peak height of 10, with a full width at half maximum of 1.0, has an intensity of 1,000 U.

Results

Construction of Clones Secreting Anti-IgG Antibodies. Spleen cells from individual, unmanipulated pathogen-free female MRL/Mp *lpr/lpr* mice, ages 4, 8, 14, and 15 wk and a 32-wk-old female of the congenic strain MRL/Mp *+/+* were fused with X63-Ag8.653 nonsecreting myeloma cells (29) and plated into ten 24-well tissue culture plates (240 wells per fusion). After 2–3 wk, hybrids developed in 192 wells each from the 4 and 8 wk spleens, and 9.8% of the hybrids secreted IgM antibodies directed against IgG. Hybrids developed in all 240 wells from both the 14 and 15 wk spleens, and 2.5% produced IgM directed against mouse IgG. Fusion with the MRL/Mp *+/+* spleen yielded 142 hybrids,

TABLE I
Binding of Monoclonal Anti-IgG to Proteolytic Cleavage Fragments of Mouse IgG

Group	Clone	Age*	IgM (ng/ml)	Fc	Fab	(Fab') ₂
I	252H6	15	100	90 ± 3.4	—	—
	295A4	14	100	90 ± 2.8	—	—
II	253B2	15	250	17 ± 1.2	—	—
	295C6	14	250	19 ± 0.6	—	—
III	146C6 [‡]	8	500	—	48 ± 9.3	24 ± 3.0
	154B5	4	500	—	39 ± 3.6	20 ± 5.0
IV	291C4	14	500	—	—	—
	294D6	14	100	—	—	99 ± 3.5
	MRL +/+	32	500	—	—	—

Data represent the percent binding to proteolytic fragments of mouse IgG compared with the binding to mouse IgG. Percent binding = [(mean cpm proteolytic fragment - mean cpm BSA control)/(mean cpm intact IgG - mean cpm BSA)] × 100 ± [(SD of triplicates/mean of triplicates) × 100].

* Age of mice (wk) from which each clone was derived.

[‡] Both clones in Group III were IgM λ. All other clones were IgM κ.

only 1 (0.7%) of which secreted IgM anti-IgG. Hybrids from each fusion that showed the strongest reactivity with mouse IgG as determined by ELISA (OD >0.8) were cloned by limiting dilution and subcloned at least four more times to insure clonality and stability. Hybridomas generated from the MRL/Mp *lpr/lpr* mice were generally unstable and 60% were lost due to cell death, low cloning efficiency, or loss of antibody production. Antigen-binding properties and IgM concentrations of supernatants obtained from each initial cloning were compared with supernatants from each subclone to monitor against the selection of an undesired mutant during the multiple reclonings.

Binding Specificities of Anti-IgG Hybridomas. MRL/Mp *lpr/lpr* mice develop an arthritis with circulating RF similar to RA (25, 26). Since RF in the sera of RA patients generally display a broader range of binding specificities than non-RA-associated RF, we sought to determine whether or not our anti-IgG-producing clones from MRL mice had a broad or restricted range of antigen-binding capabilities. We therefore tested the capacity of each monoclonal to bind to different proteolytic cleavage fragments of IgG, subclasses of mouse IgG, and IgG from other mammalian species.

IgG Fragments. The results of the binding studies with proteolytic cleavage fragments of mouse IgG [Fc, Fab and (Fab')₂] and intact pooled mouse IgG are shown in Table I. Ages of the mice from which each clone was derived are also noted. Two clones, 252H6 (15 wk) and 295A4 (14 wk), reacted strongly with the Fc fragment of IgG but did not bind to other fragments. In contrast, clones 253B2 (15 wk) and 295C6 (14 wk) bound weakly to the Fc fragment and not to other IgG fragments. Two clones, 146C6 (8 wk) and 154B5 (4 wk), bound moderately to Fab and (Fab')₂ fragments but not to Fc. Both of these clones were IgM λ, while all other clones were IgM κ. Clone 294D6 (14 wk) bound to (Fab')₂ only and probably recognizes a determinant in or near the hinge region.

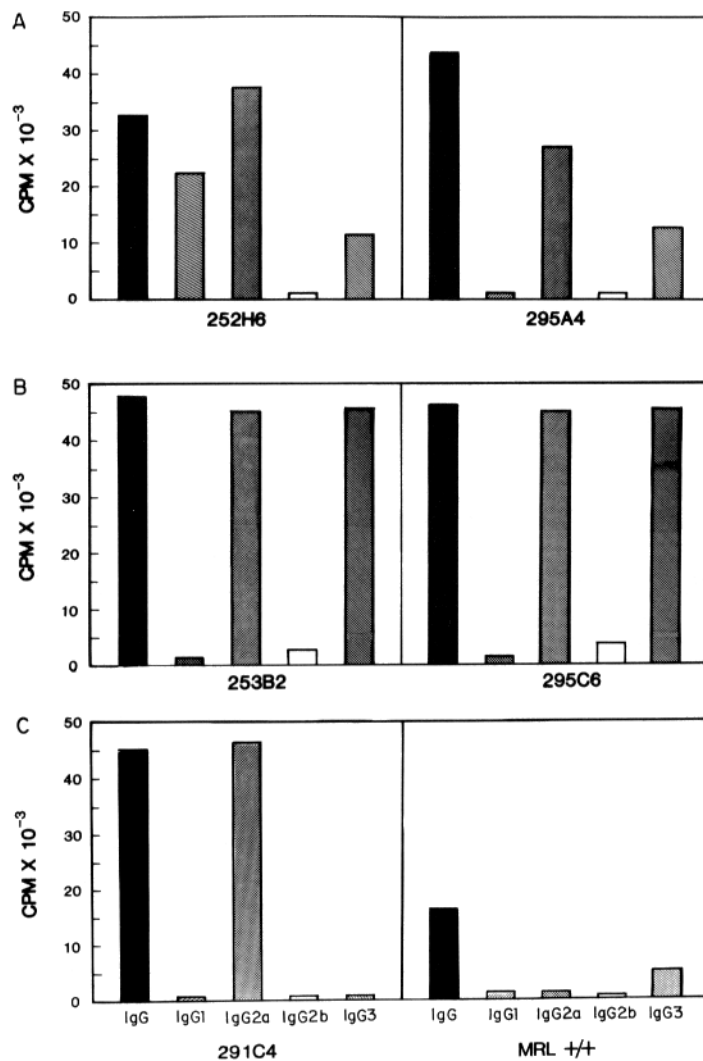


FIGURE 1. Culture supernatants (100 ng/ml) from individual clones were added to microtiter plates coated with mouse IgG (polyclonal) or monoclonal IgG subclasses. Histograms represent mean cpm binding to IgG or subclasses after subtraction of background binding of each sample to BSA (<800 cpm in each case). Assays were done in triplicate and standard deviations for each sample were <5% of mean.

Two clones, 291C4 (14 wk) and MRL +/+ (32 wk), reacted with intact IgG only. Based on this analysis, the clones could be divided conveniently into four groups. Group I clones showed strong binding only to Fc, while group II clones bound less avidly to Fc. Group III members reacted with both Fab and (Fab')₂ fragments, and group IV members recognized only intact IgG or (Fab')₂.

Murine IgG Subclasses. Both clones in group I recognized IgG2a and IgG3, and clone 252H6 also reacted with IgG1 (Fig. 1A). Group II clones each reacted preferentially with IgG2a and IgG3 (Fig. 1B). Two members of group IV displayed restricted binding to a single but distinct subclass; 291C4 preferentially

TABLE II
Binding of IgM Anti-IgG to Heterologous IgG

Group	Clone	Pig	Rat	Goat	Horse	Human	Rabbit	Bovine	Guinea Pig
I	252H6	—	—	—	—	21 ± 4.3	—	—	—
	295A4	—	—	—	—	—	—	—	—
II	253B2	73 ± 2.4	75 ± 4.0	81 ± 5.6	87 ± 2.5	81 ± 4.1	93 ± 3.9	80 ± 3.0	75 ± 6.5
	295C6	73 ± 3.2	73 ± 1.8	78 ± 2.4	85 ± 2.1	86 ± 2.1	93 ± 2.2	84 ± 5.0	74 ± 5.0
III	146C6	—	—	—	—	—	—	—	—
	154B5	—	—	—	—	—	—	—	—
IV	294D6	—	—	—	—	—	82 ± 2.0	—	—
	291C4	—	—	—	—	—	—	—	—
	MRL +/+	—	—	—	—	—	—	—	—

Data represent percent binding to heterologous IgG compared with mouse IgG at the same concentration of IgM (100 ng/ml). Percent binding = [(mean cpm heterologous IgG - mean cpm BSA control)/(mean cpm mouse IgG - mean cpm BSA control)] × 100 ± [(SD of triplicates/mean of triplicates) × 100].

reacted with IgG2a, while MRL +/+ bound to IgG3 (Fig. 1C). None of the anti-IgG antibodies exhibited significant binding to IgG2b. Group III clones and clone 294D6 of group IV did not show distinct subclass or L chain-associated binding (data not shown).

Heterologous IgG. The binding of each monoclonal anti-IgG antibody to pig, rat, human, horse, goat, cow, guinea pig, and rabbit IgG is shown in Table II. Only one member of group I, 252H6, reacted with heterologous IgG, and it crossreacted weakly with only human IgG. The clones that made up group II exhibited broad crossreactivity with heterologous IgG. In contrast, the members of group III reacted only with homologous IgG. One member of group IV, 294D6, reacted with rabbit IgG, whereas the other members did not bind to IgG of any species tested. Thus, clones residing within the same group displayed similar binding characteristics. These binding studies indicate that the members of group II in our panel of anti-IgG antibodies possess a broad binding pattern similar to RF in patients with RA, whereas members of the other groups show more restricted binding patterns.

Reactivity of anti-IgG mAbs with DNA. In some autoimmune diseases, patients synthesize antibodies directed against DNA, RNA, and/or protein-nucleic acid complexes. In fact, some RF in RA crossreact with nucleic acids either alone or as protein complexes (37). To determine whether or not any of our MRL anti-IgG antibodies also displayed such crossreactivity, we used a solid-phase RIA to test their reactivity with Z DNA (left-handed), B DNA (right-handed), and denatured (single-stranded) DNA (Table III). A monoclonal IgM κ hybridoma antibody (Za) specific for Z DNA and generated from an MRL/Mp *lpr/lpr* mouse served as a positive control.² The two clones in group I (295A4 and 252H6) both reacted strongly with Z DNA, and one, 295A4, also recognized B DNA; neither recognized single-stranded DNA. None of the other monoclonal anti-IgG antibodies at concentrations of 1.0 μ g/ml exhibited binding to DNA. Thus, a subset of our anti-IgG antibodies also exhibits crossreactivity with DNA.

²Bergen, H. R., M. J. Losman, T. O'Connor, W. Zacharias, J. E. Larson, R. D. Wells, and W. J. Koopman. Anti-Z-DNA monoclonal antibodies from unimmunized MRL/Mp-*lpr/lpr* mice. Manuscript submitted for publication.

TABLE III
Crossreactivity of Anti-IgG with Various DNA

Group	Clone	IgM (ng/ml)	Z DNA	Double-stranded DNA	Single-stranded DNA
	anti-Z DNA*	50	21,562	582	357
		100	26,846	529	565
		250	30,494	612	784
I	252H6	100	10,219	828	499
		250	17,359	1,014	1,037
	295A4	100	26,890	20,180	429
		250	35,065	29,924	1,020
II	253B2	1,000	1,952	1,301	475
	295C6	1,000	3,964	2,443	700
III	146C6	1,000	1,025	538	219
	154B5	1,000	687	373	371
IV	291C4	1,000	504	312	134
	294D6	1,000	3,875	1,536	637
	MRL +/-	1,000	3,737	2,555	503
	Background (no sample)		483	314	210

Microtiter wells were precoated with poly-L-lysine then coated with various DNA preparations at 2.5 µg/ml. Culture supernatants were diluted to 1,000; 250; and 100 ng IgM/ml and 100 µl was added to the plates. RIA was done in triplicates, and data represent (mean cpm with each DNA preparation - mean cpm BSA control). SD for each triplicate set was <5% of total cpm in each case.

* The positive control for Z DNA binding was a monoclonal IgM-κ (Za).

Identification of V_H Gene Families that Encode Anti-IgG mAbs. Since the MRL anti-IgG antibodies recognized a wide range of antigenic determinants, it seemed likely that they were encoded by many different V_H gene segments. To determine how diverse the codes for these V_H regions were, we hybridized probes from seven of the eight known murine V_H families with RNA extracted from each clone (Figs. 2-4). In all cases, a probe for C_μ was used as a positive control to estimate the quantity of H chain mRNA present for each clone, and hybridization intensities between C_μ and V_H were compared by laser densitometric scanning of the autoradiographic films. Six clones hybridized with only the 36-60 V_H family probes, pB6.3R1 (2.3 kb) and 460-42 (0.22 kb). The histograms in Fig. 2 depict intensities of hybridization with the C_μ probe and the 36-60 probes for five of the six clones that hybridized exclusively with probes from the 36-60 V_H family. The numbers below each set of histograms represent the ratio of the intensity of hybridization with the 36-60 probes to the intensity of hybridization with the C_μ probe. Filters hybridized with the C_μ (1.0 kb) or pB6.3R1 (2.3 kb) probes (Fig. 2, lower panel) were exposed 16 h, and the filter hybridized with the 460-42 (0.4 kb) probe (Fig. 2, upper panel) was exposed 48 h to compensate for the differences in the lengths of the probes (probes <0.6 kb were exposed to film for 48 h). All clones hybridized better with the pB6.3R1 probe than with

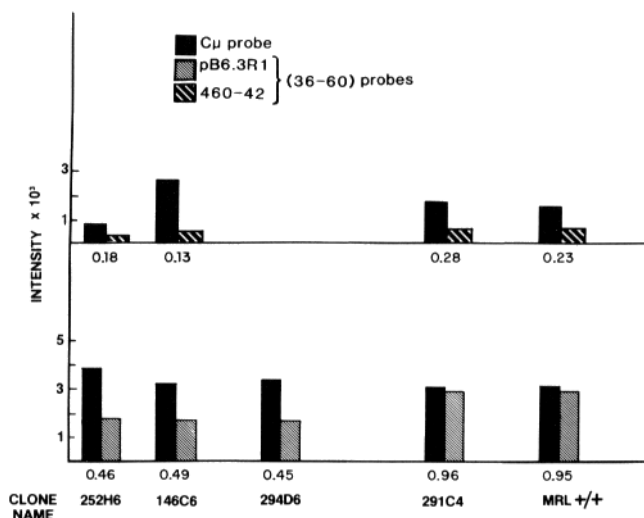


FIGURE 2. RNA was extracted from each clone and dot-blotted onto nitrocellulose filters. Separate filters were used for hybridization with each V_H probe. After exposure to x-ray film, 16 h for pB6.3R1 and 48 h for 460-42, each filter was stripped, rehybridized with the C_μ probe, and exposed for 15 h. Densitometric scans were performed on autoradiograms and intensities were calculated with the GELSCAN program. The histograms in the *upper panel* represent hybridization intensities with the 460-42 and C_μ probes and the histograms in the *lower panel* represent hybridization intensities with the pB6.3R1 and C_μ probes on a separate filter. Numbers below each histogram represent (hybridization intensity with the V_H probe/hybridization intensity with the C_μ control probe).

the 460-42 probe, as indicated by the intensity comparisons of the 36-60 probes to the C_μ probe. Two clones, 291C4 (0.96) and MRL +/- (0.95), are more homologous to the pB6.3R1 probe than are the others and are both members of group IV. Since both clones, 291C4 (0.28) and MRL +/- (0.23), also display similar intensity ratios with the 460-42 probe, they may use the same germline V_H gene. Three other clones (252H6, 146C6, and 294D6) display similar homology with the pB6.3R1 probe and are probably encoded by another member(s) of the 36-60 V_H family, even though they are each members of different binding groups. Clone 154B5 (group III) also hybridized with both 36-60 family probes (data not shown).

Three clones exhibited crosshybridization with probes from two different families, 36-60 and, to a lesser extent, J558. A representative scan of one clone in group I (295A4) is shown in Fig. 3. The lower scan in Fig. 3 exemplifies negative hybridization with the Q52 probe. The histograms in Fig. 4 represent intensities of hybridization with the V_H family probes and the C_μ control probe for each of the three clones that exhibited V_H crosshybridization. Numbers below each set of histograms represent the ratio of hybridization intensities obtained with the V_H family probes compared with the intensities obtained with the C_μ probe. A summary of antigen-binding specificities and the V_H family used by all clones is presented in Table IV. Data from these experiments indicate that the germline codes for a majority (6/9) of the anti-IgG clones represented in our panel are clustered in a small genomic locus containing only five homologous V_H gene segments. The other three clones may also have derived their codes from

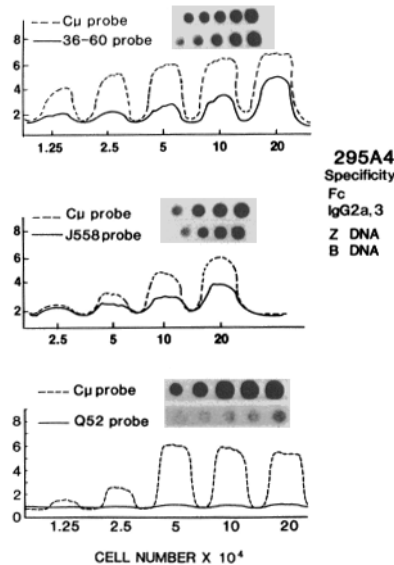


FIGURE 3. Representative densitometric scans of autoradiograms from clone 295A4 that hybridized with $C\mu$ probe (---) and probes from two V_H families (—): pB6.3R1 (36-60 family probe, *top*) and pBV_HJ558 (J558 family probe, *bottom*). *Top right* shows autoradiogram dots and far right side delineates the antigen-binding specificity of the clone.

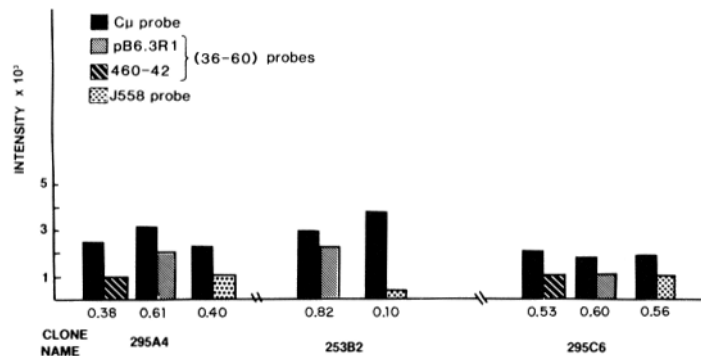


FIGURE 4. Histograms represent hybridization intensities of the three clones that displayed crosshybridization with probes from two different families (36-60 and J558). Hybridized filters were exposed as follows: pB6.3R1 (2.3 kb), 16 h; J558 (1.0 kb), 24 h; 460-42 (0.4 kb), 48 h; and $C\mu$ (1.0 kb), 15 h. Numbers below the abscissa represent comparisons of hybridization intensity with V_H family probes to intensity of hybridization with the $C\mu$ probe on the same filters used for hybridization to the V_H family probe.

the 36-60 V_H family as well, but since they also crosshybridize with J558, we cannot be certain of their origin.

Discussion

MRL/Mp *lpr/lpr* mice develop an aggressive autoimmune disease characterized by immune complex-mediated glomerulonephritis, polyarteritis, and panus-forming erosive synovitis. Associated with the autoimmune pathology are

TABLE IV
Summary of V_H Family Usage and Specificities for Anti-IgG Antibodies

Group	Clones	V _H Family	Fragments	IgG sub-classes	Heterologous IgG [‡]	DNA
I	252H6	35-60	Fc (+4)*	2a, 1, 3	H (+2)	Z
	295A4	36-60/J558	Fc (+4)	2a, 3	None	Z, B
II	253B2	36-30/J558	Fc (+1)	2a, 3	G, P, H, Rb (+4)	0
	295C6	30-60/J558	Fc (+1)	2a, 3	Ho, Gp, C (+4)	0
III	146C6	36-60	Fab (+3)	—	None	0
			(Fab') ₂ (+2)	—		
	154B5	36-60	Fab (+3)	—	None	0
			(Fab') ₂ (+2)	—		
IV	291C4	36-60	Intact IgG	2a	None	0
	MRL +/+	36-60	Intact IgG	3	None	0
	294D6	36-60	(Fab') ₂ (+4)	—	Rb (+4)	0

* Numbers in parentheses indicate binding to proteolytic fragments of mouse IgG and heterologous IgG expressed as percent binding compared with mouse IgG control (+4, >75%; +3, 50–74%; +2, 25–94%; +1, 10–24%).

‡ H, human; Gp, guinea pig; G, goat; P, pig; R, rat; Ho, horse; C, cow; Rb, rabbit.

polyclonal B cell activation (38) and the production of multiple autoantibodies, including those directed against DNA and IgG (24, 25). The precise relationship of autoantibody production to the development of autoimmune disease is unclear. In the MRL/Mp *lpr/lpr* mouse, the presence of the *lpr* gene, which is responsible for generalized lymphoproliferation and accelerated onset of autoimmune disease, is also associated with enhanced autoantibody expression (26). The introduction of the *lpr* gene into nonautoimmune mice or stimulation of normal strains with LPS results in lymphoproliferation, polyclonal B cell activation, and the production of anti-DNA and RF antibodies without autoimmune pathology (e. g., glomerulonephritis; 39–41). However, the simultaneous administration of two immunostimulators, *lpr* and LPS, to mouse strains with otherwise normal genetic backgrounds results in severe autoimmune manifestations in addition to autoantibody production (42). Taken together, these studies indicate that germ-line genes that can encode autoantibodies associated with autoimmune pathology are present in normal mice.

There is little information concerning the molecular basis of autoantibody production. Amino acid and nucleotide sequence analysis of a limited number of anti-DNA mAbs derived from autoimmune mice revealed the use of V_H gene segments highly homologous to germ-line genes in normal mice encoding antibodies directed against PC or to the hapten DNP (43, 44). These data cast doubt on the likelihood of anti-DNA autoantibodies being derived from a unique subset of V_H genes. To investigate this issue in another autoantibody system, we have constructed a panel of hybridomas secreting antibodies with anti-IgG activity from unmanipulated spleen cells of MRL/Mp *lpr/lpr* and the congenic strain, MRL/Mp +/+ mice. We obtained eight clones from the *lpr/lpr* strain and one from the +/+ strain and analyzed the binding of each antibody to proteolytic

fragments of mouse IgG [Fc, Fab, (Fab')₂], mouse IgG subclasses, and heterologous IgG, and determined which V_H gene family was used to encode these anti-IgG antibodies. We found that the anti-IgG antibodies produced by these clones bound a heterogeneous array of epitopes on the IgG molecule. This pattern is similar to that seen with anti-IgG antibodies associated with human RA, including the existence of a subset that also bound to DNA (37). Interestingly, the two IgM λ clones that were derived from young mice, 154B5 (4 wk) and 146C6 (8 wk), recognized the Fab/(Fab')₂ portion of the IgG molecule and exhibited a much more restricted binding specificity (i. e., to murine IgG alone) than the clones generated from older mice. Autoantibodies that bind the (Fab')₂ portion of human IgG have also been detected in sera from RA patients (45).

Since little is known about the molecular mechanisms underlying RF production in RA, we initiated a study with our clones from the MRL mouse to determine which families of V_H gene segments contributed codes for this heterogeneous population of autoantibodies. Our finding that the V_H portion of these antibodies was encoded predominantly by members of the 36-60 family regardless of the binding specificity was striking, because the 36-60 family comprises only five members, while J558 has at least 60 and probably 1,000 members (46). The frequency of IgG-binding hybridomas generated from the fusions of unmanipulated spleen cells from 4- and 8-wk-old mice was ~10%. Although many of the hybridomas were unstable and could not be rescued for analysis, if a similar proportion of those clones that were not analyzed was also encoded by the 36-60 family, then the representation of that family is clearly disproportionate to its size. Recent studies (47) using IgM-secreting hybridomas derived from 5-mo-old MRL/Mp *lpr/lpr* mice showed that at least three other V_H families (J558, Q52, and 7183) can encode antibodies that react with IgG at later stages of development. Since the mice from which we prepared the hybridomas were young and raised under pathogen-free conditions, and since they displayed only early disease manifestations (24),³ we feel that this pattern of preferential 36-60 V_H expression may well reflect the germline genetic contribution to subsequent immunopathology in these animals.

Comparison of the ratio of hybridization intensities obtained with the C_μ probe to the intensities obtained using the 36-60 probe suggested that at least two gene segments of the 36-60 family were represented in our panel of anti-IgG antibodies.

Two clones, 291C4 and MRL +/+, probably use the same germline gene, since their ratios of hybridization intensities of C_μ to 36-60 (pB6.3R1) are 0.96 and 0.95, respectively. Another gene(s) in the 36-60 family is likely to encode 252H6 (0.46), 146C6 (0.49) and 294D6 (0.45); the numbers in parentheses are the ratios for the pB6.3R1 probe. It is difficult to determine whether or not the remaining three clones (295A4, 253B2, and 295C6) used yet another member of the 36-60 gene family, since they all showed some degree of crosshybridization with the probe from the J558 family. The crosshybridization these clones exhibited with both 36-60 and J558 probes is not readily explainable. A trivial explanation would be that the hybridomas were biclonal. However, the repro-

³Fine, J. D., F. X. O'Sullivan, S. Gay, and W. J. Koopman. Ultrastructural localization of skin immunoreactants in the autoimmune animal strain MRL/*lpr*. Manuscript submitted for publication.

ducibility of these results despite five subclonings of each of these clones renders this explanation unlikely. Alternative explanations include somatic mutations involving random base pair substitutions or gene conversion between a 36-60 sequence and a J558 sequence.

Recent evidence suggests that the location of a V_H gene family within the V_H locus influences its time of expression during development, with those V_H families most proximal to the C_μ region being expressed earliest (32, 48). Since the 36-60 family is one of the families most distal to the C_μ locus, clonal expression of 36-60-encoded products may occur later in B cell ontogeny. It is conceivable that aberrations in the programmed expression of V_H gene families could contribute to abnormalities in the B cell repertoire characteristic of autoimmunity. Alternatively, similar abnormalities might be anticipated if defects occurred in proteins (antiidiotype network) or cells (Th or Ts) that regulate V_H gene family expression.

Since a subset of MRL *lpr/lpr* T cells that display a variety of functional and phenotypic abnormalities (49-54) is the major cell population responsible for the massive lymphadenopathy and splenomegaly seen in this strain it is possible that regulation of products contributes to the immunopathology in these animals. Neonatal thymectomy of MRL *lpr/lpr* mice markedly reduced lymphoproliferation of the phenotypically abnormal T cell population, decreased autoantibody concentrations, and prolonged life (55, 56), thus indicating a major role for T cells in the onset and severity of the autoimmune syndrome. The proliferation of abnormal T cells may create an imbalance between T cell suppression and enhancement and facilitate expansion of the autoreactive clones.

Our autoantibody-producing hybridomas derived from autoimmune mice do not use a unique set of V_H genes. Rather, they use V_H genes homologous to those present in normal mouse strains that may be used for the production of antibodies directed against exogenous antigens. In this regard, murine anti-DNA antibodies have been shown to share idiotypic markers with Ig lacking autoreactivity (57), and some anti-DNA mAbs from autoimmune mice have been shown (58) to bind to bacterial constituents. These results indicate that antibodies directed against environmental antigens may crossreact with self determinants. Alternatively, autoantibodies may be derived by somatic mutation of germline genes encoding antibodies directed against exogenous antigens. Direct evidence for this comes from a recent report of an anti-DNA antibody arising from a single point mutation in the V_H gene of an S107 myeloma cell line that originally produced an antibody reactive with phosphorylcholine (59). Thus, a minor alteration in a germline gene may lead to the generation of an autoreactive clone.

In summary, our results indicate that anti-IgG antibodies spontaneously expressed in young autoimmune MRL mice are preferentially encoded by genes residing within the small 36-60 V_H gene family. These data raise the possibility that human autoantibody counterparts occurring in rheumatic disease may also use a restricted number of germline variable region genes.

Summary

Antibodies directed against IgG and DNA are found in the sera of autoimmune MRL/Mp *lpr/lpr* mice. Little is known of the molecular mechanisms underlying

expression of such autoantibodies. We have investigated the binding diversity and pattern of V_H gene expression in a panel of murine anti-IgG antibodies. We constructed eight hybridoma clones secreting IgM antibodies that bound to mouse IgG by using spleen cells from MRL/Mp *lpr/lpr* mice varying in age from 4 to 15 wk; one clone was derived from a 32-wk-old MRL *+/+* mouse. The monoclonal IgM products exhibited varying binding specificities for intact mouse IgG, fragments of mouse IgG [Fc, Fab, (Fab')₂], and heterologous IgG. Two of these antibodies crossreacted with B and/or Z DNA. Probes from seven of eight identified mouse V_H gene families (7183, S107, Q52, J558, J606, 36-60, and 3609) were hybridized under high-stringency conditions with cytoplasmic RNA blots from each clone. Six clones hybridized only with the probe from the five-member 36-60 family. The remaining three clones crosshybridized with the 36-60 probe and the probe from the 60 member J558 family, perhaps reflecting somatic mutation from the original germline V_H gene resulting in recognition by a probe from another family, in addition to the probe from the original germline family. Our results indicate that spontaneous MRL *lpr/lpr* anti-IgG antibodies are encoded predominantly by the 36-60 V_H gene family and imply a nonrandom selection of this V_H gene family in the production of these antibodies.

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