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Monoclonal Antibodies Specific for Rat IgG1, IgG2a, and IgG2b Subclasses, and Kappa Chain Monotypic and Allotypic Determinants: Reagents for Use with Rat Monoclonal Antibodies

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ABSTRACT

Mouse monoclonal antibodies to rat IgG were obtained by fusion of immune SJL mouse spleen cells to NSI myeloma cells. Seven monoclonal antibodies have been labeled with ^{125}I and studied as to specificity and avidity by using a panel of rat monoclonal antibodies both as inhibitors and target antigens in soft well plate and indirect cell binding assays. All MAb were selected for high avidity of 4×10^7 to $\geq 2 \times 10^9$ M^{-1}. Four MAb were subclass-specific. RG11/39, RG7/1, and RG7/11 were absolutely specific for the Fc′ region of IgG1, IgG2a, and IgG2b, respectively. RG9/6 showed specificity for the Fab′ region of IgG2a but crossreacted with lower avidity with IgG2c. Three MAb reacted with rat kappa chains. RG7/9 defined a monotypic (common) kappa chain determinant. RG11/15 and RG7/7 were specific for allelic kappa 1a and kappa 1b determinants, respectively. The monotypic and kappa 1a allotypic determinants are topographically separated. The antibodies can be used as screening reagents in indirect cell binding assays. They have sensitivity similar to affinity-purified rabbit anti-rat IgG and more defined specificity. They do not crossreact with mouse or human IgG, making them particularly suitable companion reagents for rat anti-mouse or anti-human MAb. One Mab, RG7/7, strongly crossreacts with Syrian hamster IgG.

INTRODUCTION

RAT monoclonal antibodies (MAb) have come into wide spread use for defining mouse cell surface antigens,[1-6] rat alloantigens,[7,8] and for obtaining large quantities of antibodies from rat × rat hybridomas grown in vivo. This has led in turn to further interest in the properties of rat immunoglobulins, and to a need for monoclonal anti-rat IgG reagents for use in conjunction with the rat MAb.

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Abbreviations: BSA: bovine serum album; DME: Dulbecco's modified Eagle's medium; FCS: fetal calf serum; MAb: monoclonal antibody(ies); PBS: 0.15 *M* NaCl, 0.01 *M* sodium phosphate, pH 7.2; RARG: rabbit anti-rat IgG, absorbed with mouse IgG.

Rat immunoglobulin subclasses IgM, IgA, IgE, IgG1, IgG2a, IgG2b, and IgG2c have been defined by their physicochemical, biological, and electrophoretic properties, and by reaction with adsorbed antisera to rat myeloma proteins.[10] Allotype markers have been found only for IgA[11] and IgG2b.[12] The IgG1 and IgG2c but not IgG2a or IgG2b subclasses bind to *S. aureus* protein A[2,3,13] The IgG subclasses differ considerably in ability to fix complement, with order of activity IgG2b > IgG2c > IgG2a.[2,3]

Rats have both kappa and lamda light chains. Greater than 90% of normal immunoglobulins and myelomas contain kappa light chains.[14,15] Two different kappa allotypes have been defined, RI-1a and RI-1b,[11,14,16-19] nomenclature of ref. 17. The kappa 1a and 1b allotypes are complex allotypes which differ in a large proportion (11 out of 107) of the amino acids in the constant region.[20]

Monoclonal antibodies to rat IgG subclasses and kappa chains would have many applications. They would be valuable for typing, quantitating, and isolating antibodies of specific subclasses, light chain type, or kappa allotype. Antibodies to kappa chains would be particularly valuable as general anti-immunoglobulin reagents for diverse immunological applications. Anti-kappa chain MAb would react with > 90% of rat MAb and hence would be valuable screening reagents. The need for such reagents is especially marked in the rat, since the IgG2a and IgG2b subclasses do not react with *S. aureus* protein A,[2,3,13] but account for 70–90% of rat IgG MAb.[2,3] Mouse antibodies to rat IgG would be particularly valuable for use in conjunction with rat anti-mouse MAb because of their lack of crossreaction with mouse cell surface or secreted IgG. Therefore, we have selected and characterized a panel of mouse MAb specific for rat IgG subclasses, kappa chains, and kappa chain allotypes. The antibodies define subclasses and allotypes in the same manner as conventional reagents, have exquisite specificity and high sensitivity in radioimmunoassays, and are highly active second reagents for antibody bound to antigen.

MATERIALS AND METHODS

Animals, Sera, and Ig: Mice were obtained from Jackson Labs, Bar Harbor, ME. Wistar/Furth (W/F) rats were from Harlan–Sprague–Dawley, Indianapolis, IN. DA and HO rats were from the colony of the Agricultural Research Council, Babraham, UK. Sera from Buffalo, Lewis (LEW), BN, F-344, and ACI rats, and from LGE, M520, KGH, and AUG rats, were the kind gifts of Dr. T. Strom and Dr. M. Dorf, respectively, Harvard Medical School. (Lewis × ACI) F_2 sera were kindly provided by Dr. Don Cramer, U. Pittsburgh, PA. Pooled rat, Syrian hamster, and human γG were from Miles Labs, Elkhart, IN. Later analysis of the rat γG revealed it contained a mixture of kappa 1a and 1b allotypes. IgG was purified by Sephadex G-200 filtration. $F(ab')_2$ fragments were prepared by digestion of rat IgG (20 mg/ml) with pepsin (Sigma) at 25:1 w/w ratio in 0.2 *M* sodium acetate buffer, pH 4.0, for 24 h at 37°C, followed by neutralization and G-200 Sephadex filtration.

Rat Mab: Rat MAb to murine cell surface antigens were obtained after fusion of NSI mouse myeloma cells to spleen cells of rat strains DA (M1 hybrids, Refs. 2, 21), LEW × BN (M3 hybrids, Ref. 22, M5 and M7 hybrids, Ref. 6), and W/F (M12, M15, M16, and M17 hybrids, unpublished). Light chain loss variants of M1 and M3 lines have previously been described.[21,23] The Y3 Louvain rat kappa chain-secreting myeloma[9] was obtained from Dr. C. Milstein, Cambridge, UK. D4,37 which apparently secretes an IgM, λ antibody[7] was the kind gift of Dr. T. McKearn, University of Pennsylvania. Monoclonal antibodies were either used in the form of supernatants from spent cultures grown in 5–10% fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DME), or purified as previously described.[24]

Immunization and Fusion: SJL male mice were immunized intraperitoneally with 100 μg and 10 μg of pooled rat IgG in complete Freund's adjuvant on days 0 and 20, respectively. Following a protocol designed to maximize hybridoma production to soluble antigens,[21] mice were boosted with 400 μg of rat IgG in saline intraperitoneally on days 24, 25, and 26. On day 27, 4 × 10^7 spleen cells

from each of three mice were fused with NSI myeloma cells, aliquoted into three 96 well plates, and grown in selective medium as previously described.[2,6]

Initial Screening: After two weeks, culture supernatants were screened in an indirect binding assay. Positive cultures which showed either broad specificity (α-kappa chain) or narrow specificity (α-heavy chain) were cloned in soft agar. The MAb from eleven lines were iodinated and tested in direct binding assays. Those with low avidity for soluble antigen, broad heavy chain specificity, or specificity similar to other RG MAb were discarded, leaving seven MAb which are described in detail. All RG (rat gamma globulin) hybrids described here are stable, cloned lines. The lines grow in DME with 5–20% FCS or 10–20% horse serum. Large quantities of antibodies were obtained by growth in spinner flasks.

^{125}I-Labeling: Antibodies were iodinated while bound to either antigen or *S. aureus* protein A, each conjugated to Sepharose, in small columns, and eluted with glycine-HCl pH 2.3, 1% BSA as described elsewhere.[21] Rabbit anti-rat IgG, absorbed with mouse IgG, was iodinated on a 10 μl bed of 1 mg rat IgG/ml Sepharose CL-4B. For RG MAb, columns were saturated with 2 ml of culture supernatants. All RG MAb were labeled on 10 μl of protein A Sepharose 4B (Pharmacia). RG7/9 and RG7/1 were also labeled on 10 μl of 1mg/ml rat IgG Sepharose CL-4B or 2mg/ml M7/14 rat IgG2a MAb Sepharose, respectively. Each antibody was iodinated with 0.5 mCi ^{125}I. After elution and neutralization, antibodies were dialyzed versus 0.15 *M* NaCl, 0.01 M phosphate, pH 7.2 (PBS), and diluted to 4 to 10 ml (9,000–4,000 cpm/μl) with 10% BSA-PBS.

Indirect Binding Assay: Initial screening utilized softwell plates coated with 10 μg/ml rat IgG. In the first step, RG supernatants (50 μl) were added for 1 h and in the second step, ^{125}I-rabbit-anti-mouse IgG (γ-specific, Miles Labs, absorbed with rat IgG) or ^{125}I-rabbit-anti-mouse IgM (μ-specific, Miles Labs, absorbed with rat IgG) were used. Other procedures were as described under Direct Binding Assay.

Direct Binding Assay: Softwell vinyl 96-well microtiter plates (Costar No. 2595 or Linbro S-MVC 96) were coated with 40 μl of pure antigen at 10 μg/ml (or 1% BSA as control) in PBS for 1 h at 20° C or overnight at 4° C. Plates were washed twice with 1% BSA in PBS. ^{125}I-anti-rat IgG antibodies (1/10 dilution in 50 μl of 10% FCS-PBS) were added and incubated 0.5 h at 20° C. Plates were washed three times with 1% BSA in PBS, dried, the wells removed with a hot wire cutter (D. Lee, Sunnyvale, CA), and γ-counted. All assays were performed in duplicate.

Equilibrium Inhibition Assay: Serial dilutions of purified inhibitor antigens in 10% FCS-BSA (50 μl) were mixed with 5 μl of ^{125}I-anti-rat IgG antibody in hardwell microtiter plates and held at 4° C for 16–20 h. Aliquots (40 μl) were transferred to antigen-coated softwell plates and incubated 0.5 h at 20° C. Other steps were as described in the direct binding assay. K_a (association constants) were determined as the inverse of the rat antigen concentration giving 50% inhibition, using the approximation that [Ag free] = [Ag total]. Since the concentration of ^{125}I-labeled mouse MAb was 5–10 × 10^{-10} *M*, K_a of $>5 \times 10^9 M^{-1}$ represent minimum values and are indicated by "≤" signs.

Competition Assay: Inhibitor antigens, as hybridoma culture supernatants or diluted in 10% FCS-PBS (50 μl), were dispensed into antigen-coated soft well plates. During shaking on a Microshaker-II (Cooke, Alexandria, VA) 5 μl of ^{125}I-MAb was added, and shaking continued for 0.5 h. Other steps were carried out as described for the direct binding assay.

Calculation of Inhibition: Percent inhibition was calculated as

$$\frac{(1 - e/t)}{(1 - c/t)} \times 100$$

where e = cpm bound in the presence of inhibitor to antigen-coated wells, c = cpm bound in the presence of 10% FCS-PBS to BSA-coated wells, and t = cpm bound in the presence of 10% FCS-PBS to antigen-coated wells.

Other Methods: Subclass determination, SDS-PAGE of internally labeled MAb chains, the cellular indirect binding assay, and other procedures were carried out as previously described.[1]

RESULTS

Specificity of Mouse Anti-Rat IgG MAb in the Direct Binding Assay: Mouse monoclonal antibodies directed to rat IgG (RG series) were purified from hybridoma culture supernatants on rat IgG or protein A immunoadsorbent columns, labeled with ^{125}I in situ, and eluted. The labeled antibodies were then tested for binding to antigen-coated wells in a liquid–solid phase radioimmunoassay (Table 1). Four MAb had specificity for heavy chain subclasses, as shown by binding to rat MAb, and the specificity for the Fc′ or Fab′ region was tested with rat IgG and rat $F(ab')_2$. RG11/39 and RG7/1 were specific for the Fc′ region of rat IgG1 and IgG2a, respectively. RG9/6 showed specificity for the IgG2a Fab′ region, with also weak binding to IgG1 and IgG2c, but none to IgG2b or IgM. RG7/11 was specific for the IgG2b Fc′ region.

Three MAb showed anti-kappa chain specificity. RG7/9 reacted with all rat Ig subclasses, free kappa chains secreted by the Y3 myeloma, and rat $F(ab')_2$. RG11/15 reacted only with MAb derived from rat strains with the RI-1a kappa chain allotype (kappa 1a). RG7/7 reacted strongly with MAb from strains expressing the kappa 1b allotype, and with Y3 kappa 1b chains, but also showed weak to almost as strong reactivity with MAb of kappa 1a allotype.

A number of general points also emerged from the direct binding assay. Purification and iodination on rat IgG Sepharose was 2- to 4-fold superior to *Staph* protein A-Sepharose in terms of the % bindable radioactivity (see RG7/1 and RG7/9). This may have been due both to selective purification of active monoclonal antibodies containing specific rather than NSI myeloma light chains, and to protection of the antigen combining site during iodination. Binding by the RG7/1 and RG7/9 MAb iodinated on rat IgG-Sepharose was higher than that of polyclonal rabbit anti-rat IgG (RARG) which was purified and iodinated by the same method. The RG MAb were also superior in their complete lack of crossreaction with mouse IgG, while RARG, despite three absorptions with mouse IgG-Sepharose, was still crossreactive (further absorptions, however, could completely remove this crossreactivity).

Plan of Monoclonal Antibody Testing: The sections below describe the testing of individual antibodies by a variety of criteria. Since MAb recognize single antigenic determinants, they were first tested for whether they defined subclasses and allotypes in the same manner as conventional polyclonal antisera. This utilized panels of MAb previously typed with conventional subclass-specific reagents (Table 2), or derived from rat strains with known kappa chain allotype markers (Table 3). To be generally useful reagents, MAb should have high avidity. The avidity of MAb for soluble antigens was tested in equilibrium inhibition assays (Figs. 1 and 3). To be effective second antibodies in immunoprecipitation, binding assays, immunofluorescence, or to enhance complement-mediated lysis, it was essential that the binding of antigen by the first antibody should not affect the binding or specificity of the second antibody. This was tested in the indirect cell binding assay (Table 4).

Monoclonal antibodies to rat heavy chains

RG11/39, Anti-IgG1 Fc′: RG11/39 was inhibited by the M3/84 IgG1 MAb but not by MAb of other subclasses or by kappa chains (Table 2, Fig. 1A). The avidity for IgG1 was $> 8 \times 10^8$ M^{-1} (Fig. 1A). Purified M3/84 IgG1 inhibited significantly better than pooled rat IgG (Fig. 1A) as expected since IgG1 is a minor rat subclass. $F(ab')_2$ inhibited only 2% as well as IgG. This suggested that RG11/39 recognized a determinant on the Fc′ portion, and that the $F(ab')_2$ preparation contained a small proportion of undigested IgG. RG11/39 bound specifically to the anti-Mac-3 M3/84 MAb in the indirect binding assay (Table 4).

RG7/1, Anti-IgG2a Fc′: RG7/1 was inhibited only by MAb of the IgG2a subclass (Table 2, Fig. 1B). It was specific for the Fc′ region (Fig. 1B). RG7/1 had a moderate avidity for soluble antigen of 4×10^7 M^{-1} (Fig. 1B). In the indirect cell binding assay, however, RG7/1 gave specific and high (7.7–43.2%) binding to cells sensitized with rat IgG2a MAb (Table 4). This suggested that RG7/1 might have a higher avidity for cell-bound rat IgG than soluble rat IgG antigen, since typical antigen con-

TABLE 1. SPECIFICITY OF MOUSE ANTI-RAT IGG MONOCLONAL ANTIBODIES IN THE DIRECT BINDING ASSAY.

		RG11/39[a]	*RG7/1*[b]	*RG7/1*[a]	*RG9/6*[a]	*RG7/11*[a]	*RG7/9*[b]	*RG7/9*[a]	*RG11/15*[a]	*RG7/7*[a]	*RARG*[b,c]
Antigen		*Radioactivity bound (%)*[d]									
I. Rat MAb											
M3/84	$\gamma 1$, κ1b	43.2	0.4	0.2	2.6	0.7	48.8	21.3	0.3	31.1	33.2
M1/84	$\gamma 2a$, κ1a	0.2	54.2	16.2	16.9	0.0	64.5	25.8	19.7	22.7	42.8
M1/42	$\gamma 2a$, κ1a	0.3	37.4	8.8	7.6	0.0	33.7	16.4	5.8	1.9	30.3
M1/9.3	$\gamma 2a$, κ1a	0.4	53.1	15.7	8.0	0.1	52.2	21.4	18.3	27.5	38.2
M3/38	$\gamma 2a$, κ1b	0.1	48.1	12.9	13.1	0.0	41.8	17.0	0.0	31.6	33.4
M7/14	$\gamma 2a$, κ1b	0.2	52.1	14.5	16.6	0.1	42.2	19.9	0.0	31.7	46.7
M1/69	$\gamma 2b$, κ1a	0.0	0.1	0.2	0.1	18.0	50.6	21.1	18.9	21.9	20.3
M1/70	$\gamma 2b$, κ1a	0.0	−0.2	−0.1	−0.2	8.1	41.2	18.6	14.8	8.0	18.5
M5/114	$\gamma 2b$, κ1b	0.0	0.0	0.1	0.0	18.2	47.2	19.2	0.0	31.5	27.7
M12/7	$\gamma 2b$, κ1b	0.1	0.1	0.2	0.0	16.3	45.7	20.6	0.1	31.4	28.7
M5/54	$\gamma 2c$, κ1b	0.0	0.1	0.2	1.7	−0.1	39.8	16.5	0.3	30.0	14.3
M1/87	μ, κ1a	0.0	−0.3	0.0	−0.1	−0.2	37.2	18.6	4.2	1.9	4.2
Y3	— κ1b	0.2	−0.2	0.1	0.0	−0.1	9.0	5.5	0.0	23.6	5.4
II. Other											
Rat IgG		13.7	52.9	15.4	11.3	13.8	39.6	15.7	9.4	26.3	43.9
Rat $F(ab')_2$		0.7	1.4	0.5	10.0	0.2	31.3	12.2	8.4	24.7	19.0
Mouse IgG		0.2	−0.1	0.2	0.0	0.1	0.1	0.0	0.2	0.1	10.7

[a] Purified and ^{125}I-labeled on Protein A-Sepharose.

[b] Purified and ^{125}I-labeled on rat IgG-Sepharose (RG7/9, RARG) or M7/14 IgG-Sepharose (RG7/1).

[c] Rabbit anti-rat IgG.

[d] The percentage of input (17,000–47,000 cpm) ^{125}I-labeled antibody bound to antigen-coated vinyl microtiter wells was determined in the direct binding assay. Nonspecific binding to BSA-coated wells (0.3–0.8%) has been subtracted.

TABLE 2. COMPETITIVE INHIBITION OF ANTI-HEAVY CHAIN RG MONOCLONAL ANTIBODIES.[a]

Inhibiting rat monoclonal antibodies[b]	*Subclass*	*RG11/39*	*RG7/1*	*RG9/6*	*RG7/11*
		Inhibition[c] *(%)*			
M3/84	$\gamma 1$	98	6	35 (−6–78)[d]	−6
M1/9.3, 42, 84; M3/38; M5/49, 69, 116; M7/7, 84	$\gamma 2a$	3 (−1–6)	68 (30–92)	97 (95–99)	−1 (−13–9)
M1/69, 70, 89.18; M5/24, 78, 113, 114, 119; M7/21, 81	$\gamma 2b$	4 (0–10)	1 (−11–10)	−9 (−19–9)	98 (93–100)
M1/22.54; 75; M5/54, 56	$\gamma 2c$	3 (−1–6)	2 (−7–9)	42 (16–69)	−6 (−2–11)
M1/22.25; M3/31; M5/26, 106, 118; M7/20, 86	μ	4 (−8–8)	4 (−21–19)	−9 (−39–0)	0 (−18–7)
Y3 (κ)	—	2	8	−10	−3

[a] Binding of ^{125}I-RG MAb to M3/84-coated (RG11/39), M1/84-coated (RG7/1, RG9/6), or M1/69-coated (RG7/11) wells was inhibited with hybridoma spent culture supernatants in the competition assay (Materials and Methods).

[b] Inhibition was tested with individual MAb; MAb are listed in abbreviated form with the fusion designation preceding only the first MAb from each fusion.

[c] mean and (range)

[d] supernatants from different dates.

TABLE 3. COMPETITIVE INHIBITION OF ANTI-KAPPA CHAIN RG MONOCLONAL ANTIBODIES.[a]

	RG7/9	*RG7/7*	*RG11/15*
A. Hybridomas from RI-lb strains[b]		*Inhibition*[c] *(%)*	
M3/31, 38, 84; M5/24, 26, 49, 54, 56, 69, 78, 106, 111, 112, 113, 114, 116, 118, 119, 120; M7/7, 8, 20, 21, 81, 83, 84, 85, 86; M12/3, 4, 5, 6, 7; M15/5; M16/1; M17/1, 4, 5, 6, 10	94 (81–99)	92 (60–100)	1 (−7–7)
M12/5; M15/32	8 (2–15)	−7 (−10–4)	ND[d]
Y3 ($\varkappa$)	93	97	0
D4,37 (IgM λ)	0	0	0
M3/31 HLK	93	94	ND
M3/31 HK (loss of rat light chain)	8	−7	ND
M3/31 L (loss of rat heavy chain and mouse light chain)	96	77	ND
B. Hybridomas from RI-1a strains[b]			
M1/9.3, 9.47, 22.54, 42, 69, 70, 75, 84, 89.1	96 (92–98)	8 (−2–16)	95 (93–98)
M1/89.18 HK?	7	0	−2
M1/69 HL (secretes rat heavy and rat light chain)	92	9	95
M1/69 HK (secretes rat heavy and mouse light chain)	−7	0	0

[a] Binding of ^{125}I-RG MAb to M1/9.3-coated (RG7/9), RG11/15) or M3/84-coated (RG7/7) wells was inhibited with spent culture supernatants from hybridoma or myeloma lines in the competition assay (Materials and Methods).

[b] Hybridomas were derived from rat strains carrying the Lewis-type RI-1b or the DA-type RI-1a kappa chain marker. Each MAb was tested individually, and is listed with the fusion designation preceding only the first MAb from each fusion.

[c] Mean and (range).

[d] Not done.

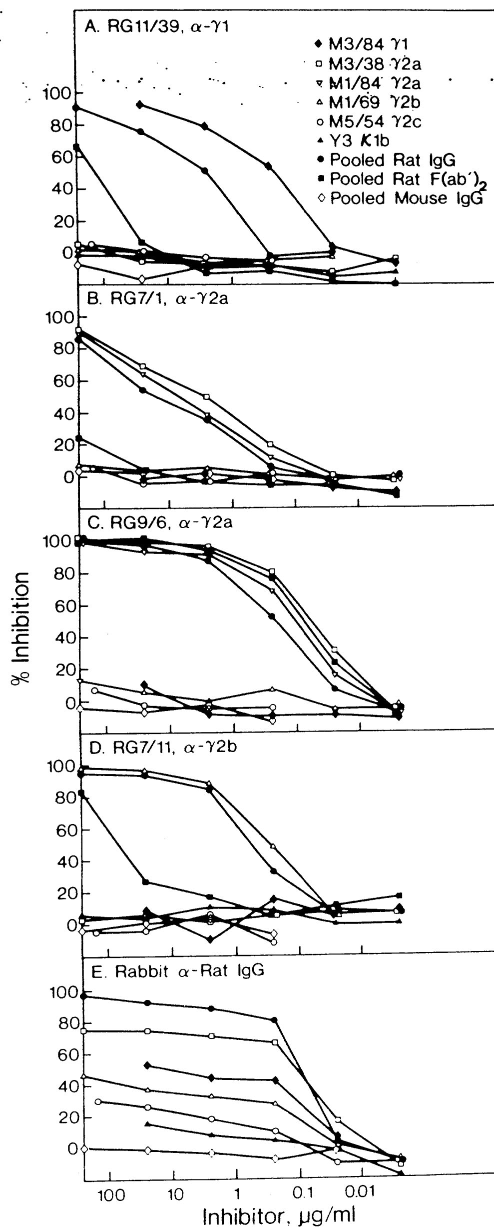

FIG. 1. Determination of specificity and avidity of anti-IgG subclass MAb and whole anti-rat IgG by the equilibrium inhibition assay. ^{125}I-anti-rat IgG antibodies were reacted with serial dilutions of IgGs for 16–20 h at 4° C, and residual binding capacity was measured after transfer to antigen-coated soft well plates as described in Materials and Methods.

centrations in the indirect cell binding assay were 1–5 × 10^{-9} M, considerably below the K_d for soluble antigen of 2.5 × 10^{-8} M. Therefore, RG7/1 was compared with other MAb with high avidity for soluble antigen, RG7/9 and RG9/6, in terms of its relative avidity for soluble and immobilized antigens in competition assays. Soluble IgG2a was 70- and 40-fold relatively less effective in competing the binding of RG7/1 to IgG2a-coated vinyl microtiter wells (Fig. 2A) or to IgG2a anti-Thy-1 MAb-

TABLE 4. ANTI-RAT IgG MONOCLONAL ANTIBODIES AS SECOND ANTIBODIES IN THE INDIRECT CELL BINDING ASSAY.[a]

Antigen specificity	1st MAb		^{125}I-labeled 2nd Antibody radioactivity bound (%)							
			RG11/39[b]	*RG7/1*[c]	*RG9/6*[b]	*RG7/11*[b]	*RG7/9*[c]	*RG11/15*[b]	*RG7/7*[b]	*RARG*[c]
A. Ly 5	M1/9.3	γ2a,κ1a	0.0	33.9	14.8	0.0	41.0	5.9	0.5	32.7
HSA	M1/69HL	γ2b,κ1a	0.0	0.1	1.3	6.8	15.0	5.7	2.9	6.2
HSA	M1/22.54	γ2c,κ1a	0.0	0.0	0.0	0.0	5.3	1.3	0.3	0.3
H-2	M1/42	γ2a,κ1a	0.0	24.5	9.4	0.0	17.7	1.4	0.0	9.9
H-2	M7/21	γ2b,κ1b	0.0	0.0	0.1	15.4	26.0	0.3	16.2	22.3
Thy-1	M5/49	γ2a,κ1b	0.0	39.1	11.9	0.0	43.0	0.3	30.0	36.5
Thy-1	M5/54	γ2c,κ1b	0.0	0.0	3.8	0.0	43.0	0.3	25.4	12.3
Thy-1	M16/1	μ, κ1b	0.0	0.0	0.0	0.0	28.3	0.0	19.2	7.2
Lyt-2,3	M12/5	γ2a,κ1b	0.0	24.1	4.4	0.0	0.0	0.1	0.0	21.2
Lyt-2,3	M12/7	γ2b,κ1b	0.0	0.0	0.2	7.7	12.8	0.2	6.6	19.2
LFA-1	M17/4	γ2a,κ1b	0.0	7.7	4.1	0.0	6.3	0.2	3.4	22.1
LFA-1	M17/5	γ2b,κ1b	0.0	0.0	0.0	3.5	5.5	0.0	3.2	9.7
Ly 6	M5/106	μ, κ1b	0.0	0.4	0.3	0.0	16.5	0.1	23.2	7.8
B. Mac-1	M1/70	γ2b,κ1a	0.0	0.4	0.4	19.0	43.3	17.8	4.8	19.5
Mac-2	M3/38	γ2a,κ1b	ND[d]	43.2	15.0	0.7	43.5	ND	33.0	35.8
Mac-3	M3/84	γ1, κ1b	8.0	0.2	0.2	0.0	11.4	0.6	6.2	12.1

[a]The percentage of input radioactivity bound to rat MAb-sensitized Concanavalin A stimulated mouse spleen cells (A) or thioglycollate-elicited mouse peritoneal exudate macrophages (B) was determined in the indirect cell binding assay. Background binding (2.3–5.0%) to cells sensitized with M1/69HK inactive MAb has been subtracted. Specific binding of greater than 2% was considered significant.

[b]Purified and labeled on Protein A-Sepharose.

[c]Purified and labeled on rat IgG-Sepharose (RG7/9, RARG), or M7/14-IgG Sepharose (RG7/1).

[d]Not done.

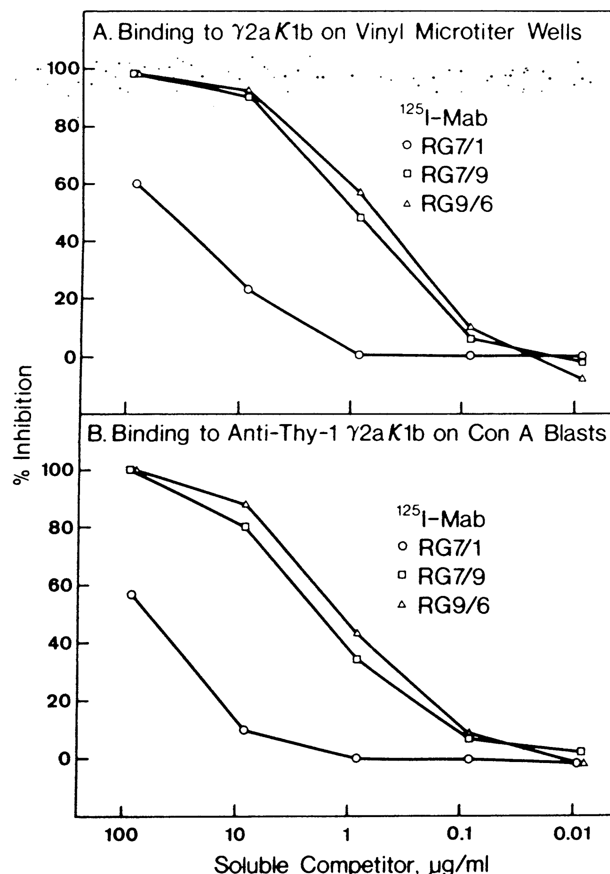

FIG. 2. Competition for binding to soluble and surface-bound antigen. Softwell plates were coated with (A) M1/84 IgG2a or (B) thymocytes were sensitized with the anti-Thy-1 M5/49 IgG2a MAb, and washed. Serial dilutions of M5/49 IgG2a MAb-containing supernatant (20 μl) were added to microtiter plates (A) or used to suspend the thymocytes (B). ^{125}I-RG MAb (5 μl) were then added, and the percent inhibition by the M5/49 IgG2a MAb of binding to soft wells or cells was determined as described in Materials and Methods.

sensitized thymocytes (Fig. 2B), respectively. The higher avidity for IgG2a bound to surfaces might be due to bivalent as opposed to monovalent binding, or to recognition of conformational differences.

RG9/6, Anti-IgG2a Fab': Competitive inhibition of RG9/6 by the panel of Rat MAb showed strong inhibition by the IgG2a subclass, weaker inhibition by the IgG2c subclass, and no inhibition by IgG2b or IgM (Table 2). Inhibition by IgG1 was weak and variable. RG9/6 was inhibited equally well by IgG and F(ab')$_2$, showing it recognizes a determinant on the Fab' portion (Fig. 1C). The equilibrium inhibition assay (Fig. 1C) showed the avidity for IgG2a was high, $> 2 \times 10^9$, but cross-reaction with IgG2c or IgG1 was not detected, suggesting an avidity of $< 10^6$. RG9/6 recognized all antibodies with the IgG2a subclass in the indirect binding assay (Table 4). It gave lower binding to an IgG2c than an IgG2a anti-Thy-1 MAb, and did not give significant binding to the M1/22.54 IgG2c MAb which bound in lower quantities to the cell surface (compare the RG7/9 anti-kappa MAb).

RG7/11, Anti-IgG2b Fc': R7/11 was highly avid for IgG2b, was specific for the Fc' portion, and did not bind to other subclasses (Fig. 1D, Table 2). Specificity for IgG2b was confirmed in the indirect binding assay (Table 4).

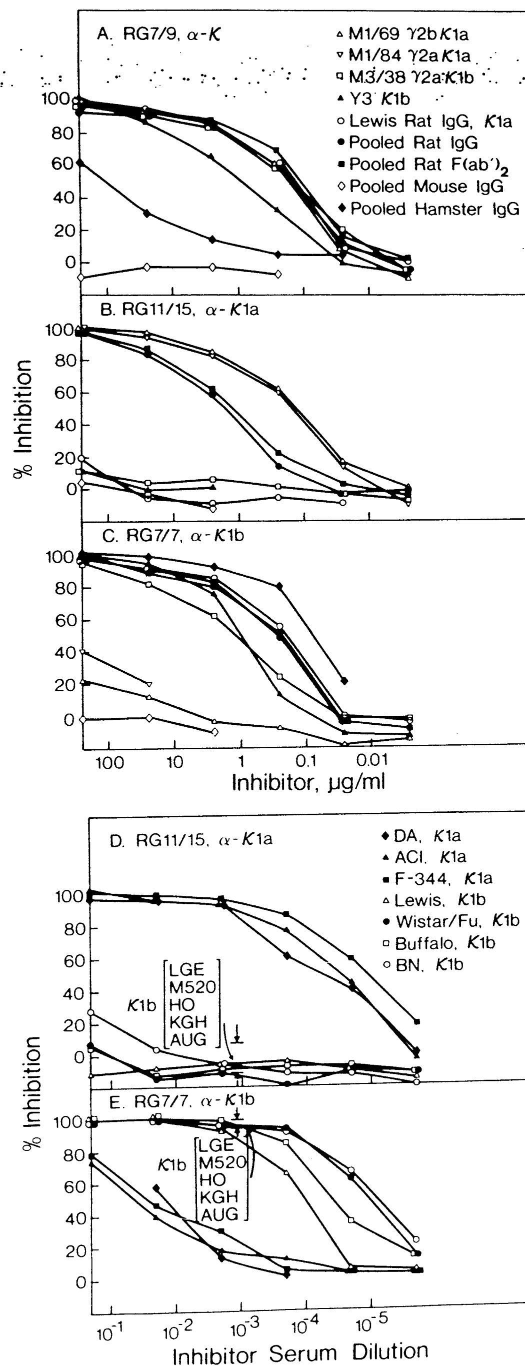

FIG. 3. Specificity and avidity of anti-kappa chain monoclonal antibodies. The equilibrium inhibition assay was carried out with serial dilutions of (A–C) purified MAb or IgG or (D,E) serum. Residual binding capacity was measured with antigen-coated soft well plates as described in Materials and Methods. In (D) and (E), inhibition by LGE, M520, HO, KGH, and AUG sera from kappa 1b rats was measured at a single dilution of 1/500.

Monoclonal antibodies to rat kappa chains

RG7/9 Anti-Kappa: This antibody was kappa-specific since it reacted with free kappa chains secreted by Y3, and with variants of the M3/31 and M1/69 lines having rat light chains, but not with those lacking rat light chains (Table 3). It did not crossreact with λ chains, since it was not inhibited by IgM,λ secreted by the D4,37 hybridoma. Furthermore, it did not react with M12/5 and M15/32 which presumably contain λ chains since they do not express the kappa 1b allotype defined by RG7/7 (see below). An extensive collection of rat monoclonal antibodies was surveyed which had been selected without bias toward light chain type, by the indirect binding assay with anti-rat IgG or by inhibition of functional activity. Of 52 MAb, 49 or 94% contained kappa chains (Table 3), in agreement with previous estimates of kappa/λ ratios in the rat. RG7/9 had a high avidity for monoclonal antibodies of both kappa 1a and kappa 1b allotype, rat IgG, and $F(ab')_2$, and a somewhat lower avidity for free kappa chains (Fig. 3A). In the indirect cell binding assay, it was a better second reagent for most MAb than affinity-purified rabbit anti-rat IgG (RARG, Table 4). RG7/9 was negative on the putative λ-containing M12/5 anti-Lyt-2,3 MAb in the cell binding assay, while the RG7/1 anti-IgG2a MAb gave strong binding to M12/5.

RG11/15 Anti-kappa 1a and RG7/7 Anti-kappa 1b: RG11/15 and RG7/7 were competitively inhibited only by MAb derived from rats with the RI-1a and RI-1b kappa chain markers, respectively (Table 3). Kappa-chain specificity of RG7/7 was shown by reaction with Y3 free kappa chains but not the D4,37 IgM,λ. RG11/15 reacted with a variant of M1/69 containing the specific rat light chain, but not with a variant containing only the myeloma light chain. RG11/15 had high avidity for kappa 1a-containing IgG's and showed no crossreaction with kappa 1b (Fig. 3B). RG7/7 showed high avidity for kappa 1b and a weak cross-reaction with kappa 1a (Fig. 3C). These results were confirmed using sera from different inbred rat strains which had previously been typed for kappa chain allotype with conventional alloantisera. RG11/15 was inhibited by sera from three kappa 1a strains but not 9 kappa 1b strains (Fig. 3D). RG7/7 was inhibited avidly by sera from kappa 1b strains, while kappa 1a sera were also inhibitory but with 1,000-fold lower avidity (Fig. 3E). The strain specificity and kappa chain specificity of RG11/15 and RG7/7 strongly suggested they recognized products of different kappa alleles. This was tested genetically with (Lewis × ACI)F_2 rats. Sera were tested at 1/500 dilution (this made RG7/7 operationally kappa 1b specific). There were 10 a/a homozygotes, 7 b/b homozygotes, 14 a/b heterozygotes, and no animals lacking both alleles. This was not significantly different by chi^2 test ($p > 0.6$) from Mendelian segregation. These results showed that the genes for the RG11/15 and RG7/7 determinants are present on homologous

TABLE 5. TOPOGRAPHIC RELATIONSHIP BETWEEN KAPPA CHAIN ANTIGENIC DETERMINANTS.[a]

	κ1b IgG		*κ1a IgG*	
^{125}I-MAb	*RG7/9 α-κ*	*RG7/7 α-κ1b*	*RG7/9 α-κ*	*RG7/7 α-κ1b*
Competing MAb	*Inhibitory Titer*[b]			
RG7/9 α-κ	60	<1	50	<1
RG7/7 α-κ1b	<1	300	<1	370
RG11/15 α-κ1a	<1	<1	<1	240

[a]To wells coated with M3/38 (κ1b IgG) or M1/84 (κ1a IgG) were added 50 μl of 10-fold serial dilutions of competing MAb in hybridoma culture supernatants. After 0.5 h, 5 μl of ^{125}I-MAb was added. Remaining procedures and calculation of inhibition were as in Materials and Methods.

[b]Inverse of dilution giving 50% inhibition.

chromosomes, and confirmed that the MAb recognize kappa chain allotype determinants. The absolute specificity of RG11/15 for kappa 1a and the higher avidity of RG7/7 for kappa 1b than kappa 1a were also reflected in the indirect binding assay (Table 4). RG11/15 bound only to MAb of kappa 1a allotype, while RG7/7 gave high binding to MAb of kappa 1b allotype but also showed low but significant binding to the M1/69 kappa 1a MAb.

To determine the relative topographic relationship of the common and allotypic determinants on kappa chains, cross-inhibition experiments were carried out (Table 5). The RG7/9 anti-kappa and RG7/7 anti-kappa 1b MAb were mutually noncompetitive and hence bound to spatially distinct sites on kappa 1b IgG. The sites recognized by these two MAb were also distinct on kappa 1a IgG. However, the two allotype-specific MAb bound to topographically related determinants, since RG11/15 inhibited the crossreaction of RG7/7 with kappa 1a IgG.

Crossreactions with Other Species: As expected since they were prepared in the mouse, none of the RG MAb crossreacted with mouse IgG (Table 1, Figs. 1 and 3). In tests on human, rabbit, guinea pig, gerbil, and Syrian hamster IgG, the only crossreactions found were with RG7/7 and RG7/9 on hamster IgG. The crossreaction with the RG7/9 anti-kappa MAb was low avidity (Fig. 3A), while that with the RG7/7 anti-kappa 1b MAb was extremely avid and appeared heteroclitic (Fig. 3C).

DISCUSSION

We have characterized a set of monoclonal antibodies defining rat heavy chain subclasses and kappa chain monotypic and allotypic determinants. Their properties are summarized in Table 6. Since the sensitivity and usefulness of antibodies in immunoassays is related to their avidity, we used inhibition assays to select for high avidity antibodies. A number of antibodies which gave high binding in soft well plate assays were found to have low avidities for soluble antigen of $< 10^{-6}\ M^{-1}$ and were discarded at an early stage in these studies. The antibodies characterized in detail had avidities of 4×10^7 to $\geq 2 \times 10^9\ M^{-1}$ (Table 6). Avidity was determined with soluble IgG as the antigen, and could reflect either monovalent or bivalent antibody-antigen interaction. The affinity for monovalent antigen of the anti-kappa chain MAb was measured with Fab′ fragments, and ranged from 4×10^7 to $\geq 10^9\ M^{-1}$ (Table 6).

Four antibodies had specificity for γ-chain determinants. Three MAb to the Fc′ portion of IgG1, IgG2a, and IgG2b showed absolute subclass-specificity, while the RG9/6 MAb to IgG2a Fab′ showed weak crossreactivity with IgG2c and possibly IgG1. The reactivity of these reagents on a large rat MAb panel was in complete concordance with conventional subclass-specific antisera, validating their use as subclass-typing reagents and confirming previous assignments of rat IgG subclasses.[10] The specificity of the MAb was superior to that of commercially available subclass-specific antisera, which sometimes give weak crossreactions with inappropriate subclasses, particularly of anti-IgG1 with IgG2a.

Aside from subclass-determination, there are a large number of applications for subclass-specific MAb. (1) We showed the subclass-specific MAb are highly active in the indirect cell binding assay. They thus may be used as screening reagents when it is desired to select for antibodies of a particular subclass. (2) The antibodies could be used to measure subclass concentrations in serum, or as immunoadsorbent columns for purification of antibodies of specific subclasses. (3) The MAb were found useful for measuring small quantities of Fc′ determinants present in $F(ab')_2$ preparations. These presumably represent small quantities of undigested IgG which are difficult to separate from $F(ab')_2$ by gel filtration. These antibodies should be useful for monitoring IgG and Fc or Fc′ removal from Fab and $F(ab')_2$ preparations, as well as for their direct removal with MAb-immunoadsorbent columns. (4) MAb specific for different subclasses could be conjugated with fluorescein and rhodamine and used in two color immunofluorescence, e.g., FITC-anti-IgG2a and TRITC-anti-IgG2b MAb could be used with IgG2a anti-Lyt-2 and IgG2b anti-Thy-1 MAb to study by immunofluorescence flow cytometry the differential expression of these antigens on lymphocytes.

Three monoclonal antibodies to rat kappa chain determinants were obtained (Table 6). RG7/9 recognized a common kappa chain epitope. RG11/15 appears absolutely specific for an allotypic determinant on kappa 1a chains. RG7/7 has 10^3 higher avidity for kappa 1b than for kappa 1a chains.

TABLE 6. PROPERTIES OF MOUSE ANTI-RAT IgG MONOCLONAL ANTIBODIES.

Monoclonal Antibody[a]		*Primary specificity*	*Avidity*[b] (M^{-1})	*Affinity*[c] (M^{-1})	*Cross-reaction*	*Avidity*[b] (M^{-1})	*Species cross-reaction*[d]	*Avidity*[b] (M^{-1})
RG11/39,	γ2b	γ1 Fc′	$\geq 8 \times 10^8$	ND[e]	None	–	–	–
RG7/1,	γ2b	γ2a Fc′	4×10^7	ND	None	–	–	–
RG9/6 HLK,	γ2b	γ2a Fab′	$\geq 2 \times 10^9$	ND	γ2c,γ1?	$< 10^6$	–	–
RG7/11,	γ2b	γ2b Fc′	$\geq 1.2 \times 10^9$	ND	None	–	–	–
RG7/9 HLK,	γ2b	κ	$\geq 10^9$	$\geq 10^9$	None	–	Hamster	2×10^6
RG11/15,	γ2a	κ1a (DA)	$\geq 1.1 \times 10^9$	4×10^8	None	–	–	–
RG7/7HL,	γ2a	κ1b (LEW)	$\geq 7 \times 10^8$	4×10^7	κ1a	3×10^5	Hamster	$\geq 1.8 \times 10^9$

[a] Presence of specific heavy (H) and light (L) and myeloma kappa (κ) chains determined by SDS-PAGE,[21] mouse subclass determined by double immunodiffusion with class and subclass-specific antibodies (Miles Labs).

[b] Determined by equilibrium inhibition with soluble antigen as described in Materials and Methods.

[c] Determined with Fab′ fragments.

[d] Tested on human, rabbit, guinea pig, gerbil, Syrian hamster, and mouse IgG.

[e] Not determined.

Specificity for kappa 1a and kappa 1b was confirmed by correlation with previously typed inbred rat strains and by codominant Mendelian segregation of the markers in F_2 rats. The common and allotypic determinants are topographically distinct, since MAb can be bound to both sites at the same time. While this manuscript was in preparation, we received preprints from two other laboratories which also report MAb to kappa chain common and 1b determinants.[26,27] However, no MAb with specificity for the kappa 1a allodeterminant has previously been reported. Kappa 1a and 1b are products of true alleles but differ by 11 amino acids in the constant region.[28] No intermediate forms of the alleles have been found in inbred strains or wild isolates of *Rattus norvegicus*.[29] We also found no intermediate expression among the inbred strains of the allodeterminants defined by our MAb. Comparative studies with alloantisera have shown that kappa 1b-like serological determinants are much more widely distributed than kappa 1a among *Murid* rodents.[29] It is interesting in this respect that the RG7/7 anti-kappa 1b MAb strongly crossreacts with Syrian hamster IgG, since anti-kappa 1b alloantisera have been found to be negative on several members of the family *Cricitidae*.[29] Since the RG7/7 xeno-MAb shows weak crossreaction with kappa 1a, this specificity may not be present in anti-kappa 1b alloantisera produced in kappa 1a-positive rats. The anti-kappa MAb, and especially RG7/7, should be useful in the analysis of rodent kappa chain genetics and evolution.

Since > 90% of immunoglobulins in the rat contain kappa chains, the anti-kappa chain MAb are valuable as general anti-immunoglobulin reagents. There is a particularly strong need for such reagents in the rat, because *S. aureus* protein A does not react with the IgG2a and IgG2b subclasses which account for 70–90% of rat IgG MAb. A few out of the many potential applications are outlined. (1) The anti-kappa MAb can be coupled with radioisotopes, fluorochromes, or enzymes and used to screen for rat MAb. We showed that the anti-kappa MAb are comparable or better than conventional affinity-purified rabbit anti-rat IgG in indirect binding assays. The anti-kappa MAb also have the advantage that binding is related to the quantity but not to the subclass of the first antibody. We now routinely use RG7/9 for screening rat anti-mouse fusions with the indirect binding assay. This antibody should also be useful for determining the molecular weight of antigens by immunodetection of sodium dodecylsulfate polyacrylamide gel blots.[26,27] The MAb have proved to be excellent linking reagents in the peroxidase anti-peroxidase method for localizing antigens in tissue sections. The lack of crossreactivity with mouse IgG is advantageous for all of these techniques. The RG7/7 MAb should be useful for screening hybrids secreting hamster antibodies. (2) Purified RG7/9 coupled to Sepharose is being used in our laboratory with excellent success for indirect precipitation of radiolabeled antigens. (3) The anti-kappa chain reagents may be used to determine the concentration of rat IgG. It has not been widely appreciated that since anti-rat IgG contains subclass specific antibodies (Fig. 1E), it cannot be used to accurately measure MAb concentrations. Measurement with anti-rat Fab is more accurate, but errors can be introduced if the unknown and standard antibodies differ in kappa allotype. The RG7/9 MAb, which shows no allotype preference, appears ideal for quantitation of kappa-containing MAb. (4) The MAb should be useful in enhancing complement-mediated lysis, since they are of the highly lytic IgG2a and IgG2b subclasses. A mixture of antibodies directed to several different epitopes should be optimal. In addition to Syrian hamster IgG, RG7/7 crossreacts with Armenian hamster but not Chinese hamster IgG.

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