MONOCLONAL IgM RHEUMATOID FACTORS DERIVED FROM ARTHRITIC MRL/Mp-*lpr/lpr* MICE*

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Studies by Kunkel and co-workers (1–4) on human monoclonal rheumatoid factors (RF)[1] spontaneously arising in patients with Waldenstrom's macroglobulinemia (5, 6) have greatly advanced our knowledge of the fine characteristics of these important anti-γ-globulin autoantibodies. For experimental purposes, availability of murine monoclonal RF will further expand our ability to assess the biologic significance of these autoantibodies in health and disease.

Recently, we observed that mice of the MRL/Mp-*lpr/lpr* (MRL/l) substrain, in addition to lupus-like syndrome and massive T cell proliferation caused by the *lpr* gene, develop an arthritic process very similar serologically and histologically to human rheumatoid arthritis (RA) (7, 8). Both IgM and IgGRF are detectable in their sera (8, 9) as well as IgG-IgGRF (9) and IgGRF-anti-DNA complexes (10).

The availability of this murine substrain has prompted us to initiate studies on the genetics, fine specificities, and biologic roles of RF in their arthritic process. As a first step, we derived several hybrids between unmanipulated MRL/l splenocytes and myeloma cells that secreted monoclonal IgMRF (mIgMRF). This report details the IgG subclass, allotypic, IgG domain, and species specificities of these monoclonal autoantibodies.

Materials and Methods

Mice. MRL/l mice (7, 11) were bred and maintained in our colony. The derivation, histologic, and serologic characteristics of this autoimmune substrain of mice have been detailed elsewhere (12).

Cell Fusion. Plasmacytomas of BALB/c origin used for the cell fusions included initially the hypoxanthine, aminopterin, thymidine (HAT)-sensitive cell line P3-X63-Ag8 expressing immunoglobulin of the chain composition γ1 and κ and, when it became available, its variant P3-X63-Ag8.653 line (13). The variant line has lost immunoglobulin expression entirely and does not synthesize γ1 or κ chains of X63 origin upon fusion with antibody-forming cells. For cell fusion, we used a modification of the technique described by Kohler

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[1] *Abbreviations used in this paper:* BBS, borate-buffered saline; BSA, bovine serum albumin; DMEM, Dulbecco's minimum essential medium; dsDNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; HAT, hypoxanthine, aminopterin, thymidine; LPS, lipopolysaccharide; mRF, monoclonal rheumatoid factors; PBS, phosphate-buffered saline; RA, rheumatoid arthritis; RF, rheumatoid factors; ssDNA, single-stranded DNA.

and Milstein (14). Briefly, splenocytes from unmanipulated 3.5-mo-old IgMRF-positive, MRL/l mice were mixed with myeloma cells (10:1 ratio; 1×10^8 spleen cells/1×10^7 myeloma cells) and a 35% final concentration of polyethyleneglycol (PEG 1000; J. T. Baker Chemical Co., Phillipsburg, NJ). After fusion, the cell suspension was mixed with 500 ml of selective HAT-Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum, glutamine, antibiotics, nonessential amino acids, sodium pyruvate, and 4.5 g glucose/liter that contained 2×10^6 fresh BALB/c thymocytes/ml. Subsequently, the cell suspension was distributed in 96-well tissue culture clusters with flat-bottomed wells (3596; Costar, Cambridge, MA). Cultures were placed in humidified 37°C, 5% CO_2 incubators and fed after approximately 8 and 14 d with HAT-DMEM containing 5×10^6/ml fresh BALB/c thymocytes. Propagation of hybridomas and subcloning at limiting dilutions were performed as described (15).

Radioimmunoassay for RF Activity. The assay for IgMRF has been described (10). Briefly, polypropylene wells (Dynatech Laboratories, Inc., Alexandria, VA) were coated by desiccation with 50 μl of a 40 μg/ml polyclonal murine IgG or 40 μg/ml of an equimolar mixture of all murine myeloma IgG subclasses (IgG1, IgG2a, IgG2b, IgG3) in borate-buffered saline (BBS), pH 8.4. After appropriate washes and coating with 200 μl of BBS containing 2% bovine serum albumin (BSA)-0.05% Tween 80, the wells were incubated overnight at 4°C with 50 μl of appropriately diluted test material. Washed wells were then incubated (4 h, 4°C) with 50 μl of affinity-purified ^{125}I-labeled (16) F(ab')$_2$ goat anti-murine μ-specific antibody (200 μg/ml BBS-BSA-Tween 80). Bound radioactivity was counted after a last series of five washes in BBS and specific counts were calculated by subtracting the counts per min associated with wells incubated with control medium. The anti-μ serum was made by immunizing a goat with MOPC-104E myeloma (μ,λ; Litton Bionetics Inc., Kensington, MD) and subsequent affinity purification on a Sepharose 4B-TEPC-183 (μ,κ; Litton Bionetics Inc.) column. The specifically bound counts of anti-μ were referred to a standard curve made by using wells coated with unlabeled anti-μ as the first reagent, TEPC-183 (μ,κ) myeloma diluted in BBS-BSA-Tween 80 at concentrations ranging from 200 to 0.4 μg/ml as the middle reagent, and ^{125}I-anti-μ as the final reagent.

Ig Isotypes and IgG Subclasses. Mouse IgM (TEPC-183), IgA (TEPC-15), IgG1 (MOPC-21), IgG2a (UPC10, RPC5), IgG2b (MOPC-195), and IgG3 (J606) myelomas were all purchased from Litton Bionetics Inc., whereas murine IgE antidinitrophenyl monoclonal antibody was a gift from Dr. F. T. Liu, Medical Biology Institute, La Jolla, CA. Polyclonal IgG of murine, bovine, rabbit, rat, goat, and human origin were all purchased from Miles Laboratories, Elkhart, IN. Human myeloma IgG subclasses (IgG1, IgG2, IgG3, IgG4) of kappa or lambda light chains and of known Gm allotype composition, and rat IgG subclasses (IgG1, IgG2a, IgG2b, IgG2c) were all a generous gift from Dr. H. Spiegelberg, Scripps Clinic and Research Foundation. Polyclonal murine IgG2a (Igh-1) of a, b, c, d, e, or j allotypes were purified from sera of pertinent murine strains (17) by absorption on staphylococcal protein A-Sepharose CL-4B columns (Pharmacia Fine Chemicals, Uppsala, Sweden) and subsequent elution with 0.1 M citrate/phosphate buffer gradient, pH 3.0–8.0, as described (18). The above proteins were used either as substrates or inhibitors in the RF radioimmunoassay.

Aggregation of Immunoglobulins. The various murine myeloma IgG subclasses and homologous and heterologous polyclonal IgG were dissolved in BBS, pH 8.4, at a concentration of 1 mg/ml. They were then heated (63°C or higher) until they became opalescent. The binding of selected mIgMRF to wells coated with an equimolar mixture of murine IgG subclasses (40 μg/ml) was measured in the presence of various concentrations of inhibitor heat-aggregated IgG diluted in BBS-BSA-Tween 80. mIgMRF were diluted to 50% of their maximal binding capacity before testing.

Fab and Fc Fragments. Murine Fab and Fc fragments were prepared as follows. A mixture of polyclonal IgG isolated from BALB/c serum and myeloma IgG2a (UPC-10) was digested with papain (Worthington Biochemical Co., Freehold, NJ) at a 100:1 substrate to enzyme ratio at 37°C for 1 h in 0.15 M phosphate-buffered saline (PBS), pH 7.0, containing 0.002 M EDTA-tetrasodium salt (J. T. Baker Chemical Co.) and 0.02 M L-cysteine (Sigma Chemical Co., St. Louis, MO). The reaction was stopped by the addition

of a slight excess of iodoacetamide. Subsequently, the sample was dialyzed against PBS and chromatographed on a Sephadex G-100 (Pharmacia Fine Chemical) column to separate undigested IgG from Fab and Fc fragments. The Fab- and Fc-containing material was then concentrated and passed through a Sepharose CL-4B-staphylococcal protein A column. The effluent and the 0.1 M citrate buffer (pH 3.5) eluate of this column, containing Fab and Fc, respectively, were dialyzed against PBS, concentrated, and used at 10 μg/ml as substrates in the IgMRF radioimmunoassay.

Human Fc (CH2-CH3 domains, residues 230–446) was obtained as above by papain digestion of a myeloma, IgG1, λ (Gm^f), except that digestion lasted 18 h instead of the 1 h used for murine IgG2a. Human pFc′ (CH3 domain, residues 335–446) was obtained by plasmin (Sigma Chemical Co.) digestion of the above myeloma protein and subsequent chromatographies as detailed elsewhere (19, 20) so as to separate the large Fabc fragment that contains all but the terminal CH3 domain from the small molecular weight fragment consisting of the free CH3 domain.

Staphylococcal Protein A. Protein A purchased from Sigma Chemical Co. as lyophilized powder was dissolved in PBS and used to localize the Fc region that mIgMRF recognized. Microtiter wells were coated with the murine myeloma pool, reacted (4°C, overnight) with an excess of protein A (250 μg/ml BBS), then washed and incubated with the mIgMRF previously diluted to 50% binding capacity. After incubation, wells were washed, and bound IgM was quantitated with radioiodinated anti-μ as described for the RF radioimmunoassay. Controls included wells coated with IgG and then incubated with mIgMRF in the absence of protein A, and wells coated with IgG and then sequentially incubated with mIgMRF, washed, and finally reacted with ^{125}I-labeled protein A.

Human C1q. Human C1q was prepared as detailed elsewhere (21). It was used for inhibition studies in which selected mIgMRF (diluted to 50% binding capacity), the substrate mouse IgG, or both were preincubated with an excess amount of C1q and then checked for their ability to interact with each other as assessed by the RF radioimmunoassay.

Cross-reactions of mIgMRF with Non-IgG Molecules. Cross-reactions of mIgMRF with mouse kidney nuclei, single-stranded DNA (ssDNA), and double-stranded DNA (dsDNA) were assessed as described (7). Reactivity with total calf thymus histones was determined by using an enzyme-linked immunosorbent assay (ELISA) modified from a solid-phase test described by Rubin et al. (22). Binding to chick collagen II was checked by the solid-phase ELISA procedure of Stuart et al. (23). Binding to a synthetic pentapeptide (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala; kindly prepared by Dr. R. Houghten, Scripps Clinic and Research Foundation), which is identical with that in the peptidoglycan of streptococci (24), was assessed by inhibition radioimmunoassays in which binding of mIgMRF to substrate Ig was assessed in the presence of increasing amounts of the peptide (500–8 μg/ml).

Results

Murine IgG Subclass and Species Specificity of Serum Polyclonal IgMRF. Before fusing MRL/l splenocytes with myeloma cells to develop monoclonal autoantibodies, we randomly selected 20 MRL/l female mice, 3–4 mo of age, and checked their sera for levels and specificities of polyclonal IgMRF for murine IgG subclasses and heterologous IgG (Table I). All sera tested contained significantly elevated amounts of IgMRF compared with sera of three normal control strains. Serum polyclonal IgMRF reacted with all murine IgG subclasses, but the degree of reactivity varied depending on the substrate subclass; the majority of the sera (12 out of 20) reacted most strongly with mouse IgG2a. In regard to heterologous IgG, extensive cross-reactions with human, cow, rat, rabbit, and goat IgG were noted.

Hybridomas Secreting mIgMRF. Four hybridizations were performed with spleen cells from individual, unmanipulated 3.5-mo-old MRL/l mice shown to

TABLE I

*Murine IgG Subclass and Heterologous IgG Specificity of Serum Polyclonal IgMRF**

MRL/l serum No.	Myeloma pool	Murine IgG subclasses				Heterologous IgG				
		IgG1	IgG2a	IgG2b	IgG3	Human	Cow	Rat	Rabbit	Goat
		μg IgMRF/ml serum								
1	30.6	10.1	41.4	5.1	11.3	4.1	5.3	7.7	3.2	4.2
2	19.9	9.3	23.1	6.2	15.2	15.3	5.4	9.8	3.6	2.7
3	69.0	12.1	77.4	8.2	11.2	10.4	4.5	13.9	8.2	6.3
4	25.6	21.6	19.5	15.2	22.9	11.0	8.1	16.4	11.3	10.1
5	61.3	17.6	69.6	17.3	32.5	14.6	5.4	17.7	11.8	4.8
6	18.7	6.1	20.8	7.7	12.9	5.5	4.7	5.2	4.9	4.7
7	80.2	88.7	69.4	24.9	37.1	10.5	16.5	82.9	9.1	10.0
8	45.7	8.7	22.5	49.6	22.2	11.9	7.9	10.4	8.0	8.6
9	51.0	37.5	59.2	24.6	27.0	16.4	14.0	36.5	14.2	17.6
10	20.7	10.4	13.7	13.7	13.9	10.0	6.8	9.8	8.6	2.0
11	14.9	8.5	15.0	7.6	8.3	8.5	5.7	4.1	3.9	7.3
12	28.3	7.0	25.2	14.5	18.4	5.1	5.7	3.0	3.5	5.0
13	55.1	17.8	79.4	5.2	14.5	4.3	3.7	6.0	5.8	4.9
14	28.9	16.4	45.8	5.2	7.8	5.1	3.9	4.9	2.5	4.7
15	27.4	34.9	14.2	18.1	11.8	9.0	16.4	34.9	7.6	12.3
16	17.5	11.7	53.4	5.4	17.3	8.6	3.3	14.7	13.2	3.1
17	49.8	21.0	22.9	47.3	35.8	17.1	12.4	20.1	13.3	11.8
18	25.8	10.8	27.4	14.1	9.4	9.7	5.9	8.6	5.8	8.7
19	30.7	19.8	17.4	46.0	21.5	14.9	10.1	13.4	34.5	9.0
20	18.8	26.7	4.9	4.6	2.6	3.0	5.1	8.8	1.6	4.3
Normal serum pools										
C3H/St	3.9	0.3	0.4	0.7	0.4	0.5	0.0	0.0	0.2	0.0
C57BL/6	4.2	0.4	0.5	0.4	0.7	0.3	0.0	0.0	0.0	0.0
BALB/c	4.6	0.4	0.7	1.4	0.9	0.7	0.3	0.0	0.3	0.2

* Microtiter wells were coated with 40 μg/ml of pooled murine myeloma immunoglobulins (all four subclasses), 10 μg/ml of individual murine myeloma subclasses, or 40 μg/ml of heterologous IgG.

contain high levels of serum IgMRF of broad specificity (mice 5, 7, 9, and 17, listed in Table I). At the completion of each hybridization the cells were cloned by limiting dilution in ~2,000 microtiter wells. After 2.5–3.5 wk, hybrids developed in 305, 218, 633, and 567 wells, respectively, for a total of 1,723 hybrids in the four fusions. Of these, 4, 3, 4, and 12 clones or 1.3, 1.4, 0.6, and 2.1%, respectively, secreted IgMRF. Positive clones from these experiments, termed RF1 through RF23, were found to secrete considerable amounts of autoantibody at the time of first testing (Table II). Poisson distribution analysis indicated that <1% of the postive wells could have contained more than one IgM anti-IgG–secreting clone. Nevertheless, to ensure that our mathematical assumption was correct, we subcloned seven clones and found that all secreted IgMRF in amounts similar to those of the parental clones. Sequential analysis of all positive clones over a period of at least 6 mo has shown stable amounts of autoantibody production.

Isotype Specificities of the mIgMRF. Classically, RF bind to IgG and very rarely to other immunoglobulin isotypes (reviewed in reference 25). Affinity-purified IgM from five of the most active clones were radioiodinated and their direct

TABLE II
*Hybridomas Secreting mIgMRF**

Clone No.	IgMRF	Clone No.	mIgMRF
	μg/ml		*μg/ml*
RF1	4.02	RF13	4.80
RF2	48.61	RF14	0.16
RF3	4.30	RF15	0.32
RF4	0.72	RF16	5.36
RF5	1.10	RF17	2.12
RF6	1.48	RF18	2.44
RF7	5.98	RF19	3.71
RF8	4.08	RF20	0.32
RF9	11.40	RF21	1.79
RF10	1.70	RF22	1.67
RF11	0.20	RF23	21.42
RF12	1.12		

* Tested on an equimolar pool of murine myeloma IgG1, IgG2a, IgG2b, and IgG3 at a total concentration of 40 μg/ml. Numbers indicate μg mIgMRF/ml culture supernatant. The first 7 positive clones were derived from the two fusions with the secretor myeloma line, and the remaining 16 were derived from the fusions with the nonsecretor line.

binding to murine IgG and IgM isotypes was ascertained. Additionally, all mIgMRF were checked for binding to IgA and IgE substrate in the same fashion as assessed for binding to IgG substrate using radioiodinated anti-μ as the final reagent. All monoclonal autoantibodies showed significant binding to murine IgG but not to IgM, IgA, or IgE (data not shown).

IgG Subclass Specificities of the mIgMRF. The IgG subclass specificities of the 23 monoclonals are listed in Table III. Eight clonotypes were identified, and the majority of the monoclonals reacted either exclusively with IgG2a (seven clones) or with all IgG subclasses equivalently (five clones). Of the remaining monoclonals, three bound to IgG2b only, four to IgG1:IgG2a:IgG3, one to IgG2a:IgG3, one to IgG1:IgG3, one to IgG1:IgG2a, and one to IgG2b:IgG3. No clone that reacted exclusively with IgG1 or IgG3 was identified, although clones RF8 and RF2 bound much better to these two subclasses, respectively, than to other subclasses.

Similar specificity patterns were observed in competition experiments with a pool of the four murine IgG subclasses used as a substrate and individual aggregated IgG subclasses as inhibitors. Representative inhibition experiments with clone RF8 are depicted in Fig. 1.

Allotypic Specificities of Anti-IgG2a mIgMRF. Six mIgMRF that bound to IgG2a only were tested for their allotypic specificities against polyclonal IgG2a isolated from the sera of mice expressing Igh-1 allotype of the a, b, c, d, e, or j alleles (Table IV). Four monoclonal autoantibodies (RF9, RF13, RF17, RF23) bound to IgG2a of the a, c, and e alleles but not those of the b, d, and j alleles. In contrast, two of these monoclonals showed additional specificities: RF14 also reacted with the b allotype and RF22 reacted with all substrate allotypes. It was noteworthy that Igh-1 antigenic specificities represented in the reactive allotypes were also represented in the nonreactive allotypes and vice versa. The only

TABLE III

*Specificities of mIgMRF for Mouse IgG Subclasses**

Clone No.	IgG1	IgG2a	IgG2b	IgG3
		μg mIgMRF/ml		
RF6	—‡	1.44	—	—
RF9	—	20.60	—	—
RF13	—	5.53	—	—
RF14	—	0.85	—	—
RF17	—	2.15	—	—
RF22	—	2.25	—	—
RF23	—	26.45	—	—
RF7	—	—	14.48	—
RF10	—	—	1.78	—
RF11	—	—	0.22	—
RF4	0.76	0.55	0.56	0.94
RF5	0.82	0.71	0.49	0.70
RF18	4.07	2.23	1.49	8.66
RF20	0.45	0.25	0.37	0.50
RF21	2.66	3.50	3.19	4.62
RF2	0.66	1.03	—	42.36
RF3	1.42	4.43	—	4.06
RF16	4.42	4.25	—	1.31
RF19	4.57	2.55	—	0.71
RF1	—	8.43	—	1.59
RF8	32.30	—	—	0.72
RF12	1.02	0.81	—	—
RF15	—	—	0.2	0.22

* Microtiter wells were coated with individual murine myeloma IgG substrates at a concentration of 10 μg/ml. Numbers indicate μg IgMRF/ml culture supernatant.

‡ Binding equal to background levels.

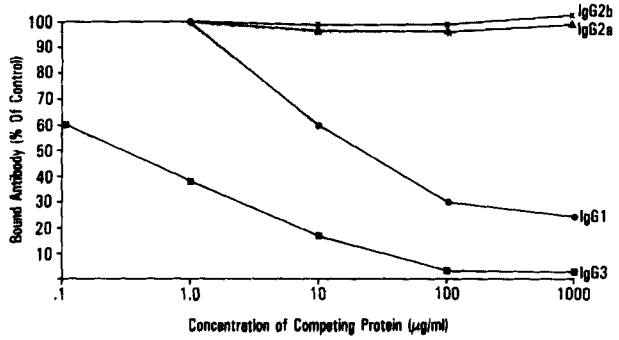

FIGURE 1. Murine IgG subclass specificity of mIgMRF. The binding of monoclonal antibody RF8 to a substrate pool of four murine IgG subclasses was assessed in the presence of various concentrations of heat-aggregated IgG1, IgG2a, IgG2b, or IgG3.

TABLE IV
Allotypic Specificities of Anti-IgG2a mIgMRF

Source of IgG2a*	Igh-1 allele‡	Igh-1 antigenic‡ specificities	Clone No.	mIgMRF
				μg/ml
BALB/c	a	1, 6, 7, 8, 26, 28, 29, 30	RF9	11.73
			RF13	3.23
			RF14	0.5
			RF17	0.18
			RF22	1.28
			RF23	13.95
C57BL/6	b	2, 27, 29	RF9	(—)§
			RF13	(—)
			RF14	0.3
			RF17	—
			RF22	0.3
			RF23	—
DBA/2J	c	3, 8, 29	RF9	25.92
			RF13	5.66
			RF14	0.25
			RF17	0.11
			RF22	2.47
			RF23	10.09
AKR/J	d	4, 6, 7, 8, 26, 29	RF9	(—)
			RF13	(—)
			RF14	—
			RF17	—
			RF22	0.35
			RF23	—
NZB	e	4, 6, 7, 8, 26, 28, 29, 30	RF9	24.00
			RF13	1.55
			RF14	0.17
			RF17	0.48
			RF22	2.12
			RF23	11.34
CBA/CaJ	j	1, 6, 7, 8, 28, 29, 30	RF9	(—)
			RF13	—
			RF14	—
			RF17	—
			RF22	0.41
			RF23	—

* Microtiter wells were coated with the various IgG2a at a concentration of 10 μg/ml.
‡ See reference 17.
§ Indicates no binding above background level; parenthesis denote very weak binding (<10 ng/ml).

exception was clone RF22, which had broad reactivity with all allotypes that shared the antigenic specificity 1.29.

Binding of mIgMRF to Heterologous IgG. The binding of the mIgMRF to human, cow, goat, rat, or rabbit IgG is shown in Table V. Four clonotypes were identified. 11 mIgMRF reacted only with homologous but not heterologous IgG, 5 with all heterologous IgG; 6 cross-reacted only with rat IgG, and 1 with all heterologous IgG except human IgG. Monoclonals with exclusively anti-mouse IgG2a or anti-mouse IgG2b activity did not cross-react with heterologous IgG. The data on heterologous IgG cross-reactions were confirmed by competition experiments

TABLE V
*Binding of mIgMRF to Heterologous IgG**

Clone No.	Levels of IgMRF with mouse IgG as substrate	Murine IgG subclass specificity	Heterologous IgG				
			Human	Cow	Goat	Rat	Rabbit
RF6	1.48	IgG2a	—‡	—	—	—	—
RF9	11.40		—	—	—	—	—
RF13	4.80		—	—	—	—	—
RF14	0.16		—	—	—	—	—
RF17	0.12		—	—	—	—	—
RF22	1.67		—	—	—	—	—
RF23	21.42		—	—	—	—	—
RF7	5.98	IgG2b	—	—	—	—	—
RF10	1.70		—	—	—	—	—
RF11	0.20		—	—	—	—	—
RF4	0.72	IgG1, 2a, 2b, 3	1.28	0.22	0.37	0.23	0.42
RF5	1.10		1.70	0.70	0.50	0.49	0.18
RF18	2.44		4.13	4.29	7.90	4.32	5.90
RF20	0.32		0.20	0.56	0.36	0.27	0.29
RF21	1.79		1.15	3.46	2.66	2.10	1.53
RF2	48.61	IgG1, 2a, 3	—	2.33	1.07	32.09	0.51
RF3	4.30		—	—	—	1.67	—
RF16	5.36		—	—	—	0.57	—
RF19	3.71		—	—	—	0.27	—
RF1	4.02	IgG2a, 3	—	—	—	1.18	—
RF8	4.08	IgG1, 3	—	—	—	8.66	—
RF12	1.12	IgG1, 2a	—	—	—	—	—
RF15	0.32	IgG2b, 3	—	—	—	0.18	—

* Microtiter wells were coated with pooled murine myeloma IgG or heterologous polyclonal IgG at a concentration of 40 μg/ml. Numbers indicate μg mIgMRF/ml culture supernatants.
‡ No binding above background levels.

with murine polyclonal IgG as a substrate and heterologous heat-aggregated IgG as competitors. Representative inhibition experiments with RF8 are depicted in Fig. 2. Five monoclonals cross-reactive with human and rat polyclonal IgG (as well as IgG from other species) and one monoclonal cross-reactive only with polyclonal rat IgG were tested for human and rat IgG subclass and human IgG allotype specificity (Table VI). Despite some variability in the degree of binding, the five human and rat IgG cross-reacting monoclonals bound to all human IgG subclasses, albeit in most instances to a lesser extent to IgG1 than to the other human subclasses, irrespective of their allotypes, and to all rat IgG subclasses. In contrast, the rat IgG cross-reacting monoclonal bound to rat IgG1 and IgG2a but not to rat IgG2b and IgG2c.

Ig Subfragment Specificity of mIgMRF. All monoclonals were tested for IgG subfragment specificity using as substrates Fab or Fc derived from BALB/c IgG. With the exception of one monoclonal, only the Fc, and not the Fab, elicited binding (Table VII), thus affirming these monoclonals' characterization as

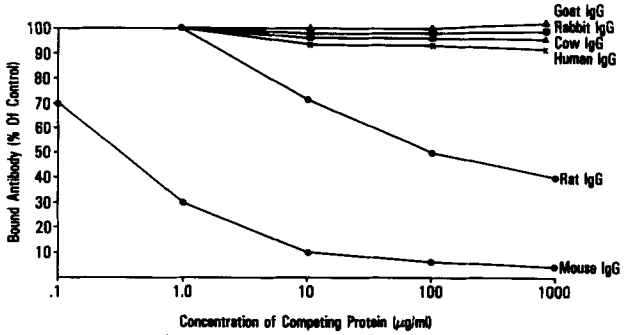

FIGURE 2. Heterologous IgG specificity of mIgMRF. The binding of monoclonal antibody RF8 to substrate polyclonal murine IgG was measured in the presence of various concentrations of heat-aggregated polyclonal IgG of homologous and heterologous (goat, rabbit, cow, human, rat) origin.

TABLE VI

Binding of mIgMRF to Human and Rat IgG Subclasses and to Human IgG Allotypes

Clone No.	Mouse IgG subclass specificity	Human IgG subclasses (allotypes)*									Rat IgG subclasses‖			
		IgG1 (Gm^a)	IgG1 (Gm^f)	IgG1 (Gm^f)	IgG2 (Gm^{n+})	IgG2 (Gm^{n-})	IgG3 (Gm^b)	IgG3 (Gm^g)	IgG4 (Gm^a)	IgG4 (Gm^b)	IgG1	IgG2a	IgG2b	IgG2c
RF4	IgG1, 2a, 2b, 3	0.27	0.12	1.05	1.11	0.2	0.37	0.33	1.22	6.94	0.80	0.40	0.80	0.10
RF5		0.26	0.47	0.55	4.76	15.07	2.51	3.60	4.12	10.04	0.60	—§	0.60	0.20
RF18		1.99	0.68	0.76	3.39	0.48	5.29	5.93	9.75	9.50	3.27	0.76	2.63	2.19
RF20		0.63	0.60	0.68	0.99	0.64	0.91	1.01	1.02	1.21	ND‖	ND	ND	ND
RF21		4.35	5.65	5.32	4.82	3.46	4.00	5.93	7.01	7.83	0.50	0.39	0.40	0.53
RF8	IgG1, 3	—	—	—	—	—	—	—	—	—	3.16	0.90	—	—

* Each human myeloma IgG subclass, derived from different individuals, was used as a substrate at a concentration of 10 μg/ml. Numbers indicate μg mIgMRF/ml culture supernatant.

‡ Polyclonal rat IgG subclasses.

§ Binding equal to background levels.

‖ Not determined.

mIgMRF. The exception was clone RF15, which bound to both substrates but better to Fc than to Fab. The amounts of IgMRF in each monoclonal, as defined by binding to the myeloma pool versus the isolated Fc, did not match. In most instances, Fc promoted better binding than did the IgG pool, perhaps because of a higher availability of Fc sites in the former. However, some of the monoclonals registered higher values for the pool of four IgG subclasses than for the Fc substrate, apparently reflecting the poorer representation of certain IgG subclasses, such as IgG3, in the Fc preparation (derived from IgG2a and polyclonal IgG) than in the equimolar pool of all four murine IgG subclasses. Examples are clone RF2, with primarily an IgG3 specificity, and clone RF8, with primarily an IgG1 specificity (Table III), both of which reacted best with the pool presumably more abundant in both subclasses.

Attempts to Define the Fc Antigenic Determinants for mIgMRF. The globular head of a C1q molecule binds to the CH2 domain of antibody (26, 27). Moreover, protein A, a constituent of the cell wall of *Staphylococcus aureus*, binds to the Fc sites on antibody molecules of all murine IgG subclasses, but does not interfere with complement binding (28). Interaction with an excess of human C1q did not inhibit binding by any of the mIgMRF to substrate IgG (data not shown). Nor

TABLE VII
*Specificity of mIgMRF for IgG Fc**

Clone No.	Myeloma IgG pool	Fab	Fc
RF1	4.02	—‡	>19.47
RF2	48.61	—	1.10
RF3	4.30	—	24.05
RF4	0.72	—	0.27
RF5	1.10	—	0.26
RF6	1.48	—	0.26
RF7	5.98	—	1.35
RF8	4.08	—	1.34
RF9	11.40	—	32.12
RF10	1.70	—	0.16
RF11	0.20	—	0.06
RF12	1.12	—	4.08
RF13	4.80	—	6.85
RF14	0.16	—	0.25
RF15	0.32	0.06	0.09
RF16	5.36	—	10.44
RF17	2.12	—	1.13
RF18	2.44	—	9.64
RF19	3.71	—	9.11
RF20	0.32	—	1.78
RF21	1.79	—	7.24
RF22	1.67	—	6.46
RF23	21.42	—	>44.55

* Microtiter wells were coated either with a pool of the four IgG subclasses (40 μg/ml), Fab (10 μg/ml), or Fc (10 μg/ml). Numbers indicate μg IgMRF/ml culture supernatant.
‡ Binding equal to background values.

did C1q inhibit this reaction when preincubated with both the mIgMRF and the substrate IgG. Thus, none of our mIgMRF recognized antigenic determinants in or around the C1q binding site at the CH2 domain. In contrast, 12 of the 23 mIgMRF were strongly inhibited (>50%) from binding to substrate IgG when protein A was used as a competitor (Table VIII). Of the remaining 11 monoclonals, 1 (RF7) showed a partial inhibition (38.4%) whereas 10 were inhibited very weakly or not at all. No consistent pattern was observed in regard to the inhibitory ability of protein A and any one murine IgG subclass specificity of the mIgMRF. We conclude that over half of our mIgMRF recognize determinants localized at the IgG CH2-CH3 junction or at sites distant to the junction that might be altered or hidden via conformational changes or contact with the protein A.

To determine which of these two possibilities is more likely, we performed the converse experiment where the IgG substrate was first incubated with the monoclonal autoantibodies and then with radioiodinated protein A. Under these conditions the protein A binding was similar to that seen in control wells where the substrate IgG had not been preincubated with the mIgMRF, *i.e.*, no inhibition was observed. Thus, we conclude that the actual binding site for this type of mIgMRF is not localized at the CH2-CH3 junction but at distant sites at the

TABLE VIII

*Inhibition of Binding of mIgMRF to Substrate IgG by Staphylococcal Protein A**

Clone No.	Murine IgG subclass specificity	Percent inhibition‡	Clone No.	Murine IgG subclass specificity	Percent inhibition
RF6	IgG2a	89.5	RF4	IgG1, 2a, 2b, 3	17.1
RF9		98.3	RF5		0.0
RF13		94.7	RF18		8.3
RF17		86.4	RF20		10.4
RF22		94.5	RF21		11.7
RF14		0.0			
RF23		0.2	RF2	IgG1, 2a, 3	98.9
			RF3		88.6
RF10	IgG2b	78.2	RF16		92.7
RF11		5.2	RF19		89.8
RF7		38.4			
RF1	IgG2a, 3	97.3	RF8	IgG1, 3	4.7
RF12	IgG1, 2a	92.6	RF15	IgG2b, 3	0.0

* Each mIgMRF, diluted to 50% binding capacity, was incubated with substrate IgG (pool of the four murine myelomas, 40 μg/ml) that had previously been coated with protein A. Following washings, the binding was assessed with radiolabeled anti-μ.

‡ Percent inhibition = [1 − (binding in the presence of protein A [cpm])/(binding in the absence of protein A [cpm])] × 100.

CH2 or CH3 domains that are simply unavailable because of shielding via close contact with the protein A chains or because of conformational changes in the IgG molecule.

Among the monoclonals not inhibited or weakly inhibitable by protein A were the five autoantibodies that cross-reacted extensively with all murine IgG subclasses and with heterologous IgG. To localize the antigenic determinant involved in the induction of this type of autoantibody, we examined their ability to interact with Fc (CH2-CH3) or pFc′ (CH3 domain) of human IgG1. All five monoclonals bound equally well to the two substrates (data not shown), strongly suggesting that the binding site for these monoclonals is at the CH3 domain.

Cross-reactions of mIgMRF with Non-IgG Substances. None of the 23 mIgMRF cross-reacted with dsDNA, chick collagen, or synthetic pentapeptide L-Ala-D-Glu-L-Lys-D-Ala-D-Ala. However, five of the mIgMRF (clone RF7, RF8, RF18, RF20, RF21) cross-reacted with ssDNA and two of the mIgMRF (clone RF7 and RF16) showed strong cross-reactions with total calf thymus histones and bound to mouse kidney nuclei. The detailed fine specificities of these six mIgMRF will be described in a separate report.[2]

Discussion

This is the first report of mIgMRF derived from the polyclonal pool of IgMRF-producing cells that arise spontaneously in MRL/l mice. Others have used similar methods to obtain monoclonal autoantibodies against erythrocytes (29), DNA

[2] Rubin, R. L., A. N. Theofilopoulos, E. M. Tan, and F. J. Dixon. Antihistone and anti-DNA activities of monoclonal rheumatoid factors produced by MRL/l mouse-derived hybridomas. Manuscript in preparation.

(30), RNA (31), and Sm (32) from spontaneous murine models of lupus.

Spontaneously occurring monoclonal anti-γ-globulins are frequently found in sera of patients with Waldenstrom's macroglobulinemia (5, 6). Kunkel and coworkers (1–4) were the first to divide such mRF into two main groups on the basis of their heavy and light chain variable region subgroups and cross-reacting idiotypes. Subsequently, these (33) and other investigators (34, 35) demonstrated share idiotypic determinants between certain of these mRF and some polyclonal RF associated with RA. Thus, these RF have proven extremely useful in defining the amino acid composition and the biochemical and idiotypic repertoires of human anti-γ-globulins, but their quantities are limited. Other mIgMRF (36) were the products of myeloma cells fused with splenocytes of 129/Sv mice preinjected with lipopolysaccharide (LPS), a polyclonal B cell activator. Still another mIgMRF originated from B cells of an individual with RA after in vitro stimulation with Epstein-Barr virus (37). Although such mitogen-induced autoantibodies and monoclonals therefrom are useful reagents, they may not represent naturally occurring and disease-associated autoantibodies. For example, spontaneously expressed RF in certain colonies of 129/Sv mice are IgA or IgM anti-IgG2a Fc (38, 39). In contrast, mIgMRF from such animals injected with LPS were almost exclusively of the IgM anti-IgG1 Fv type (36). Thus, for analysis of the fine epitopic specificities and of clonotypic and idiotypic characteristics of autoantibodies, monoclonals derived from the pool of spontaneously autoantibody-secreting cells in animals with the relevant disease might be superior to those induced by exogenous polyclonal stimuli.

In a similar fashion to what has been described for human RF (40–42), polyclonal RF in sera of MRL/l mice reacted broadly with all murine IgG subclasses and heterologous IgG, but in most instances, reactivity was stronger with murine IgG2a. Similarly, the majority of our mIgMRF had specificity for IgG2a, exclusive or in association with other murine IgG subclasses. Since immune complexes appear to play a primary role in the induction of RF (43–45), one might suggest that the high expression of anti-IgG2a RF in MRL/l mice is caused by a larger representation of IgG2a in the immune complexes that are abundantly present in sera of such mice. Indeed, most autoantibodies (anti-DNA, anti-gp70, anti-Sm) in these mice are predominantly or exclusively of the IgG2a subclass (46)[3] and the majority of their circulating immune complexes contain IgG2a as the antibody. If RF are indeed directed against antibodies complexed with antigen, they may act as enhancing antibodies similar to those described by Nemazee and Sato (47) that increase production of non-RF autoantibodies.

All our monoclonals with anti-IgG2a or anti-IgG2b activity failed to cross-react with other murine IgG subclasses or heterologous IgG. Similarly, one mIgMRF described by van Snick and Coulie (36) that exhibited anti-IgG2a activity failed to cross-react, unlike their many cross-reactive anti-IgG1 mRF. Moreover, spontaneous polyclonal RF of 129/Sv mice were exclusively anti-IgG2a without cross-reactivity with other murine IgG subclasses or heterologous IgG (38, 39). This lack of cross-reactivity by anti-IgG2a autoantibodies counters

[3] Slack, J. H., L. M. Hang, J. Barkley, R. J. Fulton, L. D. Hoostelaere, A. Robinson, and F. J. Dixon. The isotypic preferences of spontaneous B cell activators and the IgG subclasses of immune-complexed immunoglobulins of systemic lupus erythematosus. Manuscript submitted for publication.

the extensive structural homologies (65%) among various murine IgG subclasses and between murine and human subclasses (48, 49). Thus, our mIgMRF of anti-IgG2a or anti-IgG2b type seem to be directed against amino acid sequences or conformational determinants that are exposed uniquely in the aggregated forms of these murine IgG subclasses. Reviewing the sequence characteristics of the aligned CH2 and CH3 domains of the reactive IgG2a and, for example, the nonreactive mouse IgG1 (50), one finds that these two subclasses differ at amino acid residue portions 229–235, 241–254, and 287–294 of the CH2 and 357–372, 381–391, and 416–429 of the CH3 domain. Of these six portions, residues 287–294, 357–372, and particularly 416–429, which are rich in hydrophilic amino acids (lysine, arginine, aspartic acid, and glutamic acid) are more likely to be exposed at the outer surface of the folded IgG2a moleule and, therefore, more likely to stimulate RF production after their complexing with antigen. Obviously, antigenic determinants for the above mIgMRF cannot be defined accurately until the respective peptides have been synthesized and appropriately tested.

When we examined our anti-IgG2a mIgMRF for allotypic specificities, we found that the majority of them reacted with a, c, and e but not b, d, and j allotypes. Similarly, polyclonal RF of 129/Sv mice and mIgMRF from fused cells of LPS-injected 129/Sv mice were restricted in their Igh-1 allotypic specificities (36, 38). Gm (allotypic) genetic antigens of the human IgG constant homology regions of the heavy chains are often involved in human RF reactions, and several such antigens were first identified by using anti-Gm antibodies from patients with RA (51, 52). Certain allotypes are also associated with some autoimmune diseases of humans, including systemic lupus erythematosus and RA (reviewed in reference 53). Moreover, a gene linked to the Igh-C locus controls the levels and quality of RF production in some mice (54). In reviewing the known antigenic specificities of the allotypes used as substrates in our study, none can be singled out as the target determinant, since specificities in reactive allotypes also appear in the nonreactive allotypes and vice versa. On this basis, one can speculate that our anti-IgG2a monoclonals react with a new, as yet undefined, antigenic specificity. However, the known atigenic specificities of IgG2a allotypes have been defined with polyclonal homologous or heterologous antiallotypic antibodies. Therefore, the monoclonal anti-IgG2a autoantibodies could react with a subregion of a known specificity that the polyclonal antiallotypic serum does not distinguish.

In contrast to the restricted anti-IgG2a and anti-IgG2b mIgMRF, monoclonal autoantibodies reacting with all murine IgG subclasses cross-reacted with all heterologous IgG without preference for a given human IgG subclass or allotype. These results indicate that broadly cross-reacting anti-γ-globulins recognize an antigenic determinant that has been highly conserved phylogenetically.

In direct binding assays, all but one of the monoclonal autoantibodies bound to the Fc but not to the Fab portion of mouse IgG, conclusively demonstrating that these monoclonals had the sine qua non qualities of RF. The one exception was monoclonal RF15, which bound to both Fab and Fc, suggesting the presence of a common antigenic determinant in Fab and Fc. In contrast to our mIgMRF derived from the natural repertoire of the MRL/l IgMRF, 48 mIgMRF from

LPS-injected 129/Sv mice (36) did not bind to Fab or Fc, theoretically because these monoclonals were directed against the Fv region of mouse IgG. This is an unusual characteristic for RF and contrasts with the specificity for Fc of spontaneous polyclonal RF from 129/Sv mice described by the same authors (38, 39). These findings further support our contention that mitogen-induced autoantibodies may differ from natural autoantibodies in isotypic characteristics and also epitopic specificities.

With Fc identified as the fragment against which our mIgMRF were directed, inhibition studies with protein A and C1q were done to more precisely locate the antigenic determinants involved. The C1q binds via its globular heads to the CH2 domains of antibodies (26, 27) at the highly conserved amino acid sequences encompassed within residue 316–338 (55). However, C1q did not inhibit interactions of any of the mIgMRF with substrate IgG, thus ruling out this site as a responsible antigenic determinant. This finding indicates that fixation of C1q by immune complexes does not interfere with RF binding or vice versa. Yet protein A, which binds to Fc but does not interfere with complement binding (28), inhibited the interactions of over one-half of our mIgMRF with the substrate IgG. The crystal structure of the complex between fragment B of protein A and Fc fragments indicates that protein A binds at the CH2-CH3 junction (28, 56, 57). Thus, the antigenic sites for 12 of the 23 mIgMRF that were strongly inhibited by protein A could be placed at or very near the CH2-CH3 junction. However, in the converse experiment, binding of mIgMRF to substrate IgG did not inhibit subsequent binding of protein A to this substrate, indicating that the binding sites for RF and protein A are not identical. Therefore, the antigenic sites for the RF are distant to the junction but become inaccessible as tertiary IgG folding patterns change after protein A binds. Alternatively, IgG Fc sites distant to the junction in both the CH2 and CH3 domains may become sterically blocked by the protruding chains of the bound protein A, as the crystallographic studies of Deisenhofer (57) have strongly suggested. These sites may include the antigenic determinants identified above on the basis of subclass specificity and structural differences between IgG subclasses, but their exact location on the Fc domains cannot be accurately defined at this time. In contrast, the antigenic site for mIgMRF weakly or not inhibited by protein A is at the CH3 domain. This group includes the five monoclonals that reacted broadly with all murine IgG subclasses and various heterologous IgG. That the site for these five monoclonals is at the CH3 domain was demonstrated by their equal binding to human Fc (CH2-CH3) and pFc′ (CH3) pieces.

Studies with human polyclonal RF have shown that some of them cross-react with the nucleosomal histone complex or ssDNA (58). Antihistone reactivity was also described with a Waldenstrom-derived mRF (59). We found that two of our mIgMRF exhibited cross-reactions with murine IgG subclasses and certain calf thymus histones. Elution and inhibition studies to be reported[2] showed that the multiple reactivities of each mIgMRF were a property of a single antibody population. Moreover, five mIgMRF reacted with IgG and ssDNA, and one of the five had additional reactivity with histones. The biologic significance of this type of cross-reaction and the relationship, if any, of the antigenic determinants involved in the activities of these peculiar autoantibodies remain unknown.

Anticollagen autoantibodies have been described in RA (60) but possible cross-reactions of RF with collagen have not been examined. However, none of our mIgMRF reacted with chick collagen II. Finally, Bokisch et al. (24) described 7S RF-like antibodies of restricted molecular heterogeneity in rabbit immunized with streptococcal cell wall peptidoglycan. A defined pentapeptide sequence of peptidoglycan inhibited interactions of such antibodies with IgG Fc. Our studies failed to disclose any reactivity of the mIgMRF with the pentapeptide sequence of peptidoglycan, thus making it unlikely that MRL/l-associated RF form against such bacterial antigens that cross-react with IgG. Further studies of the mIgMRF that are derived from the natural repertoire of the MRL/l mice will allow us to better define their roles in immune responses and in the pathogenesis of arthritis as well as their idiotypic and epitopic specificities.

Summary

MRL/*lpr/lpr* (MRL/l) mice develop a lupus-like syndrome and a disease histologically and serologically similar to human rheumatoid arthritis. Their sera contain polyclonal IgM rheumatoid factors (RF) reactive with all murine IgG subclasses (frequently strongest with IgG2a) and several heterologous IgG. To examine the repertoire and epitopic specificities of these RF, we fused splenocytes from 3.5-mo-old seropositive MRL/l mice with appropriate myeloma partners and derived 1,723 hybridomas of which 23 secreted IgMRF.

These monoclonal IgMRF bound to murine IgG only, not to other murine isotypes. Eight murine IgG subclass-specific clonotypes were identified. Most clones reacted with either multiple IgG subclasses or with IgG2a alone. A few clones reacted solely with IgG2b but none reacted exclusively with IgG1 or IgG3. Monoclonal IgMRF with exclusively anti-IgG2a activity exhibited allotypic specificity, reacting, with few exceptions, with a, c, and e, but not b, d, or j IgG2a allotypes. Four clonotypes could be distinguished by cross-reactivity with IgG from species other than mice. Monoclonals possessing activity against several murine subclasses cross-reacted extensively with heterologous IgG, including all human IgG subclasses without allotypic restrictions. Monoclonal IgMRF specific for murine IgG2a or 2b did not cross-react with heterologous IgG. Based on the absence of cross-reactions by IgG2a-specific monoclonal autoantibodies, certain peptides of the IgG CH2 and CH3 domains appear to generate the antigenic determinants of the anti-IgG2a RF in MRL/l mice.

All of the monoclonal RF bound to Fc and, with one exception, not to Fab fragments of murine IgG. Binding of the monoclonal RF to substrate IgG was not inhibited by C1q, thus excluding the C1q-binding site at the CH2 domain as one of the responsible epitopes in the induction of MRL/l RF. mIgMRF could be categorized as strongly, weakly, or noninhibitable by protein A, which interacts with IgG molecules at or near the CH2-CH3 junction. Inhibition appears to be caused by conformational changes and/or steric shielding of certain IgG areas distant from this junction and not by identical binding sites between protein A and RF. Certain of the mIgMRF that were weakly or not at all inhibitable by protein A were found to cross-react equally well with human Fc (CH2-CH3 domains) and pFc′ (CH3 domain) fragments, indicating that the binding site for these monoclonals is at the CH3 domain. Monoclonal RF were devoid of anti-

double-strand DNA, anticollagen, or antipeptidoglycan pentapeptide cross-reactivity, but one of the monoclonals cross-reacted with histones, four with single-strand DNA, and one with both histones and single-strand DNA.

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