MULTIPLE AUTOANTIGEN BINDING CAPABILITIES OF MOUSE MONOCLONAL ANTIBODIES SELECTED FOR RHEUMATOID FACTOR ACTIVITY

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Sera from patients with rheumatoid arthritis (RA)[1] often contain rheumatoid factor(s) (RF), antibodies that bind to the Fc portion of IgG. Hannestad, Johannesson, and Stollar (1, 2) isolated monoclonal and polyclonal RF from human sera, which not only bound IgG but also were antinuclear antibodies specific for nucleohistone (1, 2). Subsequently cross-reacting autoantibodies with both RF and anti-histone activity were isolated from sera of ~10% of RA patients (3), and considerably higher incidences were suggested by other reports (4–7). RF with anti-histone activity appear to possess an idiotype that is distinct from the idiotype of RFs without anti-histone activity (5).

Sporadic reports of RF with other activities have appeared. Hannestad (8) described a RF with ability to bind nitrophenyl groups and dDNA. Cunliffe and Cox (9) detected mouse RF that bound isologous IgG as well as bromelain-treated erythrocytes.

The interpretation of these provocative findings is not unambiguous. Since these RF were part of the serum antibody, it is not possible to establish the monoclonal nature of the antibodies presumed to have multiple specificities. It is also difficult to exclude the presence of IgG with antinuclear activity which is bound to RF autoantibodies, a complex demonstrated in sera from MRL/l mice (10). The availability of cross-reacting monoclonal IgM-RF produced in tissue culture would overcome these objections, since contamination by IgG antibodies would be excluded.

We recently obtained 23 mouse hybridomas producing RF by selection for IgM antibody to mouse IgG (11). These hybrids were derived from MRL/lpr mice, a lupus strain whose natural history includes production of RF and polyarthropathy resembling human RA (12). The present report describes the

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; dDNA, denatured (single-stranded) DNA; ELISA, enzyme-linked immunosorbent assay; mRF, monoclonal rheumatoid factor; PBS, phosphate-buffered saline; poly(A), polyadenylic acid; poly(C), polycytidylic acid; poly(dA), polydeoxyadenylic acid; poly(I), polyinosinate; poly(T), polythymidylic acid; poly(U), polyuridylic acid; RA, rheumatoid arthritis; RF, rheumatoid factors; RIA, radioimmunoassay; SLE, systemic lupus erythematosus.

capacity of some of these monoclonal RF to bind multiple autoantigens in addition to IgG.

Materials and Methods

The origin and characteristics of the hybridomas producing monoclonal RF, the radioimmunoassay for RF, and the methodology for aggregating mouse IgG have been described (11).

The enzyme-linked immunosorbent assays (ELISA) were performed using antigens immobilized onto Immulon 2 microtiter plates (Dynatech Laboratories, Alexandria, VA) at ~2 μg/ml in phosphate-buffered saline (PBS), pH 7.2 and obtained from the following sources: total histones and heat-denatured DNA (dDNA) (Calbiochem-Behring Corp., La Jolla, CA), mouse IgG subclasses (Litton Bionetics, Kensington, MD), re-purified protamines, polyuridylic acid (Poly U) and polyinosinate (Poly I) (Sigma Chemical Co., St. Louis, MO), polylysine and polyarginine (Miles Laboratories, Elkardt, IN), polycytidylic acid (poly C), polyadenylic acid (poly A), polydeoxyadenylic acid (poly dA) (P-L Biochemicals, Milwaukee, WI). Individual histones were purified from calf thymus chromatin by the method of Bohm et al. (13) and their purity was >95% as assessed by polyacrylamide gel electrophoresis as previously described (14). The assays were performed as previously reported (15) using monoclonal antibody preparations isolated by ammonium sulfate precipitation of hybridoma culture fluid.

Affinity purification of antibody preparations was performed with various antigens immobilized in microtiter plates. Antibody was diluted in standard ELISA solvent and incubated in replicate antigen-coated wells. After appropriate washes, antibody was eluted by incubation at room temperature in a solution consisting of 0.1 N NH_4OH, 1 mg/ml BSA, 0.05% Tween-20 (pH 10.9) for 1.5 h. The eluates were pooled and neutralized by the addition of phosphate buffer and HCl and assayed as described above.

The mouse IgM concentration in each monoclonal antibody preparation was determined by ELISA using wells coated with affinity-purified anti-mouse immunoglobulin (polyvalent, Tago, Inc., Burlingame, CA). A parallel set of wells was incubated with increasing concentrations of a mouse myeloma IgM (MOPC 104E, Litton Bionetics) in order to generate a standard curve. The IgM concentration in an antibody preparation was estimated from the standard curve after appropriate correction for dilution.

Immunofluorescence studies were performed using Hep-2 cells (Bion, Ridge, IL) or a rabbit kidney cell line (Electro-Nucleonics, Bethesda, MD) grown and fixed on microscope slides. Antibody preparations were diluted in PBS and 25 μl was incubated with the substrate for 1 h at room temperature. After appropriate washes, fluorescein-conjugated $F(ab')_2$ anti-mouse IgM (Cappel Laboratories, Cochranville, PA) was added and incubated for 30 min. Positive cells were visualized by fluorescence microscopy.

Results

Anti-DNA and Anti-histone Activities of Monoclonal RF. Mouse hybridomas were selected for secretion of IgM RF as previously described (11). Antibody produced in tissue culture from these hybridomas was concentrated by ammonium sulfate precipitation and tested for binding to total histones and dDNA by ELISA. Monoclonal RF (mRF) that were reactive with either dDNA or histone, as well as their binding to IgG subclasses, are shown in Table I. Of the 23 mRF, three displayed highly elevated binding to dDNA and one was weakly, but significantly, reactive. Two mRF bound histones and one of these (mRF7) was also a dDNA reactor.

The antibodies that displayed anti-histone activity were assayed for binding to individual histones and basic polypeptides, as shown in Table II. Clone 7 bound histones H1 and H4 predominantly, as well as polylysine, polyarginine, and

TABLE I

*Antigen Binding Specificities of Multireactive Monoclonal Antibodies**

Clone	IgM bound to test antigen:					
	μg IgM/ml				OD	
	IgG1	IgG2a	IgG2b	IgG3	Total histone	dDNA
mRF7	0	0	14.5	0	2.3	16.7
mRF16	4.4	4.3	0	1.3	6.7	0
mRF20	0.5	0.3	0.4	0.5	0	6.2
mRF21	2.7	3.5	3.2	4.6	0	5.8
mRF18	4.1	2.2	1.5	8.7	0.1	0.6

* A total of 23 stable monoclonal antibodies selected for binding to mouse IgG were produced (11). Shown above are the five clones that were reactive with total histones, individual histones, or dDNA at a 200-fold dilution of tissue culture supernatant concentrated by ammonium sulfate precipitation. The anti-IgG activity was determined by RIA and the anti-histone and anti-dDNA activities were assayed by ELISA.

TABLE II

*Reactivity of Histone-Binding mRF with Various Histone Classes and Basic Polypeptides**

Clone	IgM bound to test antigen (OD)							
	H1	H2A	H2B	H3	H4	Polylysine	Polyarginine	Protamines
mRF7	12.1	0.1	1.1	0.8	9.2	11.9	4.6	3.6
mRF16	0	17.3	0	0	>22	0	0	0

* Concentrated tissue culture supernatants from hybridoma clones 7 and 16 were diluted 100-fold and tested for reactivity with various antigens. With the exception of mRF21, which had a weak reaction with histone H1, none of the other mRF displayed reactivity with any of the individual histones.

TABLE III

*Reactivity of dDNA-binding mRF with Various Polynucleotides**

Clone	IgM bound to test antigen (OD)						
	dDNA	Poly(dA)	Poly(A)	Poly(T)	Poly(I)	Poly(U)	Poly(C)
mRF7	7.4	0	0	0	6.1	0	0
mRF18	0.1	0	0.2	0.1	0.1	0	0
mRF20	1.9	0	0	1.8	2.1	0	0
mRF21	1.5	0	0.5	2.4	0	0	0

* Binding to polynucleotides was determined at an IgM concentration of 6 μg/ml for mRF7 and mRF21, and at 0.6 μg/ml for mRF20 due to its limited supply. The binding of mRF18 was negligible at 6 μg/ml and results at 60 μg/ml are shown above. Five other mRF tested, including mRF16, did not bind to any polynucleotide at 60 μg/ml.

protamines. Clone 16 also bound H4 but differed from mRF7 in displaying binding to H2A and to no other histone or basic polypeptide.

The four mRF with anti-dDNA activity were examined for reactivity with synthetic polynucleotides. Each monoclonal displayed a unique binding profile with the six polynucleotides tested (Table III). Clone mRF20 bound to poly(I) and poly(T), whereas mRF21 did not interact with poly(I) but displayed affinity for poly(A) and poly(T). Clone mRF7 bound only to poly(I). In contrast, mRF18

showed weak binding to poly(A), poly(I), and poly(T). It appears, therefore, that the mRF that displayed cross-reactions with dDNA have subtle but significant differences in their polynucleotide binding specificities.

Monomolecular Nature of the Multiple Activities of mRF7 and mRF16. The hybridomas were grown under conditions that should insure monoclonality, and this was supported by the observation that upon subcloning each well containing a growing clone had the entire spectrum of antibody activities. In addition direct proof was sought that the multiple reactivities of each mRF preparation resided in the same molecule. Six serial adsorptions of mRF7 in mouse IgG-coated wells resulted in sequential, concomitant loss of 82% of the anti-histone activity. As a control for nonspecific loss of antibody, an IgM monoclonal antibody to dDNA, which was devoid of RF activity, was subjected to adsorption by mouse IgG, but no loss of anti-DNA activity was observed.

To further substantiate that the multiple reactivity of the antibody preparations resided within a single population, antibodies that were affinity purified on each cross-reacting antigen were examined for their binding specificities. Fig. 1 shows the antibody activities of three affinity-purified preparations of mRF16. Antibody eluted from H4-coated wells displayed reactivity with H4, H2A, and most importantly, mouse IgG. Similar binding properties were observed with the H2A eluate. Of greatest importance was the observation that antibody eluted from mouse IgG-coated wells bound histones H4 and H2A. It appears, therefore, that mRF16 antibody affinity purified on each relevant antigen displayed binding properties that resembled those of the untreated preparation.

Fig. 2 shows the results of a similar experiment using mRF7. Five preparations, affinity purified on H1, polylysine, H4, IgG2b, and dDNA, were tested for binding to all these antigens. With each antigen used for immunoadsorption, the eluted antibodies displayed similar antigen binding profiles, indicating that the same antibody population was isolated regardless of the antigen used for affinity purification.

Evaluation of Multiplicity of Molecular Interactions by Inhibition Studies. Of the

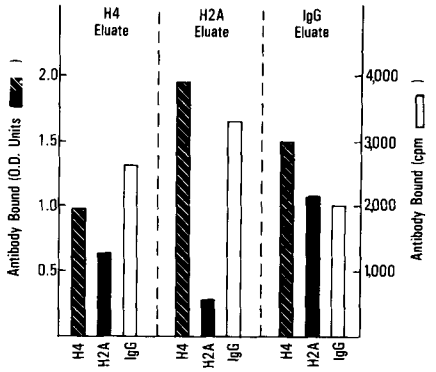

FIGURE 1. Reciprocal cross-reactions of affinity-purified mRF16. Hybridoma antibodies produced in tissue culture were bound to histones H4 or H2A or to mouse IgG and subsequently eluted from the solid phase antigen. The capacity of each eluate to bind H4 and H2A was tested by ELISA, and the binding to mouse IgG was assessed by solid phase radioimmunoassay.

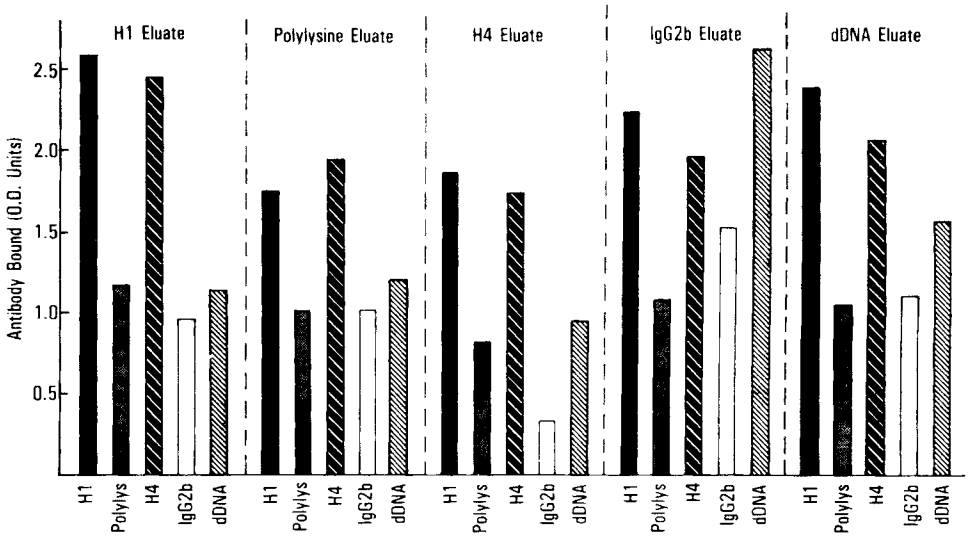

FIGURE 2. Reciprocal cross-reactions of affinity-purified mRF7. Hybridoma antibodies produced in tissue culture were bound to H4, H1, polylysine, IgG2b, or dDNA. Antibody was subsequently eluted from each solid phase antigen. The capacity of each eluate to bind the relevant antigens was determined by ELISA.

five multi-reactive monoclonal RF, mRF7 possessed the most complex antigen binding properties. By examining the ability of mRF7 to bind to dDNA or IgG2b in the presence of various soluble antigens, we sought to obtain some insight into the molecular basis of this phenomenon.

Inhibition studies involving different classes of histones could not be performed because of the strong tendency for histones to interact with various antigens, including polyanions such as DNA, IgG at histone concentrations above 10 μg/ml (unpublished observations), and self associations (histone-histone interactions). As a result of these secondary interactions, competition studies are hampered and commonly show enhancement of binding rather than inhibition. Therefore, the relationship between the histone binding site and the DNA and IgG binding sites could not be ascertained. However, inhibition studies were possible with DNA, IgG, and polynucleotides where such secondary interactions do not occur under appropriate conditions.

Fig. 3 shows the ability of various nucleic acids and of aggregated IgG2b to affect the binding of mRF7 to solid-phase dDNA. Inhibition of 90% of the anti-dDNA activity of mRF7 was achieved with 0.02 μg/ml poly(I), 0.2 μg/ml dDNA, and 2 μg/ml aggregated IgG2b. No effect of poly(A) at the highest concentration used (50 μg/ml) was observed, consistent with the absence of detectable direct binding to poly(A) (Table III). The corollary experiment, that is the capacity of various soluble proteins and polynucleotides to inhibit the binding of mRF7 to IgG2b, is shown in Fig. 4. Aggregated IgG2b, the homologous antigen, blocked 50% of the antibody binding at 0.25 μg/ml and 90% at 1 μg/ml. Poly(I) and dDNA were even more potent competitors on a weight basis, displaying 50% inhibition of anti-IgG2b activity at <0.006 μg/ml and <0.02 μg/ml, respectively, and approaching complete inhibition at ~0.5 μg/ml. Poly(A) had only a slight

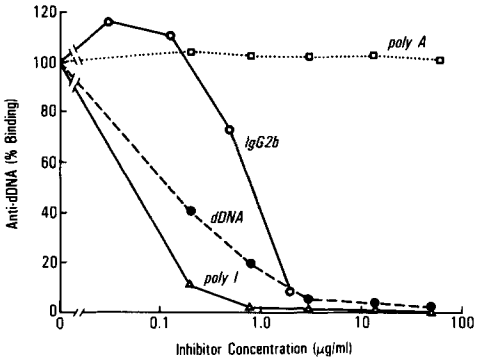

FIGURE 3. Inhibition of anti-dDNA activity of mRF7 by various antigens. Increasing concentrations of poly(I), poly(A), dDNA, or IgG2b were added to mRF7, and the mixtures were immediately plated into wells coated with dDNA and subjected to the standard ELISA.

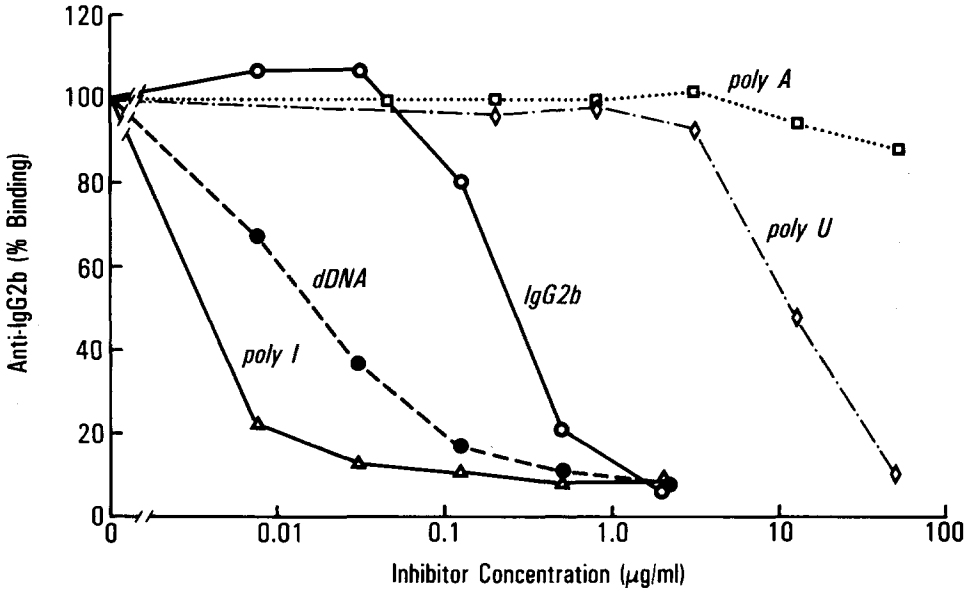

FIGURE 4. Inhibition of RF activity (anti-IgG2b) of mRF7 by various antigens. Increasing concentrations of poly(I), poly(A), poly(U), dDNA, or IgG2b were added to mRF7, and the mixtures were immediately plated into wells coated with IgG2b and subjected to the standard ELISA.

inhibitory effect on the anti-IgG2b activity at 50 μg/ml. However, poly(U), a polynucleotide for which we did not detect binding of mRF7 by direct ELISA, inhibited anti-IgG2b activity at a concentration >5 μg/ml and brought about 50% inhibition at 12 μg/ml. It should be noted that since the molecular weights and valencies of the various inhibitors are unknown, their potency as inhibitors cannot be used to accurately estimate their relative binding affinities.

As indicated above, H4 showed non-specific binding to immobilized mouse immunoglobulins, thereby limiting inhibition studies with this antigen. Below 10 μg/ml H4 did not block anti-IgG2b activity of mRF7.

As shown in Table III, mRF20 and mRF21 displayed strong binding to dDNA. Table IV shows that the RF activities of both mRF20 and mRF21 were blocked by increasing concentrations of soluble dDNA. The anti-IgG activity of mRF8, an antibody that did not have anti-dDNA activity, was not affected by the presence of dDNA up to 50 μg/ml.

Immunofluorescence Studies. The 23 mRF were tested for binding to intracellular antigens in fixed Hep-2 cells, as detected microscopically using a fluorescein-conjugated anti-mouse IgM. Nine of these mRF displayed immunofluorescence staining, and representative patterns are shown in Fig. 5. A cytoplasmic filamentous staining was displayed by mRF4,mRF5, mRF18, and mRF21; the latter two antibodies also gave an underlying "ground glass" or fine speckled staining in the cytoplasm. It is unlikely that binding of these mRF to what appears to be intermediate filaments (16) was nonspecific or mediated through the Fc region of IgM, because 19 of 23 mRF at similar concentrations and an IgM myeloma protein at 250 μg/ml did not bind cytoskeletal components.

A speckled pattern of cytoplasmic fluorescence was displayed by mRF20 and mRF7, the latter showing fluorescence that tended to concentrate around the periphery of the nucleus. Nuclear and chromosomal staining was displayed by mRF16, presumably due to its anti-histone activity. Monoclonals mRF10 and mRF11 bound to structures in the cytoplasm of Hep-2 cells that resembled discontinuous thick fibers or rods. These structures appear to be mitochondria by the presence of stained spherically shaped organelles (as expected for mitochondria) in a cell line derived from rabbit kidney.

As previously shown, mRF7, mRF18, mRF20, and mRF21 possessed anti-dDNA activity and the capacity to bind a variety of homopolynucleotides (Table III). Therefore, the effect of pre-incubating these antibodies with dDNA on their intracellular staining properties was examined. Absorption with 10 μg/ml dDNA significantly reduced staining of all these mRF except mRF21 and completely blocked immunofluorescence at 50 μg/ml. It appears, therefore, that the speckled cytoplasmic staining properties of mRF7, mRF18, and mRF20 are closely related to their anti-dDNA activities. However, the staining of the non-anti-dDNA mRF (mRF 4, 5, 10, 11, and 16) was not affected by dDNA up to 50 μg/ml.

TABLE IV

*Effect of dDNA on Binding of mRF to Mouse IgG**

dDNA μg/ml	Anti-IgG (% of control)		
	mRF20	mRF21	mRF8
0	100	100	100
1	102	46	100
10	31	5	88
50	8	3	111

* Increasing concentrations of dDNA were mixed with each preparation of diluted mRF, and the mixture was assayed for binding to a pool of mouse IgG myeloma proteins. Monoclonals mRF20 and mRF21 displayed anti-dDNA, whereas mRF8 did not.

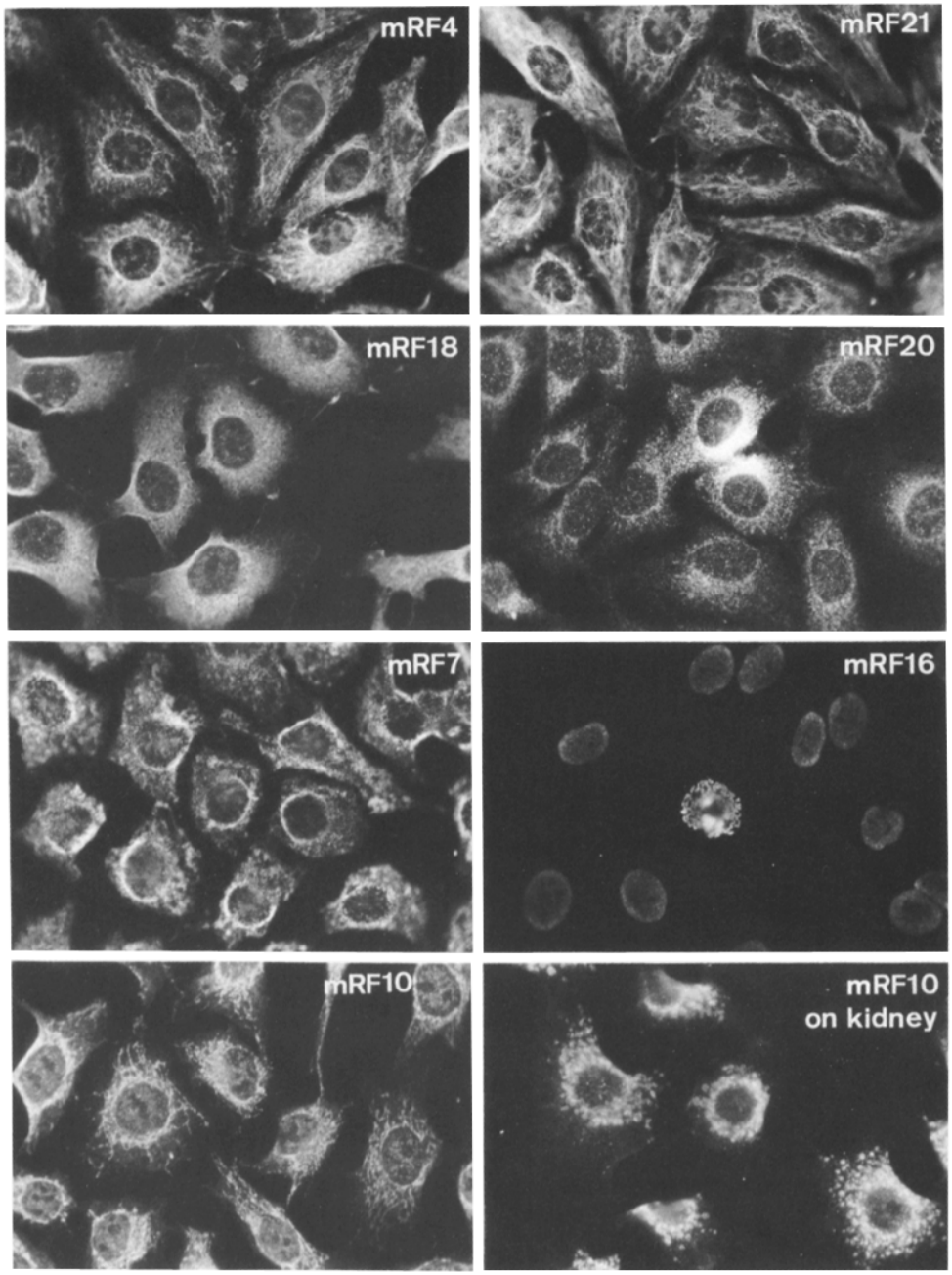

FIGURE 5. Binding of various mRF to intracellular antigens as assessed by indirect immunofluorescence. Each preparation was diluted to ~60 μg IgM/ml and incubated with Hep-2 cells grown and fixed onto microscope slides. Antibody binding was revealed by a fluorescein-conjugated $F(ab')_2$ anti-mouse IgM at 500× original magnification. The staining of mRF10 is shown on a cell line derived from rabbit kidney as well as on Hep-2 cells. Not shown are mRF4, which appeared similar to mRF5 and mRF11, which was similar to mRF10.

Discussion

In this study we have described a number of mRF from a lupus and arthritis animal model (i.e., MRL/l mice) that cross-reacted with a variety of cellular antigens. Of the 23 hybridomas obtained, ~¼ produced antibodies that had other activities in addition to the anti-IgG activity for which they were selected. The predominant cross-reactions were against histones and basic homopolypeptides and against dDNA and homopolynucleotides. No two mRF showed exactly the same profile of antigen binding specificities, indicating that each hybridoma originated from a different B cell. The relatively high incidence of cross-reacting mRF is consistent with previous reports on cross-reacting polyclonal RF from human sera (1–8) and provides strong support for the existence of such antibodies in patients with rheumatoid arthritis. Work in progress with human mRF derived from RA and lupus patients has also demonstrated similar cross-reactions.[2]

Numerous reports of monoclonal antibodies and antibodies from polyclonal sources with capacity to bind more than one antigen have appeared. Mouse and human monoclonal antibodies selected for anti-DNA activity bound a variety of polynucleotides and phospholipids (17) and many of the antibodies possessed cross-reactive idiotypes, even if derived from different mice (18) or humans (19). A monoclonal antibody was produced by Pillemer and Weissman (20) that bound Thy-1 antigen on T lymphocytes and the idiotypic determinant of TEPC-15 myeloma protein. A number of studies reported the production of monoclonal antibodies that bound microfilaments or intermediate filaments as well as other antigens with no known structural homology (21–25). Reports of monoclonal antibodies displaying cross-reactions between viral proteins and a nuclear antigen (26) or other intracellular components (27) have recently appeared. Antibodies from human sera with the capacity to bind dDNA and nitrophenyl groups have been reported (28). In rabbits antibodies were raised to AMP that bound menadione and caffeine but not the much more structurally similar GMP (29), and to κ-light chain that cross-reacted with β_2-microglobulin (30). Particularly relevant to the present report are studies with human RF with a variety of multiple specificities (1–8). The present findings of multireactive monoclonal antibodies produced in tissue culture with similar properties support the previous studies and may add impetus to the search for the immunochemical basis, the pathological significance, and the genesis of such antibodies.

Another aspect of the present studies that may be relevant to previous observations concerns the immunofluorescence findings. The capacity of the polynucleotide binding mRF to display a "ground-glass" pattern of cytoplasmic staining may be due to binding to RNA in ribosomes, since this activity was blocked by dDNA, a heterogenous polynucleotide that may consist of conformations of the phosphodiester backbone that cross-react with ribosomal RNA. The staining of cytoskeletal components by four other mRF cannot be explained by any known antigen binding activity of these mRF. However, patients with certain autoimmune diseases such as the CREST syndrome (32), Sjögren's syndrome (33), and particularly, rheumatoid arthritis (34, 35), show a high incidence of antibodies

[2] Theofilopoulos, A. N., R. L. Rubin, R. S. Baladeras, E. M. Tan, and F. J. Dixon. Specificities of human IgM monoclonal rheumatoid factors derived from patients with rheumatoid arthritis and systemic lupus erythematosus. Manuscript in preparation.

to cytoskeletal elements. The current observations raise the possibility that in human sera, RF activity and binding to cytoskeletal elements may be a property of the same antibody population.

Inhibition studies are commonly used to gain insight into the nature of the interactions involved in antibodies exhibiting multiple reactivities. Using mRF7 as a prototype of this phenomenon, we demonstrated that soluble dDNA blocked the IgG binding activity and that soluble, aggregated IgG inhibited the anti-DNA activity. A similar result was observed with mRF20 and mRF21 but not with control mRF that did not display anti-dDNA activity. Competition between DNA and IgG suggests that a single region or two regions with partially overlapping amino acid contacts in the antibody combining site are responsible for both activities. However, a combining site within an antibody with capacity to bind both IgG and dDNA is unexpected, since there is no apparent similarity in primary structure between the phosphodiester backbone of denatured DNA (the presumed determinant [31]) and a peptide within IgG2b. The affinity of the DNA-binding RF for polynucleotides with different nucleotide bases such as poly(I), poly(T), and poly(A) suggests that the nucleotide base is not the primary binding site, but that a special conformation of the nucleic acid backbone is required to generate the epitope to which the antibody is directed. Recognition by a single region within the antibody combining site of two epitopes having no known similarity in primary structure, e.g., DNA and IgG or H4 and IgG, is generally explained by a conformational epitope presumed to be common to the cross-reacting antigens.

Alternative explanations for the molecular basis underlying multireactive antibodies have been proposed (36–38), based on the notion that antibody combining sites are considerably larger than that needed to be complementary to a single epitope. A relatively large binding site may consist of separate subregions with capacity to react with different epitopes. Antigens may display co-inhibition because of overlapping spatial requirements rather than the same amino acid contacts within the antibody combining site. The present findings suggest that multireactive antibodies may not be uncommon and, therefore, may be of significance as a mechanism for generating antibody diversity.

Summary

We report that ~¼ of monoclonal rheumatoid factors produced by hybridomas derived from fusions of spleen cells from MRL/lpr/lpr mice with systemic lupus erythematosus (SLE) and arthritis exhibited multiple reactivities with other autoantigens, including dDNA, histones, and/or cytoskeletal-cytoplasmic elements. The patterns of reactivities of most of these clones differed, indicating that each had a separate B cell ancestor. Studies with eluted antibodies demonstrated that a single species of antibody molecules was responsible for the observed multiple reactivities. Inhibition experiments suggested that an antibody combining site may be large enough to accommodate dissimilar epitopes. These findings may provide further insights into the generation and extent of antibody diversity as well as the etiopathogenesis of systemic autoimmune diseases.

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