ISOTYPIC AND ALLOTYPIC SPECIFICITY OF MOUSE RHEUMATOID FACTORS*

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It is only recently that rheumatoid factor (RF)[1]-like anti-IgG autoantibodies have been studied in the mouse. They were first detected in high titers in the mutant strain MRL/l, which develops a severe autoimmune disease characterized by massive T lymphocyte proliferation and autoantibody production against many different self-antigens (1). Soon thereafter the presence of RF was also recognized in the serum of many normal strains from various colonies (2, 3). In most of these strains, the titers of RF increase with age and in some, like the 129/Sv, they reach levels comparable to those of the MRL/l. In addition to these RF of spontaneous origin, RF were also detected in the serum of several normal mouse strains after injection of lipopolysaccharide (LPS) (4). This polyclonal activator of B lymphocytes proved to be a potent stimulus for the induction of RF: it was found by plaque-forming cell techniques that a high proportion of the B lymphocytes stimulated to IgM production by LPS produce IgM with RF activity (5, 6, 7). In addition, the hybridomas derived from spleen cells polyclonally activated in vivo frequently secreted IgM with anti-IgG activity (8).

The specificity of mouse RF has mainly been studied in the 129/Sv strain. The autoantibodies spontaneously produced by these mice in certain colonies preferentially reacted with IgG2a (2). In contrast, the monoclonal RF derived from 129/Sv spleen cells after polyclonal activation in vivo had a very different spectrum of specificities, since most were specific for IgG1. It thus seemed that, in this strain, RF may be selectively activated in vivo on the basis of their subclass specificity. It was therefore of interest to find out whether subclass specificity is a peculiarity of certain 129/Sv RF or is a fundamental characteristic of all mouse RF. Accordingly, we tested the specificity of RF found in the serum of various mouse strains including autoimmune strains like NZB and MRL/l. We also examined the specificity of RF secreted in vitro by spleen cells of several strains upon stimulation with LPS. In addition, we generated a large number of monoclonal RF from four different strains under a variety of conditions including polyclonal activation in vitro. The results indicate that whatever

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; DNP, dinitrophenyl; DS, dextran sulfate; FBS, fetal bovine serum; GBS, glycine-buffered saline; LPS, lipopolysaccharide; RF, rheumatoid factor; SRBC, sheep erythrocytes; TNP, trinitrophenyl.

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the strain or mode of induction, all the mouse RF that were analyzed reacted with subclass-specific structures.

Materials and Methods

Mice. 129/Sv, C57Bl/6, BALB/c, and CBA/Ht mice were maintained under specific pathogen-free conditions in the colony of our Institute by Dr. Guy Warnier. The first three strains were derived from breeders given by Dr. J. L. Guénet, Institut Pasteur, Paris, the last from breeders obtained from Dr. H. Hewitt. NZB/Binj, MRL/l, RIIIS/J, and CE/J mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and A/HeJ from the Central Animal Laboratory, University of Nijmegen, The Netherlands.

Polyclonal Stimulation In Vitro. Spleen cells were teased in Hanks' balanced salts solution containing 5% fetal bovine serum (FBS). Cell suspensions were filtered through a nylon mesh gauze (80 μm; Nylon Swiss; J. Staniar and Co., Manchester, United Kingdom [UK]), centrifuged at 100 *g* for 10 min and resuspended at 5×10^5 cells/ml in Iscove's medium supplemented with 5% FBS, 1-glutamine (1.5×10^{-3} M), 1-asparagine (2.4×10^{-4} M), 1-arginine (5.5×10^{-4} M), 2-mercaptoethanol (5×10^{-5} M), and either 25 μg/ml LPS from *Escherichia coli* (055:B5; Difco Laboratories, Detroit, MI) or 25 μg/ml LPS plus 200 μg/ml dextran sulfate (DS) (500,000 mol wt; Pharmacia Fine Chemicals, Uppsala, Sweden).

Cell Fusion and Culture. Polyclonally activated or untreated normal spleen cells were fused with SP2/0-Ag-14 myeloma cells at a 5:1 ratio according to the method described previously (8). The myeloma cells were grown in Iscove's medium enriched with 10% FBS.

Coupling of Proteins to Polystyrene Particles. Proteins were coupled to carboxylated polystyrene particles, 0.8 μm, (Rhône-Poulenc, Courbevoie, France) after activation of the latter with 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide as described by Masson et al. (9). Briefly, 250 μg of protein was added to 50 μl of a 10% (vol/vol) suspension of carbodiimide-activated particles. After overnight incubation at 4°C and appropriate washing the particles were resuspended in 1 ml of glycine-buffered saline (GBS) (0.1 M glycine, 0.15 M NaCl, pH 9.2) containing 1% bovine serum albumin (BSA). Before use, these suspensions were further diluted 10 times in the same buffer and sonicated at 25 W for 15 s (Branson Sonic Power Co., Danbury, CT). By this method, the number of molecules coupled per particle was, respectively, 10–15,000 for human IgG and mouse IgG1, IgG2a, and IgG2b; 3,000 for mouse IgM; 7,000 for ovalbumin; and 25,000 for lysozyme.

Detection of RF-secreting Clones. The large numbers of clones generated by the fusion of polyclonally activated spleen cells were screened for antibody activity by testing the ability of their secretion products to agglutinate polystyrene particles coated with various proteins. Aliquots (20 μl) of medium were mixed with an equal volume of particles in round-bottomed microtiter plates (Greiner, Nurtingen, Federal Republic of Germany [FRG]). By this system agglutinations were readily screened visually after 2 h of incubation at room temperature (Fig. 1). The limit of IgM antibody detection by this assay was ≃0.1 μg/ml.

Inhibition of Agglutination with Heat-aggregated IgG. RF preparations were incubated for 1 h at 37°C with heat-aggregated IgG before addition of the IgG-coated particles. After 1 h more at 37°C, the inhibition of agglutination was measured by counting the number of nonagglutinated particles with a particle counter as described previously (2).

Radioimmunoassays. Polyvinyl flexible microtiter plates (Flow Laboratories, Inc., Rockville, MD) were coated by overnight incubation with 25 μl of a 10 μg/ml protein solution in 1:5 diluted GBS. After washing in saline containing 0.01% Tween 20 (Technicon Chemicals, Orcq, Belgium), they were incubated for 3 h at 37°C with monoclonal RF. Nonspecific binding was drastically reduced by subsequently soaking the plates for 7 min in saline containing 1% Nonidet P-40 (Fluka AG, Buchs, Switzerland), before further incubation (2 h at 37°C) with ^{125}I-labeled affinity-purified goat antibodies specific for mouse IgM.

Protein Preparations. Monoclonal and polyclonal mouse IgG subclasses were purified by sequential elution from protein A-Sepharose as described (10). Monoclonal IgM was isolated from ascitic fluid by ammonium sulfate precipitation, gel filtration on AcA22 Ultrogel (LKB Produkter, Bromma, Sweden), and preparative Pevikon-block electrophoresis (SERVA Feinbiochemica GmbH & Co., Heidelberg, FRG). Mouse transferrin was purified by chromatography on DEAE-cellulose (D32; Whatman Ltd., Kent, UK), and gel filtration on Ultrogel

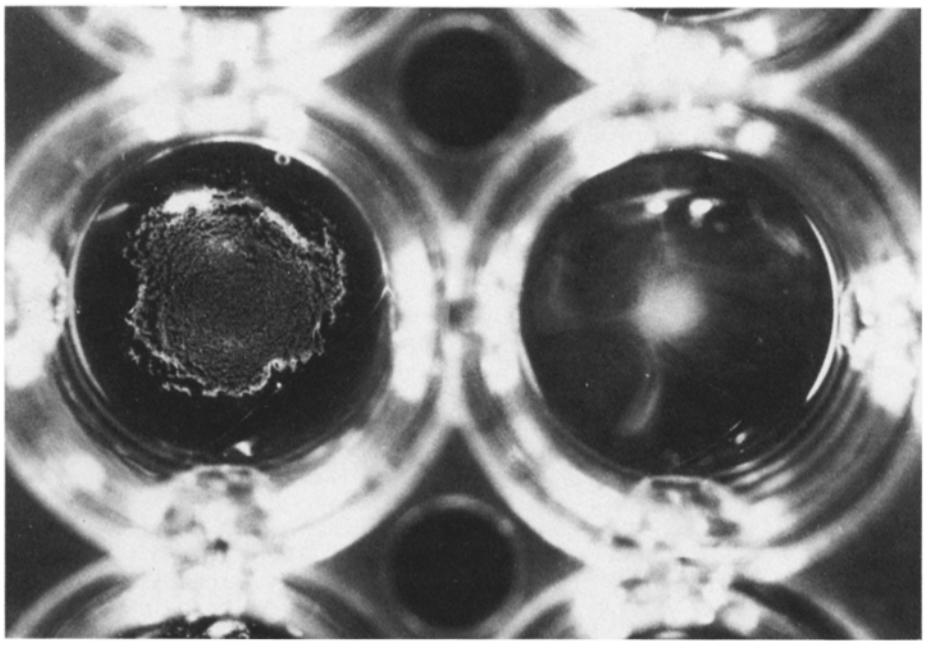

FIG. 1. Agglutination of IgG1-coated polystyrene particles in microtiter plates. Culture medium from an RF-secreting hybridoma (left well) and control medium (right well) were incubated for 2 h with a 1% suspension of polystyrene particles coated with IgG1.

AcA44. Human IgG was isolated from a pool of human serum by ammonium sulfate precipitation and chromatography on DEAE-cellulose. Hen egg white lysozyme was purchased from Koch-Light Laboratories Ltd., Berkshire, U.K., and ovalbumin from Sigma Chemical Co., St. Louis, MO.

Results

Subclass Specificity of Polyclonal RF of Spontaneous Origin. RF are regularly found in the serum of various normal and autoimmune strains of mice. We tested by competition experiments the specificity of the RF of two strains with autoimmune diseases (MRL/l and NZB/BinJ) and of three "normal" strains (C57Bl/6, CBA/Ht, and 129/Sv) whose sera were found to strongly agglutinate polystyrene particles coated with either IgG1 or IgG2a.

Addition of graded quantities of heat-aggregated IgG1 or IgG2a to these diluted sera resulted in a complete inhibition of the agglutination when the competing IgG and the IgG on the particles had the same isotype. No inhibition was observed when the subclass of the inhibiting IgG differed from that of the IgG present on the particles (Table I). These anti-IgG1 and anti-IgG2a autoantibodies therefore belonged to distinct antibody populations. Unlike the sera of the three normal strains, those of MRL/l and NZB/BinJ mice also agglutinated particles coated with IgG2b. The latter agglutination was again subclass specific since it was inhibited by heat-aggregated IgG2b but not by IgG1 or IgG2a (Fig. 2).

Subclass Specificity of Polyclonal RF Induced by In Vitro Stimulation with LPS. Spleen cells of 129/Sv and C57Bl/6 mice were stimulated in vitro with LPS and the secreted anti-IgG autoantibodies were analyzed for subclass specificity by competition exper-

TABLE I

Specificity of Polyclonal Mouse RF of Spontaneous Origin

Particles coated with	Strain*	Serum dilution	Control agglutination	Concentration of heat-aggregated IgG‡ inhibiting agglutination by 50%	
				IgG1	IgG2a
				mg/ml	
IgG1	C57Bl/6	1:10	84§	0.003	>1
	CBA/Ht	1:2	57	0.050	>1
	MRL/l	1:30	85	0.016	>1
	NZB/BinJ	1:5	70	0.003	>1
	129/Sv	1:2	76	0.004	>1
IgG2a	C57Bl/6	1:15	85	>1	0.025
	CBA/Ht	1:135	78	>1	0.001
	MRL/1	1:135	58	>1	0.017
	NZB/BinJ	1:5	82	>1	0.009
	129/Sv	1:135	76	>1	0.003

* For each strain pooled sera from 5 to 10 mice >6 mo old were used. After appropriate dilution in GBS containing 1% BSA and heating at 56°C for 30 min to destroy any thermolabile agglutinators, sera were incubated with various concentrations of heat-aggregated IgG for 1 h at 37°C. Polystyrene particles coated with IgG1 or IgG2a were then added to the mixture and incubated with shaking at 37°C for 1 h. Finally, residual nonagglutinated particles were counted instrumentally (2).

‡ MOPC-31C was used as competing IgG1 and MOPC-173 as competing IgG2a. To ensure the subclass specificity of the inhibitions, the polystyrene particles were coated with different monoclonal proteins of 129/Sv origin (108B7 for IgG1 and 1103G4 for IgG2a).

§ Percent agglutinated particles measured in the absence of inhibitor.

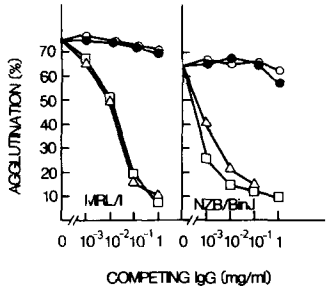

FIG. 2. IgG2b-specific RF in MRL/1 and NZB/BinJ mice. Sera of 4-mo-old MRL/l and NZB/BinJ mice diluted 1:5 in GBS were incubated at 37°C with heat-aggregated IgG1 (108B7 [●]), IgG2a (MOPC-173 [○]), monoclonal IgG2b (307E3 [△]), and polyclonal IgG2b isolated from 129/Sv serum (□). 1 h later, particles coated with monoclonal IgG2b (308A8) were added and the agglutinated particles measured after a further 60 min incubation at 37°C.

iments similar to those used for the analysis of RF found in mouse sera. With this technique, no cross-reaction could be detected between antibodies reacting with either IgG1 or IgG2a (Table II).

Similar experiments were carried out with spleen cells of A/HeJ, CBA/Ht, and RIIIS/J mice. The culture media strongly agglutinated particles coated with IgG1 and to a lesser extent particles coated with IgG2a. The latter reaction however, was too weak to be properly analyzed by competition experiments. We therefore restricted the specificity analysis to the agglutination of IgG1 particles. To ensure that low affinity reactions would not be overlooked, the latter analysis was carried out by

TABLE II

Specificity of Polyclonal RF Induced by In Vitro Stimulation with LPS

Strain*	Inhibitor‡	Inhibition of the agglutination§ of particles coated with	
		IgG1	IgG2a
C57Bl/6	IgG1 (108B7)	81	0
	IgG2a (MOPC-173)	4	78
129/Sv	IgG1 (108B7)	72	0
	IgG2a (MOPC-173)	0	85

* Spleen cells of 20-wk-old C57Bl/6 and 129/Sv mice were stimulated in vitro for 7 d in the presence of 25 μg/ml LPS as described in Materials and Methods. The medium concentrated sevenfold by precipitation with 50% saturated ammonium sulfate agglutinated 60–70% of the IgG1- and IgG2a-coated particles.

‡ Inhibitors were heat-aggregated and used at a concentration of 1 mg/ml. They were preincubated with the medium for 2 h at 37°C before addition of the IgG-coated particles.

§ In percent of control agglutination.

TABLE III

Specificity of Polyclonal Anti-IgG1 Autoantibodies Induced by In Vitro Stimulation with LPS

Strain*	Residual‡ anti-IgG1 agglutinating activity after absorption with particles coated with		
	IgG1 (108B7)	IgG2a (1103G4)	IgG2b (308A8)
A/HeJ	10	89	94
C57Bl/6	6	97	97
CBA/Ht	17	103	115
RIIIS/J	38	99	97
129/Sv	16	99	100

* Media from LPS-stimulated spleen cell cultures were obtained as described in Table II.

‡ Media were incubated at room temperature for 2 h with a constant volume of particles coated with various IgG preparations. After centrifugation, the residual agglutinating activity towards IgG1(108B7)-coated particles was determined by using a volume of particles ten times smaller than that used for absorption. This residual activity was expressed in percent of the control agglutination measured after absorption with particles coated with an unrelated protein (mouse transferrin).

absorption rather than competition experiments. Accordingly, the residual anti-IgG1 activity was determined in medium incubated with a 10-fold excess of particles coated with various IgG preparations. Only IgG1-coated particles removed anti-IgG1 activity (Table III). Similar results were obtained with particles coated with two different monoclonal IgG1 preparations (MOPC-31C and 108B7).

Subclass Specificity of Monoclonal RF. Monoclonal RF were obtained from three different sources: (*a*) mice that spontaneously produce RF in vivo, (*b*) mice injected with LPS, or (*c*) spleen cells after polyclonal activation in vitro. Clones that produced RF were detected by screening culture media for agglutination of particles coated with IgG1, IgG2a, or IgG2b in microtiter plates. Positive clones were expanded and their medium was retested for agglutination of IgG-coated particles by monitoring

the reaction with a particle counter as described (2). The specificity of these reactions was checked by testing the ability of heat-aggregated monoclonal IgG to inhibit the agglutination. Different Ig of the same isotype were used to coat the particles and inhibit the reaction so as to restrict this analysis to antibodies specific for constant IgG regions. Clones yielding agglutinations that could not be inhibited by heat-aggregated IgG were found to produce IgM molecules that bound readily to plastic surfaces whatever their protein coat. These clones were discarded. The specificity of positive clones was further checked by solid-phase radioimmunoassay using flexible polyvinyl plates coated with different IgG preparations. Typical specificity data are shown in Fig. 3 and Table IV.

MONOCLONAL RF OBTAINED WITHOUT POLYCLONAL ACTIVATION. Seven hybridizations were performed with spleen cells of adult 129/Sv mice (≃12 wk old). They yielded a total of ≃3,000 clones, 26 of which specifically reacted with autologous IgG. Of these 26 anti-IgG clones, 25 were specific for IgG2a and 1 for IgG1. A similar hybridization carried out with BALB/c mice produced two anti-IgG2a clones out of 550 hybridomas.

MONOCLONAL RF OBTAINED AFTER POLYCLONAL ACTIVATION IN VIVO. We have previously shown that, after intraperitoneal injection of LPS into 20-wk-old 129/Sv mice, it is possible to obtain, at a high frequency, hybridomas with RF activity most of which are specific for IgG1. Here we repeated these experiments with 4-wk-old 129/Sv mice and with 4- or 20-wk-old C57Bl/6 mice. Of ≃3,000 clones, 56 were found to react with IgG. No significant difference in the frequency of these clones was detected whatever the strain or age of the animals. 11 of these clones were subcloned

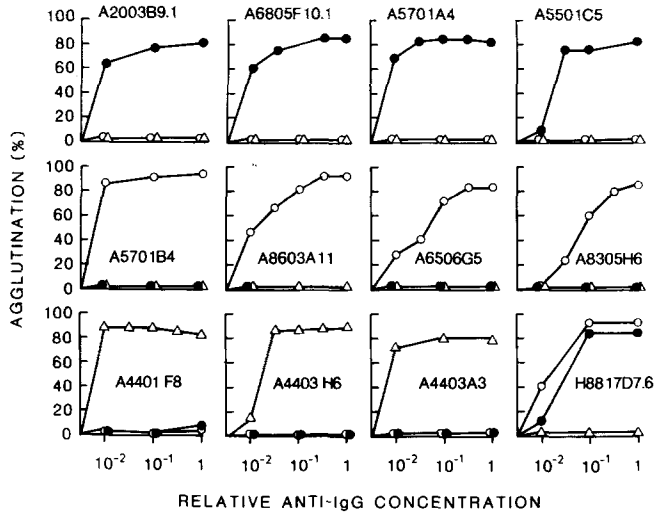

FIG. 3. Agglutination of IgG-coated polystyrene particles by monoclonal RF. Culture media from various hybridomas were incubated for 1 h at 37°C with particles coated with IgG1 (108B7 [●]), IgG2a (1103G4 [○]), or IgG2b (308A8 [△]). The agglutination was measured as described (2). The strains of origin of the monoclonal RF were as follows: C57Bl/6 for A2003B9.1; BALB/c for A6805F10.1, A8603A11, and A8305H6; CBA/Ht for A5501C5; and 129/Sv for A5701A4, A5701B4, A6506G5, A4403A3, A4403H6, A4401F8, and H8817D7.6. Relative anti-IgG concentrations refer to dilutions of the RF-containing culture media.

TABLE IV

Subclass-specificity of Monoclonal RF

Monoclonal RF			Binding* to wells coated with			
Specificity	Origin‡	Clone	IgG1§ (108B7)	IgG2a (1103G4)	IgG2b (308A8)	IgG3 (FLOPC -21)
Anti-IgG1	B6, LPS in vivo	A2003H11	32	0.9	0.4	1.9
		H6314G12	41	1.3	0.5	1.9
	129/Sv, spontaneous	A6505C6	10	0	0	0.1
	129/Sv, LPS + DS in vitro	A5701A4	46	0.1	0	0.2
		A5703F12	48	0.1	0.9	0.7
	BALB/c, LPS + DS in vitro	A6805F10	38	0	3.7	0.5
		A6803F6	16	0	0.6	0.3
	CBA/Ht, LPS in vitro	A5501C5	33	0	0.9	0.2
		A9804F4	30	0	0.6	0.2
Anti-IgG2a	129/Sv spontaneous	A2901D2	0.6	29	0.3	0.6
		A6107C6	0.2	31	0.2	2.7
		A6501A3	0.4	35	2.3	4.7
		A6608F9	0.9	40	4.5	1.1
	129/Sv, LPS + DS in vitro	A5701B4	0.8	42	0	1.2
		A5705A5	2.8	26	4.2	1.6
	BALB/c, spontaneous	A8305B4	0.1	14	0	0
	BALB/c, LPS + DS in vitro	A8601A2	1	27	0	1.6
		A8602B4	1.2	28	0	1.4
Anti-IgG2b	129/Sv, LPS + DS in vitro	A4401F8	0.6	0.3	37	0.3
		A4403H6	0.5	0	31	0.4
		A4403A3	0.2	0	38	0.2

* Measured as described in Materials and Methods. Figures represent the mean of triplicate measurements expressed in cpm $\times 10^{-3}$ after subtraction of the radioactivity bound to wells coated with BSA.

‡ See explanations in the text.

§ To remove any contaminating IgM molecules, IgG preparations were ultracentrifuged over a sucrose gradient. Only the 7S fractions were used to coat the wells.

and used for specificity studies. 10 were specific for IgG1, and 1 reacted equally well with IgG1 and IgG2a (H8817D7.6) (Fig. 3).

MONOCLONAL RF OBTAINED AFTER POLYCLONAL ACTIVATION IN VITRO. It is relatively difficult to control the degree of polyclonal activation obtained after injection of LPS in vivo. Potential limitations to the activation stem from the toxicity of LPS, which varies from one strain to another, and from regulatory influences of T lymphocytes (11). Because in vitro polyclonal activation overcomes these difficulties, spleen cells of 4-wk-old mice were activated in vitro with LPS or with a mixture of LPS and DS, which has been reported to activate 80% of B lymphocytes in vitro (12). 3 d later the cells were fused with the SP2/0-Ag-14 myeloma cell line and the resulting hybridomas were tested for anti-IgG activity. Three hybridizations were carried out with CBA/Ht and 129/Sv mice and two with BALB/c mice. The total number of clones generated varied considerably from one experiment to another. In some, the fusion frequency was so high that it became difficult to accurately determine the number of clones obtained. The latter could therefore only be roughly estimated at ≃9,000 clones. 34 had strong anti-IgG activity; of these, 16 were derived from 129/Sv

spleen cells, 8 with anti-IgG1 specificity, 4 with anti-IgG2a specificity, and 4 with anti-IgG2b specificity. Six anti-IgG clones were derived from CBA/Ht cells; of these, five were anti-IgG1 and one reacted with both IgG2a and IgG2b. Of the 12 BALB/c anti-IgG clones, 3 were anti-IgG1 and 9 anti-IgG2a. Like the anti-IgG clones activated in vivo either spontaneously or after injection of LPS, the monoclonal RF derived from spleen cells polyclonally activated in vitro thus also show a preferential if not exclusive reactivity towards IgG subclass-specific structures.

Allotypic Specificity of Monoclonal RF. We have previously reported that the anti-IgG2a RF spontaneously produced by certain mouse strains has an allotypic specificity in the sense that it does not react with IgG2a of the b allotype (3). The allotypic specificity of the monoclonal anti-IgG2a RF obtained here was tested by measuring the ability of heat-aggregated IgG2a molecules of the a and b allotypes to inhibit the agglutination of $IgG2a^a$-coated particles. Of the 26 anti-IgG2a clones derived from 129/Sv spleen cells without prior polyclonal activation, 6 reacted indistinctly with IgG2a of both allotypes, but 20 failed to react with IgG2a of the b allotype. The two anti-IgG2a clones derived from BALB/c mice under similar conditions showed the same allotypic specificity. After polyclonal activation in vitro, both kinds of anti-IgG2a clones were detected (Table V). No allotypic specificity was detected for anti-

TABLE V

Allotypic Specificity of Anti-IgG2a Autoantibodies

Origin	Clone	Control* agglutination	Inhibition by heat-aggregated‡			
			$IgG2a^a$		$IgG2a^b$	
			MOPC-173§	A6202F4	A7708F11	Polyclonal
129/Sv, spontaneous	A2901D2	90	91	91	7	0
	A6107C6	85	100	98	0	0
	A6506G5	56	100	100	9	0
	A6608D6	81	100	100	0	0
	A7105D4	69	100	100	0	0
	A7601E5	74	100	100	0	0
	A6501A3	85	100	100	100	78
	A6504C12	67	100	100	100	85
	A6507A10	89	100	96	96	81
BALB/c, spontaneous	A8305H6	85	88	90	28	8
BALB/c, LPS + DS in vitro	A8603A11	72	86	100	0	0
	A8607C1	86	86	65	0	0
129/Sv, LPS in vitro	A5701B4	91	100	100	100	90
	A5705A5	77	95	100	95	87
	A5705B8	84	100	100	100	100

* Agglutination of particles coated with 1103G4 ($IgG2a^a$ of 129/Sv origin).

‡ Measured as described in Materials and Methods and expressed in percent inhibition of control agglutination.

§ The polyclonal $IgG2a^b$ was purified from the serum of SJL/J mice. MOPC-173, A6202F4, and A7708F11 are monoclonal IgG2a proteins of BALB/c, 129/Sv, and C57Bl/6 origin, respectively. All four preparations were used at 1 mg/ml.

IgG1 RF, which reacted equally well with polyclonal IgG1 of the b (C57Bl/6) and d (AKR) allotypes (13) and with IgG1 of the a allotype (data not shown).

Binding of Monoclonal RF to Antigen-Antibody Complexes. The hybridomas obtained after in vitro polyclonal activation were not only tested for anti-IgG activity but also for possible reactivity with mouse IgM, human IgG, ovalbumin, and hen egg white lysozyme. Although some 40 clones reactive with mouse IgG were obtained in these experiments, no hybridoma was found to react specifically with the other tested proteins.

In view of this high relative frequency it was important to examine the possibility that the anti-IgG antibodies were directed against artefactual antigenic determinants that could result from the adsorption of IgG onto the plastic solid phase used in the detection and specificity analyses described above. We therefore tested the reactivity of some anti-IgG1 and anti-IgG2a clones with IgG immobilized in a more physiological way. The RF were mixed with monoclonal IgG1 and IgG2a anti-dinitrophenyl (DNP) monoclonal antibodies in the presence of trinitrophenylated sheep erythrocytes (SRBC). Strong binding of the RF was observed only when the SRBC were coated with IgG of the appropriate isotype. No binding was observed when IgG1 and IgG2a without anti-DNP activity were used (Table VI).

Differential Reactivity of Monoclonal RF with Aggregated and 7S IgG. Circulating RF in both man and mouse characteristically have a low affinity for IgG. This is well illustrated by the fact that the agglutination of IgG-coated particles by RF is easily inhibited by heat-aggregated IgG but very little by 7S IgG. In patients with rheumatoid arthritis, it has been reported that high affinity RF is actually produced but is not detectable in the serum (15).

TABLE VI

*Binding of Monoclonal Anti-IgG Autoantibodies to Antigen-Antibody Complexes**

Monoclonal IgM		IgM bound to TNP-SRBC in the presence of‡			
Clone	Specificity§	IgG1 anti-DNP	IgG1	IgG2a anti-DNP	IgG2a
8815B5‖	Anti-IgG1	22	1	3	1
A5706D9	Anti-IgG1	23	1	2	1
A6005B9	Anti-IgG2a	1	1	33	1
A6501A3	Anti-IgG2a	1	1	43	1
A5701B4	Anti-IgG2a	1	1	8	1
8817D7‖	Anti-(IgG1 + IgG2a)	33	1	27	1
101B11‖	Unknown	1	1	2	1

* SRBC were trinitrophenylated as described in (14). Equal volumes (25 μl) of a 10% cell suspension, of monoclonal IgG1 or IgG2a solutions (100 μg/ml), and of appropriately diluted medium containing monoclonal RF were mixed and incubated at room temperature for 2 h. All dilutions were in Tris-buffered saline, pH 7.4, containing 5% FBS. After washing, the cell pellet was resuspended in the same diluent containing affinity-purified goat anti-mouse IgM antibodies labeled with ^{125}I. After further incubation at room temperature for 2 h and extensive washing, the radioactivity associated with the pellet was measured. The figures correspond to specifically bound cpm $\times 10^{-3}$ (obtained after subtraction of cpm bound to SRBC incubated with the monoclonal IgM in the absence of any IgG). All tests were performed in triplicate.

‡ The following hybridomas of 129/Sv origin were used: A6204G12, IgG1 anti-DNP; A6202F4, IgG2a anti-DNP; 108B7, IgG1; 1103G4, IgG2a. The last two proteins have no known specificity.

§ As determined by latex agglutination and solid phase radioimmunoassays.

‖ These monoclonal IgM proteins were used in a purified form at a concentration of 10 μg/ml in Tris-buffered saline containing 5% FBS.

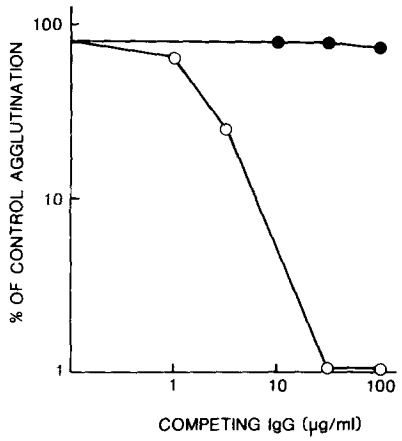

FIG. 4. Inhibition of the agglutination of IgG1-coated particles in the presence of antigen-antibody complexes. Antigen-antibody complexes were made by mixing 400 μg of a purified monoclonal IgG1 anti-DNP antibody (A6204G12) with 20 μg of BSA DNP_{10}. Complexed (○) and free (●) A6204G12 (25 μl) were incubated with an equal volume of a monoclonal IgG1-specific IgM RF of 129/Sv origin (A8007A3). After 1 h at 37°C, 25 μl of a 1% suspension of IgG1-coated particles were added and the agglutination measured after 1 h more at 37°C. The control agglutination, measured in the absence of IgG1, was 80%.

TABLE VII

Reactivity of Monoclonal RF with Aggregated and 7S IgG

Monoclonal RF		Inhibition* of the agglutination of IgG-coated particles in the presence of	
Specificity	Clone	Aggregated IgG	7S IgG‡
Anti-IgG2a	A2901D2	100	0
	A6107C6	100	3
	A6501A3	100	5
	A6608F9	77	0
	A5701B4	100	4
	A8305B4	100	0
Anti-IgG2b	A4401F8	100	77
	A4403A3	100	71
	A4403H6	100	100
	A4409G3	100	82

* For anti-IgG2a clones, the particles were coated with 1103G4, a 129/Sv IgG2a monoclonal protein, and the competing IgG was MOPC-173, a BALB/c IgG2a myeloma protein. For anti-IgG2b clones, the particles were coated with 308A8, a 129/Sv IgG2b hybridoma protein, and the competing IgG was MOPC-141, a BALB/c myeloma protein. The inhibitions, which were carried out as described in Materials and Methods, are expressed in percent of the control agglutination. Inhibitors were used at 1 mg/ml.

‡ Obtained by ultracentrifugation over a sucrose gradient.

Mouse monoclonal RF produced in vitro in the absence of mouse serum should give access to such high affinity autoantibodies. We therefore compared the ability of a 7S or complexed IgG1 anti-DNP monoclonal antibody to inhibit the agglutination

of IgG1-coated particles by anti-IgG1 clones. All anti-IgG1 monoclonal antibodies tested were strongly inhibited by the complexed IgG1, but none was affected by the IgG1 used in the absence of antigen. Typical data are shown in Fig. 4. Similar results were obtained for all anti-IgG2a clones, which reacted well with IgG2a only when the latter was aggregated by heating. In contrast, with all four monoclonal anti-IgG2b RF, similar inhibitions were obtained with 7S and heat-aggregated IgG2b (Table VII).

Discussion

The RF spontaneously produced by normal mouse strains were found to consist of two distinct antibody populations, one specific for IgG1 and one specific for IgG2a. This subclass specificity seemed very strict as it was impossible by agglutination-inhibition experiments to detect any cross-reaction between these two RF species. Even the NZB and MRL/l strains, which produce numerous autoantibodies against a large number of self-antigens, had RF with a similarly strict subclass specificity. However, their serum contained an additional variety of RF that reacted specifically with IgG2b.

Competition and absorption experiments also resolved the RF secreted in vitro by polyclonally activated spleen cells into distinct subpopulations, each specific for one IgG subclass. Within the sensitivity limits of these experiments, virtually all mouse RF appeared to be directed against subclass-specific sites on mouse IgG. This conclusion was corroborated by the results obtained with the monoclonal RF that were derived from various strains under different conditions: of the 73 clones tested, 71 had a strict subclass specificity.

In addition to their subclass specificity, some anti-IgG2a clones were allotype-specific in the sense that they failed to react with IgG2a of the b allotype. This suggests that the antigenic determinants recognized by these RF are located in the hinge region or the CH3 domain of the IgG2a heavy chain, where most amino acid substitutions between the a and b allotypic forms of mouse IgG2a have been found (16, 17). No such allotypic specificities have so far been detected with anti-IgG1 and anti-IgG2b clones. This is probably due to the fact that the divergence between γ2a alleles is much more important than, for example, that between γ2b alleles. It actually appears that the third domain of the $\gamma 2a^b$ gene has diverged so much from the corresponding $\gamma 2a^a$ domain that "it may have derived from elsewhere in the heavy chain family" (18).

Subclass and allotype specificities do not seem to be a peculiarity of mouse RF. Most rheumatoid arthritis patients produce RF that react with an antigen, called Ga, which is present on IgG1, IgG2, and IgG4 but not on IgG3 (19), and a minority of human RF specifically recognize the "γ4 non-a" antigen, which is only found on IgG4 molecules (20). In addition, many human RF are specific for allotypic structures. A number of Gm markers have even been identified with rheumatoid sera, e.g., Gm(a), (x), (b^1), and (g) (21–24).

Although such similarities between the specificities of human and mouse RF are intriguing, little is known about their biological significance. The present results, however, show that in the mouse the subclass specificity of RF depends on the stimuli responsible for its induction. After in vitro polyclonal activation with LPS and DS, spleen cells gave rise to a broad spectrum of subclass-specific RF: 16 were specific for

IgG1, 13 for IgG2a, and 4 for IgG2b. In contrast, of the 28 monoclonal RF derived from BALB/c and 129/Sv mice without prior polyclonal activation, 27 were specific for IgG2a and 1 for IgG1, which indicates that under certain conditions anti-IgG2a clones can be selectively activated in vivo. In 129/Sv mice, this spontaneous production of IgG2a-specific RF is apparently induced by an infectious agent primarily located in the intestinal tract. Contamination of RF-negative 129/Sv mice by a single intragastric injection of intestinal fluid from RF-positive animals is indeed sufficient to trigger a chronic production of this autoantibody (manuscript in preparation). However, because the causative infectious agent has not yet been isolated, it is difficult to analyze the mechanisms that result in such selective activation of IgG2a-specific RF. It would therefore be interesting to examine whether it is possible to induce the production of RF in the mouse in the course of immunizations with well-defined antigens that do not behave like polyclonal activators.

Immunization of rabbits with protein antigens has been shown to induce significant RF levels (25, 26). If similar responses could be obtained in the mouse, a comparison between the subclass specificity of these RF and the isotypic pattern of the specific antibodies induced in such experiments might help to solve the still controversial issue of the role of autologous IgG in the induction of RF.

The hybridomas obtained here after polyclonal activation in vitro were screened not only for RF activity but for binding to mouse IgM, human IgG, hen lysozyme, and ovalbumin as well. Although some 40 IgM-producing clones were found to react with mouse IgG subclasses, not a single clone displayed any specific activity towards the other proteins. This raised the possibility of artefactual interactions between mouse IgM and mouse IgG when the latter is adsorbed onto plastic surfaces. However, the narrow specificity of all these clones for individual IgG subclasses made this possibility rather unlikely. In addition, the ability of antigen-antibody complexes to successfully compete with plastic-bound IgG for the binding of the anti-IgG clones indicated that the antigenic determinants recognized in these reactions are not generated by the mere adsorption of IgG on polystyrene or polyvinyl surfaces.

It has been shown that the RF present in the serum of patients with rheumatoid arthritis has a much lower avidity for IgG than the RF directly secreted by the cells, as it can be detected with a plaque-forming cell assay (15). It has been assumed that this high affinity RF precipitates in the extravascular space and never reaches the serum. It was therefore of interest to test the affinity of the monoclonal RF derived from mice, such as the 129/Sv, which spontaneously produce RF in vivo. Of the 25 anti-IgG2a clones tested, none reacted significantly with monomeric IgG2a, as indicated by the failure of 7S IgG2a to inhibit the agglutination of IgG2a-coated particles. Also, the anti-IgG1 clones obtained after in vitro polyclonal activation showed no detectable reaction with monomeric IgG1 whereas complete inhibition of agglutination was observed with antigen-bound IgG1. In contrast, a strong inhibition of agglutination with monomeric IgG could be obtained for anti-IgG2b clones, indicating that higher affinity RF are present in the mouse although they seem to represent only a minor fraction of the mouse RF repertoire.

Summary

The specificity of polyclonal mouse rheumatoid factors (RF) was analyzed by competition experiments with heat-aggregated mouse IgG subclasses. The RF spon-

taneously produced by three normal mouse strains (129/Sv, CBA/Ht, and C57Bl/6) and by two strains with autoimmune diseases (MRL/l and NZB) were found to consist of distinct non-cross-reactive antibody subpopulations each specific for one IgG subclass. The sera of the normal strains contained IgG1- and IgG2a-specific RF. The autoimmune strains produced an additional variety of RF that was specific for IgG2b. Also, the RF secreted by spleen cells of various normal strains after in vitro polyclonal activation with lipopolysaccharide could be resolved into distinct subpopulations specific for IgG1 or IgG2a.

These results were confirmed by the analysis of monoclonal RF derived from BALB/c, C57Bl/6, CBA/Ht, and 129/Sv mice: of 73 hybridomas with RF activity, 71 displayed a strict subclass specificity. The subclass predominantly recognized depended on the origin of the spleen cells used to generate the hybridomas. After polyclonal activation in vitro, a broad spectrum of different specificities was obtained with 16 RF specific for IgG1, 13 for IgG2a, and 4 for IgG2b. In contrast, 27 of 28 monoclonal RF derived from 129/Sv and BALB/c mice without prior polyclonal activation were specific for IgG2a, and of these 75% were allotype specific since they failed to react with IgG2a of the b allotype. These results demonstrate the importance of subclass specificity in the production of RF in vivo.

With the exception of the IgG2b-specific clones, all these monoclonal RF reacted preferentially with heat-aggregated or antigen-bound IgG. Among the hybridomas generated by the fusion of in vitro polyclonally activated spleen cells of 4-wk-old mice, the frequency of clones with RF activity was at least 40 times higher than that of clones specific for mouse IgM, human IgG, ovalbumin, and hen lysozyme.

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References

1. Andrews, B. S., R. A. Eisenberg, A. N. Theofilopoulos, S. Izui, C. B. Wilson, P. J. McConahey, E. D. Murphy, J. B. Roths, and F. J. Dixon. 1978. Spontaneous murine lupus-like syndromes. *J. Exp. Med.* **148:**1198.
2. Van Snick, J. L., and P. L. Masson. 1979. Age-dependent production of IgA and IgM autoantibodies against IgG2a in a colony of 129/Sv mice. *J. Exp. Med.* **149:**1519.
3. Van Snick, J. L., and P. L. Masson. 1980. Incidence and specificities of IgA and IgM anti-IgG autoantibodies in various mouse strains and colonies. *J. Exp. Med.* **151:**45.
4. Izui, S., R. A. Eisenberg, and F. J. Dixon. 1979. IgM rheumatoid factors in mice injected with bacterial lipopolysaccharides. *J. Immunol.* **122:**2096.
5. Dresser, D. W., and A. M. Popham. 1976. Induction of an IgM anti-(bovine)-IgG response in mice by bacterial lipopolysaccharide. *Nature (Lond.).* **264:**552.
6. Dresser, D. W. 1978. Most IgM-producing cells in the mouse secrete auto-antibodies (rheumatoid factor). *Nature (Lond.).* **274:**480.
7. Dziarski, R. 1982. Preferential induction of autoantibody secretion in polyclonal activation by peptidoglycan and lipopolysaccharide. 1. In vitro studies. *J. Immunol.* **128:**1018.
8. Van Snick, J. L., and P. Coulie. 1982. Monoclonal anti-IgG autoantibodies derived from lipopolysaccharide-activated spleen cells of 129/Sv mice. *J. Exp. Med.* **155:**219.
9. Masson, P. L., C. L. Cambiaso, D. Collet-Cassart, C. G. M. Magnusson, C. B. Richards,

and C. J. M. Sindic. 1981. Particle-counting immunoassay (PACIA). *Methods Enzymol.* **74:**106.

10. Ey, P. L., S. J. Prowse, and C. R. Jenkins. 1978. Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. *Immunochemistry.* **15:**429.
11. Izui, S., R. A. Eisenberg, and F. Dixon. 1981. Subclass-restricted IgG polyclonal antibody production in mice injected with lipid A-rich lipopolysaccharides. *J. Exp. Med.* **153:**324.
12. Wetzel, G. D., and J. R. Kettman. 1981. Activation of murine B lymphocytes. III. Stimulation of B lymphocyte clonal growth with LPS and dextran sulfate. *J. Immunol.* **126:**723.
13. Parsons, M., P.-A. Cazenave, and L. A. Herzenberg. 1981. Igh-4d, a new allotype at the mouse IgG heavy chain locus. *Immunogenetics.* **14:**341.
14. Henry, C. 1980. Anti-hapten plaques. *In* Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shigi, editors. W. H. Freeman and Co., San Francisco. 95–102.
15. Robbins, D. L., T. L. Moore, D. A. Carson, and J. H. Vaughan. 1978. Relative reactivities of rheumatoid factors in serum and cells: evidence for a selective deficiency in serum rheumatoid factor. *Arthritis Rheum.* **21:**820.
16. Schreier, P. H., A. L. M. Bothwell, B. Mueller-Hill, and D. Baltimore. 1981. Multiple differences between the nucleic acid sequences of the $IgG2a^a$ and $IgG2a^b$ alleles of the mouse. *Proc. Natl. Acad. Sci. USA.* **78:**4495.
17. Dognin, M. J., M. Lauwereys, and A. D. Strosberg. 1981. Multiple amino acid substitutions between murine γ2a heavy chain Fc regions of Ig1a and Ig1b allotypic forms. *Proc. Natl. Acad. Sci. USA.* **78:**4031.
18. Ollo, R., and F. Rougeon. 1982. Mouse immunoglobulin allotypes: post-duplication divergence of γ2a and γ2b chain genes. *Nature (Lond.).* **296:**761.
19. Allen, J. C., and H. G. Kunkel. 1966. Hidden rheumatoid factors with specificity for native γ-globulins. *Arthritis Rheum.* **9:**758.
20. Natvig, J. B., and M. W. Turner. 1971. Localization of Gm markers to different molecular regions of the Fc fragment. *Clin. Exp. Immunol.* **8:**685.
21. Grubb, R., and A. B. Laurell. 1956. Hereditary serological human serum groups. *Acta Pathol. Microbiol. Scand.* **39:**390.
22. Harboe, M., and J. Lundevall. 1959. A new type in the Gm system. *Acta Pathol. Microbiol. Scand.* **45:**357.
23. Harboe, M. 1959. A new hemagglutinating substance in the Gm system, anti-Gm^b. *Acta Pathol. Microbiol. Scand.* **47:**191.
24. Natvig, J. B. 1966. Gm(g)-A "new" gamma-globulin factor. *Nature (Lond.).* **211:**318.
25. Aho, K., and O. Wager. 1961. Production of "anti-antibodies" in rabbits. *Ann. Med. Exp. Biol. Fenn.* **39:**80.
26. Williams, R. C., Jr., and H. G. Kunkel. 1963. Antibodies to rabbit γ-globulin after immunizing with various preparations of autologous γ-globulin. *Proc. Soc. Exp. Biol. Med.* **112:**554.