Detection of Immune Complexes

THE USE OF RADIOIMMUNOASSAYS WITH Clq AND MONOCLONAL RHEUMATOID FACTOR

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ABSTRACT This study describes two sensitive, rapid, relatively simple, competitive inhibition radioimmunoassays for detecting immune complex. The tests are based on the inhibition of I^{125}-Clq or I^{125}-monoclonal rheumatoid factor (mRF) binding to an insoluble substrate, IgG-Sepharose. The assays can be performed in 5 h utilizing 10 μl of serum. Heating of serum is not required and polyclonal rheumatoid factors do not interefere. With the two assays, a wide range of complexes of various size and complement fixing activity can be detected. The Clq test can detect complement fixing Ig complexes larger than 19S, while the mRF tests detect complexes of IgG as small as 8S irrespective of their complement fixing activity. Mouse, rabbit, and human aggregated IgG (agg IgG) can be detected in the Clq test, and human and rabbit agg IgG in the mRF test. As low as 4 μg/ml of isolated human agg IgG can be detected in the Clq test and 0.5 μg/ml in the rheumatoid factor test. Sensitivity is greater for mouse agg IgG. For pathologic sera which must be diluted to eliminate interfering factors, the sensitivity of the assay is approximately 10 times less. The Clq test showed marked inhibition by systemic lupus erythematosus sera with close correlation with CH50 levels and disease activity. The mRF test showed better correlation with rheumatoid arthritis sera. In addition, anionic macromolecules known to react with Clq and other Clq reactants that occur in pathologic sera such as the "low molecular weight" substances in systemic lupus erythematosus are also detected. These reactants are not detectable in the mRF test and can be eliminated in the Clq test by performing the test at higher ionic strength. The tests can be applied to the study of a variety of pathologic states where immune complexes appear to play a role.

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INTRODUCTION

At present there continues to be a great need for sensitive quantitative tests for the detection of antigen-antibody complexes. The possible role of immune complexes in a variety of basic immune mechanisms is currently under intensive study (1–4) and in addition immune complexes have been shown to have an important role in a wide variety of diseases (5–12).[1]

Various techniques have been described for detecting immune complexes (13, 14) but none so far have achieved the sensitivity, quantitative capacity, and simplicity for wide application. A major approach to the detection of immune complexes has been the use of reagents which will react preferentially with complexed immunoglobulins but not monomeric immunoglobulins. Two such reagents are the Clq component of complement (15, 16) and monoclonal anti-IgG (monoclonal rheumatoid factor, mRF) (17).[2] These two reagents have been used for the detection of immune complexes in a variety of technical procedures, including more recently radioimmunoassays (18–23). Tests with either reagent have limitations (14); however, when radioimmunoassay methods utilizing both reagents are run in parallel, the system has the capability of detecting both complement-fixing and noncomplement-fixing immune complexes over a wide range of molecular weights.

[1] Jerry, L. M., L. S. Adams, J. P. Sladowski, and M. G. Lewis. Direct detection of immune complexes in sera of patients with malignant melanoma. Manuscript submitted for publication.

[2] *Abbreviations used in this paper*: agg IgG, aggregated IgG; BSA, bovine serum albumin; JRA, juvenile rheumatoid arthritis; m mho/cm, conductance expressed in reciprocal milliohms per centimeter; mRF, monoclonal rheumatoid factor (anti-IgG); NDNA, native or double-stranded DNA; RA, rheumatoid arthritis; RF, rheumatoid factor; RIA, radioimmunoassay; SDNA, single-stranded DNA; SLE, systemic lupus erythematosus.

This approach has been taken in this study and two new radioimmunoassays are described, which present considerable improvement over the current methods.

METHODS

Reagents

Monoclonal anti-IgG. A mRF from a patient with Waldenstrom's macroglobulinemia was selected for specificity for aggregated IgG (agg IgG) as demonstrated in gel diffusion (Fig. 1). A broad reactivity with variety of different heated gamma globulins was observed, suggesting no allotype specificity. The mRf was isolated by affinity chromatograph with a 5/35-cm IgG-p-azobenzamidoethyl Sepharose-6B column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), prepared according to the method of Bing (24). The protein was eluted with 2 M glycine-HCl, pH 2.8. The peak obtained was pooled, dialysed against phosphate-buffered saline, aliquoted, and stored at −70°C. Just before use the samples were thawed and centrifuged at 2,000 rpm for 10 min at 4°C.

Clq component of complement. Clq was isolated from fresh normal serum by precipitation with deoxyribonucleic acid (DNA, Worthington Biochemical Corp., Freehold, N. J.) (14) and further purified by chromatography in Sephadex G-200 column (Pharmacia Fine Chemicals, Inc.) and zone electrophoresis (25). Purity of preparation was ascertained by radical immunodiffusion against anti-IgG, anti-IgM, and anti-IgA (Tri-Partigen Behring Diagnostics, American Hoechst Corp., Somerville, N. J.). There was less than 2% contamination with IgM and IgG. Just before use the samples were thawed and centrifuged at 2,000 rpm for 10 min at 4°C.

IgG-Sepharose. The procedure of Bing (24) was used to covalently link human IgG (Cohn FII, Research Products Division, Miles Laboratories, Inc., Elkhart, Ind.) to Sepharose-6B. 80% of the IgG added to the activated beads was bound. The final concentration was 16 mg IgG/g of Sepharose (dry weight). Just before use the IgG-p-azobenzamidoethyl Sepharose was washed and equilibrated with the binding buffer (0.075 μM Tris-HCl 0.01 M Na_2H_2EDTA, bovine serum albumin 1% pH 7.4).

Aggregated IgG. Human, rabbit, sheep, bovine, and mouse IgG aggregates were prepared according to the method of Dickler (26). Extent of aggregation of proteins was determined by density gradient ultracentrifugation analyses.

DNA preparations. To minimize contaminating single-stranded DNA (SDNA) in native DNA preparations (Worthington DNA), solutions were filtered through a 0.45-mm cellulose filter (Millipore Corp., Bedford, Mass.) which selectively binds SDNA. Denatured DNA (SDNA) was prepared by heating 0.5 mg/ml solution of DNA in boiling water for 15 min and immediately plunging the tube into ice water. DNAse treatment was performed as previously described (16).

Soluble antigen-antibody complexes. Immune serum was obtained from a rabbit hyperimmunized with human serum albumin (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio). Soluble immune complexes were prepared as previously described (16).

Radioiodination

Lactoperoxidase-catalyzed radioiodination was routinely conducted in isotonic veronal buffer, 0.01 M, pH 7.4, by a modified method of Heusser et al. (27). Briefly, the Clq and mRF were dialysed against the buffer and kept ice cold. 30% H_2O_2 (Mallinckrodt Inc., Saint Louis, Mo.) was diluted to 10^{-5} immediately before use; Na ^{125}I (New England Nuclear, Boston, Mass.) was diluted to 10 mCi/ml; and lactoperoxidase (sp act 18.2 IU/mg, Calbiochem, San Diego, Calif.) to 45 IU/ml. The reagents were added in the following order and amounts: Clq (10 mg/ml) or mRF (2.3 mg/ml), 50 μl; buffer, 50 μl; $Na^{125}I$, 50 μl; lactoperoxidase 200 μl; and H_2O_2, 200 μl. The labeled protein was separated from free iodide on a 1/30 cm Sephadex G-100 column. The sp act was approximately 0.055 μCi/μg for Clq and 0.1 μCi/μg for mRF. Bovine serum albumin (BSA, Reheis Chemical Co., Inc., Berkley Heights, N. J.) to a final concentration of 1% was added and the samples aliquoted and stored at −70°C. Just before use the reagents were thawed and centrifuged at 20,000 rpm for 30 min at 4°C and only the upper half of supernate was utilized in the test.

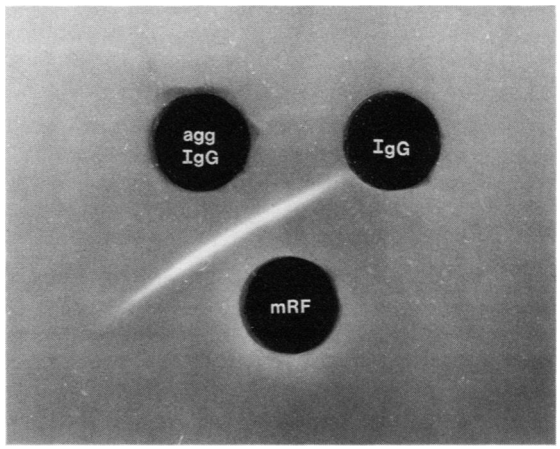

FIGURE 1 Gel diffusion plate showing precipitation of an isolated monoclonal anti-IgG (mRF) with agg IgG (1 mg/ml). Neither precipitation nor inhibition of the mRF-agg IgG line occur with monomeric IgG (3 mg/ml).

Density gradient ultracentrifugation

5–40% sucrose density gradient ultracentrifugation was carried out in 0.075 μM Tris-HCl 0.01 M Na_2H_2EDTA, pH 7.4 as previously described (16). Gradients were spun at 35,000 rpm for 18 h at 4°C in a Beckman Ultracentrifuge model L5-65, in an SW 50 rotor. (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) 0.2-ml samples were collected from the top in a density gradient fractionator model 640 (Instrument Specialities Co., Inc., Little Falls, N. J.). Alkaline phosphatase (Type IIIR *Escherichia coli*, Sigma Chemical Co., St. Louis, Mo.) and IgM were used as the 6.5S and 19S markers, respectively.

Radioimmunoassays

Both tests, Clq inhibition and mRF inhibition tests, consist of a two-step inhibition reaction between I^{125}-Clq or I^{125}-mRF with IgG-Sepharose. In the first step, 0.4 ml of the binding buffer (0.075 M Tris HCl, 0.01 M Na_2H_2EDTA, BSA 1%, pH 7.4, conductance 7.0 m mho/cm [reciprocal milliohms per centimeter]) was added to 10/75-mm Falcon plastic tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.); 0.1 ml of a 2-μg/ml of I^{125}-Clq or mRF were added.

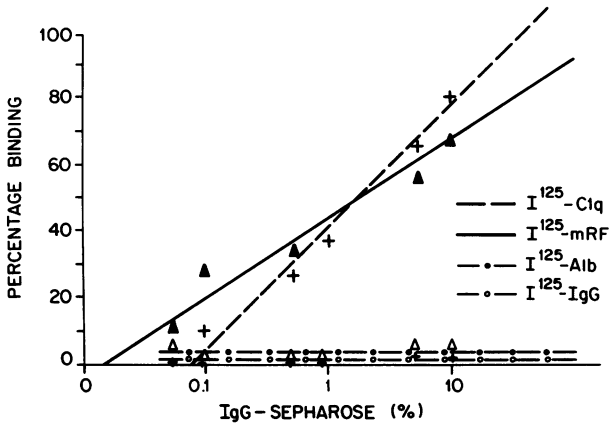

FIGURE 2 Comparative binding curves (regression analyses) for 0.2 μg of various I^{125}-labeled proteins with increasing amounts of IgG-Sepharose.

The mixture was incubated for 3 h at 4°C. In the second step of the reaction, 0.1 ml of a concentration of IgG-Sepharose which gave 50% binding (usually 5%; see Results) was added to each tube and the volume completed to a final volume of 1 ml with the binding buffer. The tubes were stoppered and incubated at 4°C for 2 h with continuous rotation on a radioimmunoassay (RIA)-pack rotator (Pharmacia Fine Chemicals, Inc.) for 2 h. After that the tubes were centrifuged for 2 min at 500 rpm and washed three times with the washing buffer (0.4 M NaCl, 0.1 M Na_2H_2EDTA, pH 7.4, conductance 45 m mho/cm) utilizing an aspirator pump (RIA aspirator, Pharmacia Fine Chemicals, Inc.). The radioactivity of the pellet was determined in a Beckman Gamma Counter (Beckman Instruments, Inc., Fullerton, Calif.). In all tests, a tube without inhibitor and IgG-Sepharose (0% binding or radioactivity bound to the tube) and a tube without inhibitor (100% binding) were always included. The percent of inhibition was calculated as follows: percent inhibitors = 1-cpm tube-cpm 0%/cpm 100%-cpm 0% × 100 and the result compared to standard curves. Standard curves were performed for each preparation of reagents. Binding curves, in a one-step reaction, between I^{125}-Clq or I^{125}-mRF and IgG-Sepharose were performed to find the 50% binding. Inhibition curves, in a two-step reaction, by adding different amounts of agg-IgG alone or dissolved in normal serum were prepared. These standard curves were submitted to regression analyses and plotting of the regression curve done by computer program (Hewlett Packard Co. 9810-A calculator, Palo Alto, Calif.).

Clinical specimens. All sera were allowed to clot at 25°C for 45 min and were stored at −70°C. All synovial fluids were treated with hyaluroindase (Worthington Biochemical Corp.) and also stored at −70°C. Just before use, the samples were thawed and centrifuged at 2,000 rpm for 10 min at 4°C. Rheumatoid factors from sera of patients with rheumatoid arthritis were isolated in the same way as the mRF reagent.

Other tests. Antibodies to native or double-stranded DNA (NDNA) were quantitated by a modified Millipore filter assay, described by Ginsberg and Keiser (28). The antigen utilized was Poly dA-dT^{C14} (Grant Island Biological Co., Grand Island, N. Y.). Total hemolytic activity (CH50) was measured according to Kent and Fife (29). Rheumatoid factor was quantitated by latex agglutination method. Serum immunoglobulin concentrations were determined by an automated immunoprecipitin procedure (Technicon Instruments Corp., Tarrytown, N. Y.).

Statistical methods. Correlation coefficients (r) were calculated by a standard program on a Hewlett-Packard 9810-A calculator.

RESULTS

Conditions for binding of I^{125}-Clq and I^{125}-mRF to IgG-Sepharaose. Binding of I^{125}-Clq was studied as a function of composition and ionic strength (conductance) of buffers and temperature and time of incubation. Maximal binding of I^{125}-Clq to IgG-Sepharose was obtained at 4°C after 1 h of incubation. Prolonging the period of incubation up to 24 h increased the binding by only 10%. The maximal binding of I^{125}-Clq to IgG-Sepharose was obtained with 0.075 M Tris-HCl, 0.01 M Na_2H_2-EDTA, pH 7.4, 1% BSA, conductance 7.0 m mho/cm, which is consistent with that previously reported (22). It was necessary to add 1% BSA to the buffers to prevent nonspecific sticking of I^{125}-Clq to the tube.

A washing step was found to be essential to increase the specificity of the inhibition step of the assay. A high ionic strength buffer 0.4 M NaCl, 0.1 M Na_2H_2-EDTA, pH 7.4, conductance 45 m mho/cm, was selected to eliminate nonspecific bound Clq to IgG-Sepharose as well as other contaminating proteins since it is well known that at low ionic strength, proteins can be nonspecifically bound to an insoluble phase. The wash step considerably decreased the counts bound to the gel. Clq bound to IgG-Sepharose is known to be very stable under these conditions (24) but to confirm this, unlabeled Clq in higher concentrations was incubated with a proportional amount of IgG-Sepharose, and the gel was washed. Clq could not be detected in the washed supernates.

Binding of I^{125}-mRF was studied in the same way. I^{125}-mRF binds better at higher temperatures than Clq. However, no significant difference was observed between 4° and 37°C. Optimal incubation time was similar to that for Clq binding. In contrast to the Clq the I^{125}-mRF binds without any significant difference in buffers of variable compositions and ionic strength ranging from 6 to 107 m mho/cm.

The specificity of Clq and mRF binding to IgG-Sepharose is shown in Fig. 2. Maximal binding of I^{125}-Clq and I^{125}-mRF occurs around 10% IgG-Sepharose. In contrast, no nonspecific binding was observed with other labeled proteins such as I^{125}-albumin or I^{125}-IgG. Optimization of the sensitivity in the inhibition assays was obtained with the concentration of gel that showed 50% binding as has been previously shown for competitive RIA (30, 31).

Inhibition of I^{125}-Clq and I^{125}-mRF binding to IgG-Sepharose. The condition for the inhibition of Clq

and mRF binding to IgG-Sepharose was studied utilizing a range of concentration of aggregated IgG. For the Clq test, maximal inhibition was obtained after 3 h of incubation. Increasing this incubation period progressively decreased the inhibitory capacity probably as a consequence of denaturation of the Clq. For the mRF, the same period of time was observed for maximal inhibition but no changes were observed up to 24 h of incubation. Agg IgG inhibited the mRF binding to the insoluble phase irrespective of incubation temperature. Clq showed no significant difference between 4° or 25°C; however, a marked decrease was observed at 37°C. Varying the ionic strength of the buffer showed maximal inhibition at the same low ionic strength as for the binding of Clq to the insoluble phase. No inhibition was observed in buffer with conductance greater than 65 m mho/cm. In contrast, for the mRF test no significant difference was observed from 6 to 107 m mho/cm. To facilitate performing both tests in parallel, the same buffers and period of incubation were utilized.

It was essential to examine the effect of 7S IgG on the assays because of known reactivity of mRF and Clq with monomeric IgG (17, 32) and its presence in large amounts in serum. The specificity of Clq and mRF for agg IgG is shown by comparative inhibition studies with agg IgG and monomeric IgG (Fig. 3). The Clq test can detect as low as 0.4 μg of agg IgG and 50% inhibition was observed with 2 μg. The monomeric IgG can inhibit the test; however, 100 times the concentration that produced 50% inhibition with agg IgG showed only a slight inhibition. In the mRF test, agg IgG can be detected as low as 0.05 μg, and 50% inhibition was observed with 0.7 μg. The monomeric IgG also inhibits the mRF test, but also only in concentrations about 100 times greater than agg IgG. To minimize the inhibition by monomeric IgG in normal serum a 1:10 dilution was required. Although the Clq test was less sensitive to monomeric IgG, a 1:20 dilution was needed to achieve approximately the same minimum. This was most likely due to the additional interference of endogenous Clq on the Clq binding assay as shown below.

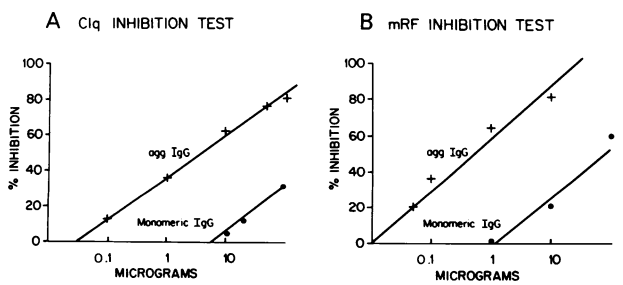

FIGURE 3 Comparative inhibition by increasing amounts of agg IgG and monomeric IgG of binding of 0.2 μg Clq (A) or mRF (B) to IgG-Sepharose.

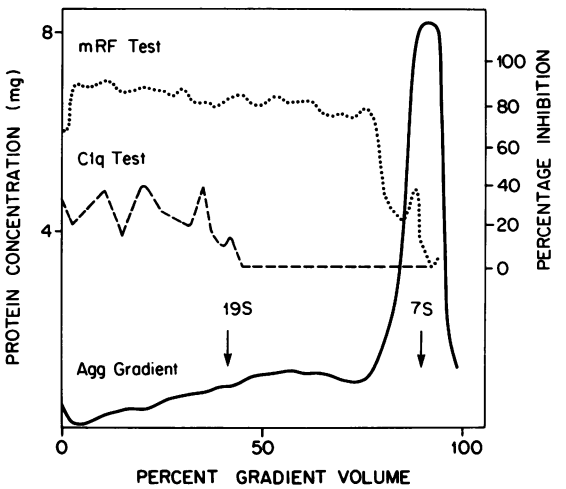

FIGURE 4 10–40% sucrose density gradient fractionated of heat-aggregated IgG. The 7S and 19S markers are shown by the arrows. The percent inhibition of various size aggregates in the Clq and mRF inhibition tests is shown in the upper part of the figure.

The inhibition by agg IgG in the presence of normal serum was tested by diluting different amounts of human agg IgG in normal serum diluted 1:20 for the Clq and 1:10 for the mRF. For the mRF test, agg IgG dissolved in normal serum showed the same level of inhibition as compared to agg IgG in buffer. However, for the Clq test, the amount of agg IgG necessary to produce 50% inhibition increases approximately 10 times, when utilizing agg IgG dissolved in normal serum. Dilution of normal serum greater than 1:100 did not produce any decrease in sensitivity.

To examine this phenomenon normal serum was treated in different ways before the addition of agg IgG. Treatments consisted of: (*a*) heating at 56°C for 30 min; (*b*) adding EDTA to a final concentration of 0.2 M; and (*c*) adding 0.01 M EDTA and anti-Clq, followed by centrifugation at 20,000 rpm for 20 min and filtration of the supernate through a 0.4-mm cellulose filter to specifically deplete Clq (Clq R reagent). The same levels of sensitivity were obtained only with the Clq R reagent serum. There was a relative improvement of sensitivity with heating for the upper range of agg Ig tested, in the lower range there was increased inhibition. The latter observation is most likely explained by the fact that the amount of aggregated IgG produced on heating would be detectable only in the lower range of the assay. 0.2 M EDTA had no significant effect. From these data, it is most likely that endogenous Clq binding to agg IgG is responsible for the decreased sensitivity of the assay for agg IgG in normal serum.

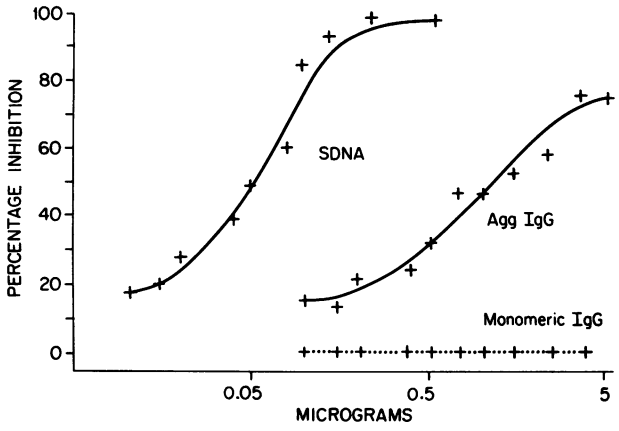

FIGURE 5 Comparative studies of the inhibition by SDNA, agg IgG, and monomeric IgG on the binding of I^{125}-Clq to IgG-Sepharose.

Bovine, rabbit, sheep, and mouse aggregated IgG were tested in both systems. Aggregation of each protein used was confirmed by sucrose density gradient analysis. The same inhibition was achieved for human and rabbit agg IgG, in both systems. Mouse agg IgG inhibits the Clq test, but not the mRF test. Amounts as low as 0.2 μg could be detected (twice more sensitive than for human agg IgG). Monomeric mouse IgG inhibited the assay only in concentrations approximately 150 times greater. No inhibition in either test was observed with bovine or sheep agg IgG.

Different classes of Ig and subclasses of IgG were also studied. The Clq binding as expected was inhibited by agg IgM and IgG 3, 1, and 2 but not IgA and IgG 4. The mRF binding was inhibited by all subclasses of human IgG. The effect of size of complexes in these inhibition tests is shown in Fig. 4. Clq can detect complexes greater than 19S and the mRF test detects complexes as low as 8S, as previously demonstrated (14). Similar results were obtained with preformed immune complexes. Clq detected complexes produced from 1 to 10 times antigen excess whereas mRF detected complexes produced from 1 to 200 times antigen excess.

Inhibition of the Clq binding to IgG Sepharose by anionic substances. The Clq component of complement is known to bind to various polyanionic substances such as DNA, lipopolysaccharide, heparin, carrageenan and trinitrophenol conjugated to BSA. Studies on NDNA and denatured DNA (SDNA) were carried out in detail. Inhibition capacity of SDNA (Fig. 5) is considerably higher than agg IgG in the Clq test. 50% inhibition is observed with 50 ng of SDNA and as little as 100 ng could be detected. DNAse treatment completely abolished this inhibiting activity. No significant difference was observed between NDNA and SDNA inhibitory capacity at low ionic strength (conductance 7.0 m mho/cm).

The minimal amount detectable of some other anionic substances was: carrageenan, 1 μg; heparin, 0.001 IU; lipopolysaccharide, 0.1 μg; and trinitrophenol conjugated to BSA, 0.01 μg. No inhibition by polyanionic substances was observed in the mRF test.

The binding of Clq to these anionic substances in many instances, depends on the ionic strength. When performing the test in ionic strength lower than 7 m mho/cm (Fig. 6), little differences in inhibition capacity are observed for SDNA, NDNA, and agg IgG. However, at the ionic strength of normal human serum (conductance = 9 m mho/cm) no inhibition is observed by NDNA but SDNA and agg IgG inhibition persists. At ionic strengths progressively greater than conductance <9 m mho/cm, the inhibitory capacity of SDNA decreases. Only weak inhibition persists at conductance = 13 m mho/cm. The agg IgG inhibitory capacity also decreases with increasing ionic strength of the buffer, but this decrease is less accentuated, with considerable inhibition remaining at conductance = 13 m mho/cm. No inhibition was observed with SDNA at conductance equal or greater than 64 m mho/cm. Inhibition by other anionic substances was completely abolished when the conductance of the buffer was increased to 9 m mho/cm.

Potential interfering reactors. To study the interference of endogenous Clq bound to circulating immune complexes in the mRF inhibition test, the following experiment was performed: agg IgG was added to increasing amounts of unlabeled Clq and these complexes utilized in the mRF test. No significant difference was observed in the percentage of inhibition between agg IgG and agg IgG treated with Clq. The effect of polyclonal rheumatoid factor (RF)

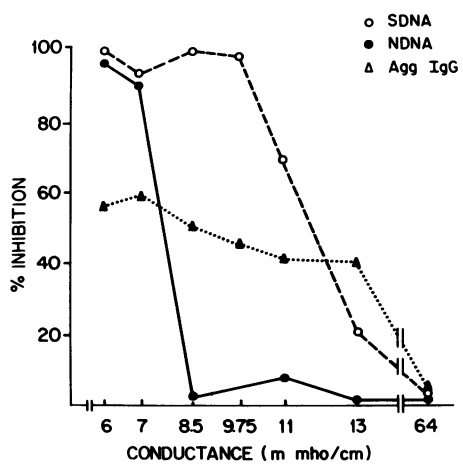

FIGURE 6 Effect of ionic strength on the inhibition of SDNA, NDNA, and agg IgG or the binding I^{125}-Clq to IgG-Sepharose.

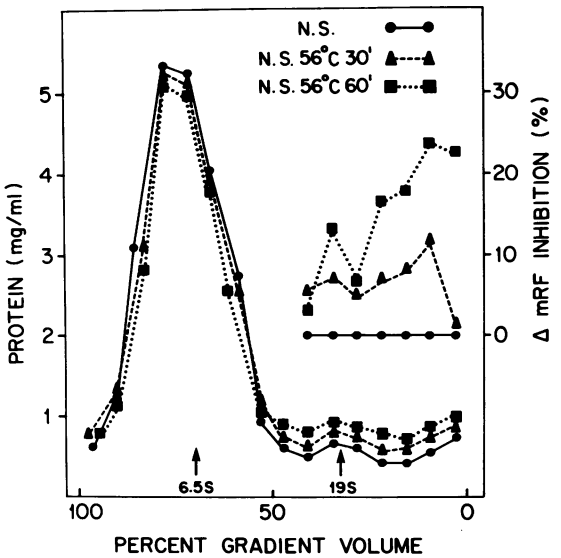

FIGURE 7 Comparative study of sucrose density gradient fractionations of normal serum (N.S.), N.S. heated at 56°C for 30 and 60 min. At the right of the figure the increase in inhibition in the mRF test which resulted from heating is shown for the fractions in the bottom half of the gradient.

on the assays is considered under Clinical studies (below).

Reproducibility of the RIA and effect of heating and thawing. Decomplementation by heating at 56°C for 30 min was carried out in four normal and four known positive sera and the results in both tests compared to those of fresh sera. The inhibition increased in normal sera and was variable, higher or lower for the pathologic sera. The effect of heating normal serum was further studied by the following experiments: I^{125}-Clq or I^{125}-RF was added to normal serum or normal serum heated at 56° for 30 min and fractionated on a sucrose density gradient. In the unheated sera the Clq sedimented in its usual 11S location and the mRF just beyond the 19S marker (probably 22S). In the heated sera both the Clq and RF were found in the lower part of the gradient. Fig. 7 shows the quantitative studies with the mRF assay on two heated samples. Similar inhibition though in lesser amounts was found in the Clq assay. The findings are consistent with increasing aggregation of IgG with progressive heating at 56°C.

Freezing at −70°C and thawing at 4°C normal and pathologic sera four times had no effect in either test. The reproducibility of the assays on specimens stored at −70°C was tested by repeating the Clq and mRF test five times utilizing aliquoted normal and positive controls over a 3-mo period. The variation of the results was 10% for the Clq assay and 14% for the mRF assay.

Clinical studies. The Clq and mRF inhibition tests

Clq INHIBITION TEST: PERCENTAGE OF INHIBITION BY PATHOLOGIC SERA

Disease	SLE	RA	JRA	MEL	HAA	CRYO	NL	Total
Patients	31	55	24	8	2	2	20	142
Sera	72	64	24	8	6	2	20	196
Positives (%)	49 (68)	31 (48)	10 (40)	2 (25)	4 (67)	1 (50)	1 (5)	
Negatives (%)	23 (32)	33 (52)	15 (60)	6 (75)	2 (33)	1 (50)	19 (95)	

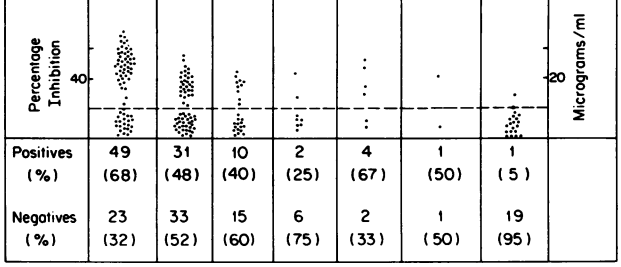

FIGURE 8 Results of screening normal and a variety of pathologic sera with the Clq inhibition test. The dotted line indicates the upper limit of normal (20% inhibition, 10 μg/ml). MEL, malignant melanoma; HAA, hepatitis antigen (B); CRYO, cryoglobulinemia; NL, normal.

were used to screen normal sera and sera from patients with active diseases of the types where circulating immune complexes are thought to be present (Figs. 8, 9). For most specimens both tests were run in parallel. In these initial studies only 1 of 20 normal sera gave greater than 20% inhibition in the Clq inhibition test. In the mRF assay all gave less than 15% inhibition. These values were taken as the upper limits of normal for evaluating the results in pathologic sera. Inhibition levels higher than normal were found in all disease groups tested. Comparison of the results of the two assays on the same specimens showed differences in both the incidence of positives and the level of positivity. The correlation of the level of positivity in the two assays for all specimens studied was low ($r = 0.17$).

mRF INHIBITION TEST: PERCENTAGE OF INHIBITION BY PATHOLOGIC SERA

Disease	SLE	RA	JRA	MEL	CRYO	NL	Total
Patients	31	55	24	8	2	20	140
Sera	72	64	24	8	2	20	190
Positives (%)	35 (48)	36 (56)	6 (25)	6 (75)	0 (0)	0 (0)	
Negatives (%)	37 (52)	28 (24)	19 (75)	2 (25)	2 (100)	20 (100)	

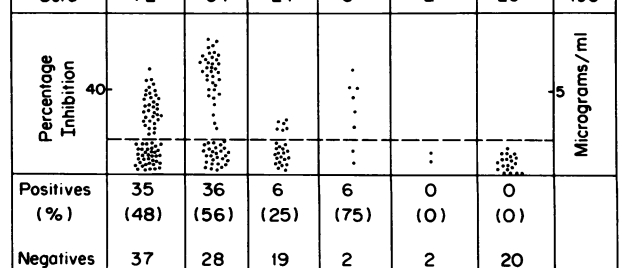

FIGURE 9 Similar study as Fig. 8 using the mRF inhibition test. The upper limit of normal is 15% inhibition, 2 μg/ml.

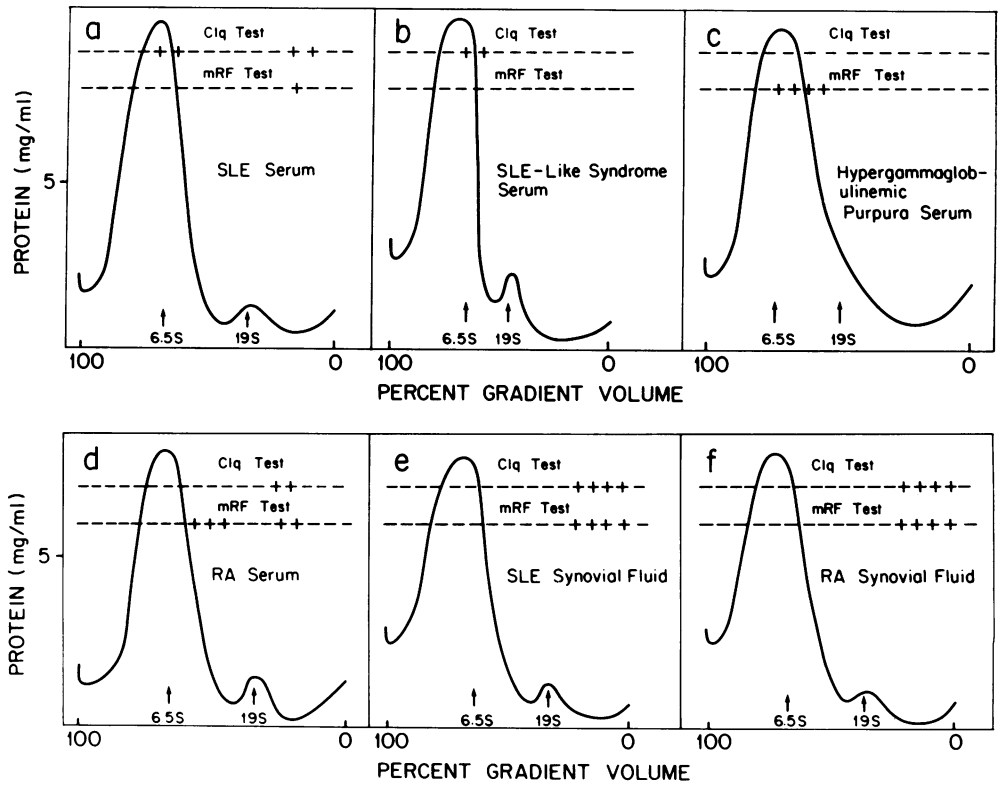

FIGURE 10 Sucrose density gradient fractionation of a variety of pathologic sera (a–f). Gradient fractions giving significant inhibition in the Clq and in RF tests are shown as plus signs at top of figure. The 6.5S and 19S markers are shown by arrows.

The Clq test showed a higher incidence of positives and higher levels of positivity than the mRF assay in systemic lupus erythematosus (SLE) and juvenile rheumatoid arthritis (JRA). Among the positive SLE sera 40% were positive in both tests, although the correlation coefficient was only 0.39; 40% were positive only in the Clq test; and 20% were positive only in the mRF. The latter finding is consistent with the greater sensitivity of the mRF assay. The greater positivity in the Clq assay found in some sera and the finding of sera positive in the Clq but negative in the mRF assay is inconsistent with the known relative sensitivity of the two assays.

The disparate results are most likely due to inherent differences in the assays to detect different types of complexes or inhibiting substances. Two types of studies support this possibility. In most SLE sera studied by sucrose density gradient, two different size inhibitors could be detected by the Clq test: One approximately 7S and the other larger than 19S. An example of such an analysis is shown in Fig. 10a. The mRF test detects only the larger inhibitor in these sera and not the low molecular weight substance that reacts with Clq. These low molecular weight substances which have been previously described in SLE (6) are similar to the low molecular weight substances found in the SLE-related syndrome (33). A sucrose density gradient analysis of a serum from a patient with this syndrome is also shown in Fig. 10b. Only low molecular weight reactants are found in these sera, and as in the SLE sera, the reactant does not inhibit the mRF test. In the second study the positive SLE sera were also tested at higher ionic strength (conductance = 9 m mho/cm) in the Clq test. The number of positive results decreased from 68% to 48%. Under these conditions it can be shown by sucrose density gradient experiments that the low molecular weight reactants in sera from patients with SLE and the SLE-related syndrome do not inhibit Clq binding.

Among the sera from patients with JRA studied, five were positive in both tests, five were positive in only the Clq test. One serum was weakly positive in only the mRF test. In those positive in both tests the correlation of levels of positivity was again low. The findings are similar to those in SLE; however, the concentrations of inhibiting substances is considerably lower. Attempts at further characterization of these substances have not been made.

In contrast with the Clq assay, the mRF assay

showed a higher incidence of positives among sera of patients with Rheumatoid Arthritis (RA) and malignant melanoma. Differences in both the number of positives and the level of positivity were found for RA sera but were not as marked as those described above for SLE sera in the Clq assay. Approximately 85% of the RA sera were positive in both tests. The correlation of the levels of positivity in two tests was low ($r = 0.36$). In the majority of positive sera the level of positivity was higher in the mRF test. Approximately 15% of the RA sera were positive in the mRF assay only. None were positive in only the Clq assay.

The increased positivity of RA sera in the mRF test could possibly be due to interference from rheumatoid factors which are present in high incidence in this disease. In one assay reported utilizing rheumatoid factors for the detection of complexes, rheumatoid factors present in pathologic sera were found to markedly interfere (20). However, in that study polyclonal rheumatoid factor was used as a reagent. In another assay utilizing a monoclonal rheumatoid factor reagent, no interference was detected by high-titered polyclonal rheumatoid factor (21). In these studies there was no apparent interference of free rheumatoid factor present in RA sera. A group of RA sera was studied as shown in Table I. There was no correlation between positivity in the mRF assay and serum rheumatoid factor titers ($r = 0.14$) or serum IgG concentrations ($r = 0.20$). In the same study there was also no correlation between positivity in the Clq assay, and serum rheumatoid factor titer ($r = 0.00$) or serum IgG concentrations ($r = 0.10$). The effect of free rheumatoid factors in these sera was further studied by isolating the rheumatoid factors from three of the sera strongly positive in the mRF assay. The isolated rheumatoid factors in the same concentration found in the starting sera did not inhibit the binding of the labeled mRF. Additional evidence of this type is shown in Fig. 11. A rheumatoid synovial fluid strongly positive for both RF and immune complexes was fractioned by sucrose density gradient. Complexes could be demonstrated in the bottom of the gradient by both the RF and Clq tests. RF present in the 19S region did not inhibit either test.

From the above studies it does not appear that free rheumatoid factors in RA sera are responsible for the increased reaction of these sera in the mRF assay compared with the Clq assay. A more likely possibility was illustrated by sucrose density gradient analysis of RA sera. In some patients reactants with both mRF and Clq were observed in the region greater than 19S but in addition substances between the 7S and 19S which inhibited only the mRF assay could be demonstrated (Fig. 10d). This finding is consistent with the intermediate complexes previously reported (17). The reactivity of "intermediate complexes" in pathologic fluids with the mRF assay but

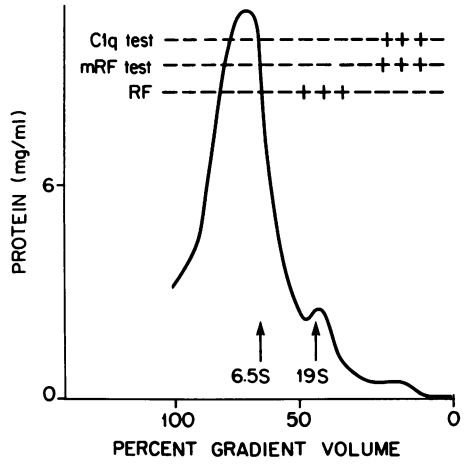

FIGURE 11 Sucrose density gradient fractionation of a RA synovial fluid. The 6.5S and 19S markers are shown. Gradient fractions positive for RF or giving significant inhibition in the Clq and mRF tests are at the top of the figure by plus signs.

TABLE I

Sera from Patients with Active Rheumatoid Arthritis: Comparison of Inhibitors of Clq and mRF Binding Tests with IgG Concentration and Rheumatoid Factor Titers

Patient	Inhibition		RF titer	IgG
	mRF	Clq		
	%			mg/ml
J. J.	93	30	1:320	17.8
H. E.	87	33	1:320	18.2
R. G.	95	30	1:640	19.9
B. D.	93	24	1:640	15.0
L. D.	68	50	1:40	12.6
P. D.	76	46	1:160	15.8
J. C.	88	56	1:40	12.3
G. B.	58	53	1:160	10.6
J. B.	75	53	1:80	16.0
E. H.	92	16	1:160	18.3
H. H.	84	28	1:320	10.6
B. G.	80	25	1:160	22.6
E. A.	77	2	1:80	5.8
M. C.	98	37	1:40	13.6
B. S.	68	53	1:640	18.7
M. P.	95	31	1:20	21.6
H. O.	39	33	1:640	17.3
S. M.	88	24	<1:20	24.0
J. L.	72	52	1:80	14.7
P. D.	58	50	1:80	17.4
Normals	30.7±4.5	12.3±6.1	<1:20	9.62±4.12

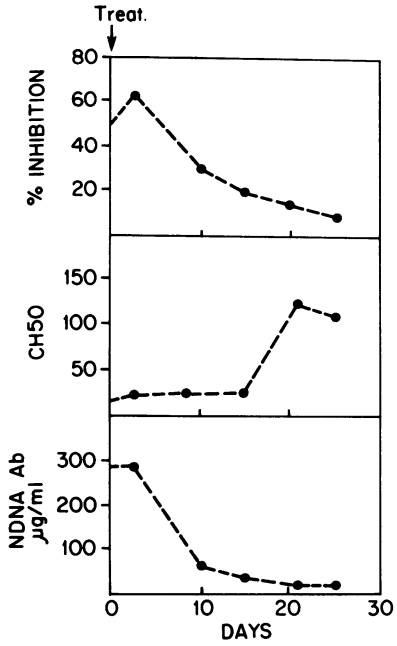

FIGURE 12 Serial study of SLE patient showing comparison of Clq inhibition test results with serum hemolytic complement (CH50) and NDNA antibody levels. Arrow indicates when treatment (Treat.) was begun.

not Clq assay was confirmed by sucrose density gradient studies on a serum of a patient with hypergammaglobulinemic purpura that previously has been shown to have high concentrations of "intermediate complexes" consisting of 7S rheumatoid factors (34). There was strong inhibition of the mRF binding between the 7S and 19S markers but no inhibition in the Clq test (Fig. 10c).

A greater incidence of positivity in the mRF assay compared to the Clq assay was also seen in the studies on sera from patients with malignant melanoma although only a few sera were tested. The disparate results were considerably greater than that seen in RA sera. The nature of the reactants remains to be determined.

A correlation between activity of disease and positive tests in both assays was observed. The Clq test appears to correlate closely with levels of complement, as observed in some serial studies of patients with antigen (B)-positive hepatitis or SLE. The phenomena is illustrated in the study of a patient with SLE in Fig. 12. Of note is that although the Clq test was more strongly positive, the results using the mRF test also correlated with the hypocomplementemia, disease activity, and levels of DNA antibody titers. Similar findings have been made in five other patients with SLE that have thus far been serially studied. Serial studies have not as yet been done for the other disease groups.

While no systematic attempt has as yet been made to study the nature of the reactant detected in pathologic fluids by these assays, a number of synovial fluids from patients with RA or SLE have been encountered that are markedly positive in both tests and which on sucrose density gradient analyses have reactive material that is in a region greater than 19S is shown in examples in Figs. 10e and f. Such specimens facilitated the further characterization of the nature of the Clq and mRF reactants. The fractions containing the reactants were pooled, concentrated and assayed for immunoglobulins. IgG and IgM could be demonstrated in RA synovial fluid whereas only IgG could be demonstrated in the SLE synovial fluid. These findings are consistent with the presence of complexes containing IgG and IgM in the RA synovial fluid and complexes of IgG in the SLE synovial fluid.

DISCUSSION

The two assays developed in this study afford a number of improvements over currently available methods. The competitive inhibition method utilizing an insoluble phase provides greater sensitivity and quantitative capacity than radioimmunoassays employing direct binding (31). The covalent linkage of IgG to the resin via the p-azobenzamidoethyl group represents an "arm" holding the protein away from the Sepharose which prevents steric hindrance and allows firmer Clq and mRF binding. Under these conditions a washing step is possible that can eliminate any contaminants or denatured labeled proteins thereby increasing the overall specificity of the assays.

In addition, heating of serum specimens as is necessary in a number of other assays is not required. Heating serum specimens as shown in these studies causes aggregation of immunoglobulins and may also destroy thermolabile antigens that possibly are present in immune complexes in unknown pathologic sera. The use of a mRF with high avidity for complex IgG minimizes the interference by monomeric IgG and polyclonal rheumatoid factors. However, the high IgG levels in serum still present an interference problem which is circumvented by using a 1:10 dilution of test sera. Sensitivity of the assay is proportionately decreased by the dilution step, but in spite of this, on the basis of the limited comparative data currently available, the test with mRF is more sensitive than any assay thus far well characterized (14).

Endogenous Clq interferes with the detection of complexes of IgG added to normal serum. The techniques used by others, heating (18, 22) or EDTA (35) to eliminate this problem were found to be ineffective in this system. Heating as noted earlier is also undesirable because of artifacts introduced. The inter-

ference can be circumvented by diluting the serum with a concomitant decrease in sensitivity. The assay is most useful clinically where Clq levels are depressed since the decrease in sensitivity seen in normal serum is not a problem. As best can be determined, even with the dilution step, the sensitivity of the assay is as sensitive or more sensitive as other assays with Clq in detecting isolated complexes and complexes in pathologic sera (14). Of note is that the assay can be used for detection of immune complex in species other than man and appears to have greater sensitivity in detecting aggregated mouse IgG.

The use of the two assays in parallel, one employing Clq and the other mRF, allows for detection of both complement-fixing and noncomplement-fixing immune complexes and a wide range of size of complexes. The poor correlation of the two assays in quantitating inhibitory substances present in pathologic sera is for the most part due to the different specificities the assays have for certain complexes and other reactants. However, these differences allow comparisons which point up the presence of anionic substances which react in the Clq test but not in the mRF assay and small complex of IgG which react in the mRF assay but not Clq assay.

The reaction of Clq with most anionic substances is markedly dependent on ionic strength. At the ionic strength used routinely in the Clq binding assay in this study, anionic substances react strongly with Clq. These conditions were used to obtain maximal sensitivity of the assay for complexes of IgG. With certain sera, particularly sera from SLE, the inhibition of binding in the Clq test was considerably greater than in the mRF assay. The difference most likely is due to the presence of anionic substances free or in complexes in addition to complexed IgG detected in both tests. This is suggested by the elimination of the differences in the two tests by performing the Clq test at higher ionic strength and by sucrose density gradient experiments which show low molecular weight reactants present along with higher molecular weight reactant in the same sera. The low molecular weight reaction can be eliminated by raising the ionic strength whereas the high molecular weight reactants persist.

It has not yet been determined if the increased reactivity in the Clq binding test compared to the mRF binding in SLE sera in all cases is due to the presence of low molecular weight anionic substances or whether in some cases complexes containing anionic substances are present. In any case, from a clinical standpoint the increased sensitivity of the test under low ionic strength is desirable since there appears to be close correlation of positive tests with clinical activity not only in SLE but in the SLE-like syndrome (33) and in denque (22). As shown, the Clq test can be done at conditions which eliminate the reaction of anionic substances but the sensitivity for detecting complexes of IgG is significantly reduced.

The reaction of SDNA with Clq is of considerable interest and may be of some importance in the immunopathology of SLE (36–38). In contrast to NDNA and the other anionic substances tested, SDNA reacts with Clq at higher ionic strength. This is consistent with previous studies which have shown that SDNA can activate complement under physiologic conditions whereas native DNA does not (39). Under the conditions which the Clq assay is performed in this study as well as in other studies, (e.g., the Clq deviation test), the inhibition of SDNA is considerably greater than that for aggregated gamma globulin on an equivalent weight basis. In studying SLE patients then, with the Clq binding test, enhanced inhibition could occur with the presence of SDNA antigen alone or to complexes containing SDNA antigen. There is yet no direct evidence for this, but the possibility is not unlikely and is under study. In addition, complexes of SDNA antigen and NDNA antibody could exist in the presence of excess NDNA antibody and provide a possible explanation for the findings demonstrated in Fig. 12.

Differences in the sizes of complexes detected is a characteristic of the two assays which can provide useful information when the tests are run parallel. This is most clearly demonstrated in evaluating certain pathologic specimens. In some SLE sera both high molecular weight and low molecular weight substances that are reactive with Clq have been detected. In sucrose density gradient analysis it can be shown that the high molecular weight substances react with both Clq and monoclonal rheumatoid factor as would be expected for complexes of gamma globulin whereas low molecular weight substances react with only Clq. Knowing that Clq reacts with certain anionic substances and does not react with complexes of gamma globulin below 19S in size, this finding would be most consistent with the presence of an anionic substance in the SLE sera that reacts with Clq but not monoclonal rheumatoid factor. Similar low molecular weight substances have been recorded in a group of patients who have an SLE-like syndrome (33) and this finding has been confirmed in this study. In hypergammaglobulinemic purpura sera where intermediate complexes are known to be present, sucrose density gradient analysis shows that intermediate complexes can be detected by monoclonal rheumatoid factor and not by the Clq test. With the two tests to study RA sera, intermediate complexes which have been reported previously can be detected by the mRF binding test but not by the Clq binding test. In addition, in a number of sera

which have been tested so far there appear to be high molecular weight substances which react in both the tests. The inability of the Clq inhibition test to detect the intermediate complexes compared to the mRF inhibition test probably explains the higher incidence of positivity in the mRF inhibition test in RA patients compared to SLE patients.

In interpreting the results of these tests, it must be kept in mind that neither assay differentiates reaction due to immune complexes from those due to nonspecifically aggregated Ig such as might occur in vivo with physical alteration of Ig or in vitro. The precautions described for the use of these tests are designed to minimize the latter problem. For definitive evidence that immune complexes are responsible for the inhibition reaction, both antigen and antibody must be identified in reactive substance. A major problem in attempting such characterization with previous techniques has been the marked dilutions that occur with isolation procedures. The increased sensitivity afforded by these new assays may possibly eliminate this problem.

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