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A reappraisal of the monoclonal rheumatoid factor test for circulating immune complexes: a comparison of two monoclonal rheumatoid factor reagents

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SUMMARY

Assays involving monoclonal rheumatoid factor (mRF) reagents are frequently used for the detection of circulating immune complexes, particularly in the rheumatic diseases. A study has been performed to investigate the interaction of two purified mRF reagents with normal sera, sera and synovial fluid from patients with rheumatic diseases and with heat-aggregated human IgG used as a model of immune complexes. Interaction has been measured by a simple laser nephelometric technique and a sensitive radiolabelled mRF precipitation assay.

Both mRF reagents showed little reactivity with normal sera but reacted strongly with many of the pathological specimens. Similarly both mRFs reacted with large molecular sized heat aggregates of IgG while a variable reactivity was found with uncomplexed or monomeric IgG. However in pathological sera, both mRFs reacted predominantly with monomeric IgG and a significant correlation was found between the two reagents. This reactivity with monomeric IgG remained after separation of pathological sera in low pH sucrose gradients suggesting it was not due to the presence of small immune complexes of the classical type. In addition no reactivity was found with either reagent with IgG–RF intermediate complexes.

It is concluded that mRF reagents are not specific for IgG containing immune complexes. They also react with monomeric IgG and this reactivity is particularly prominent in certain pathological sera. The possible nature of this reactive monomeric IgG is discussed.

INTRODUCTION

The monoclonal rheumatoid factor (mRF) test for circulating immune complexes was one of the earliest to be described and has been recommended for use particularly in the diagnosis and assessment of rheumatic diseases (Luthra *et al.*, 1975; Lambert *et al.*, 1978). The principle of the test is that mRF reacts preferentially with aggregated or complexed IgG possibly due to the increased number of exposed IgG Fc antigenic determinants in the complexed state.

The mRF test was initially developed by Winchester and colleagues following Hannestad's observation that polyclonal rheumatoid factor (pRF) precipitated with IgG aggregates in rheumatoid arthritis (RA) synovial fluid (Hannestad, 1967). Winchester, Kunkel & Agnello (1971) observed that mRFs obtained from patients with lymphoproliferative disorders gave more efficient

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precipitation than pRF. They found using agar gel double-diffusion experiments that mRF formed visible precipitin lines with 57% and 70% of RA sera and synovial fluid respectively, while no precipitation was observed with normal sera. Gel filtration studies suggested that the mRF reacted predominantly with large molecular weight complexes of IgG. Further refinements in the assay were reported by Luthra *et al.* (1975) and Gabriel & Agnello (1977) who described radioimmunoassays involving competitive inhibition of mRF binding with heat-aggregated human IgG (HAGG). The development of these radioimmunoassays greatly enhanced their sensitivity but surprisingly these assays did not correlate significantly with other tests for IgG containing circulating immune complexes and they seemed to detect immune complex like material preferentially in RA when compared with other rheumatic diseases (Lambert *et al.*, 1978). The mRF radioimmunoassay, however, confirmed Winchester's observation that the material detected in RA sera and synovial fluid was of high molecular weight consistent with it being aggregates or complexes of IgG (Gabriel & Agnello, 1977).

We have recently studied the interaction of a mRF with HAGG and sera from patients with rheumatic diseases using laser nephelometry (Roberts-Thomson & Bradley, 1979). This technique is simple and sensitive and we have confirmed the preferential reactivity of mRF with RA sera and synovial fluid. However, from our ultracentrifugation and gel chromatographic studies it appeared that mRF reacted predominantly with monomeric or uncomplexed IgG in pathological sera in contradistinction to previous studies. In the present study these findings have been confirmed and extended and a second mRF reagent has been compared with the first reagent. The results show that mRF assays are not necessarily specific for circulating immune complexes but preferentially detect uncomplexed monomeric IgG.

MATERIALS AND METHODS

Sera and synovial fluids. Sera and synovial fluids were obtained from patients with systemic lupus erythematosus (SLE) and definite or classic RA (ARA criteria). Control sera were obtained from healthy laboratory donors. Serum and synovial fluids (SF) specimens were examined either immediately before freezing or stored at $-70°C$ prior to use.

Purification of mRF. Monoclonal RF was isolated from the serum of two patients with lymphoproliferative disorders. Their sera contained mixed cryoglobulins with monoclonal IgM–RF. Both mRFs were isolated in the same way. The serum cryoglobulin was collected by centrifugation at 1,000 *g* after allowing the serum to stand for 4°C for 48 hr. The cryoglobulin was washed twice in cold phosphate-buffered saline (PBS), redissolved by warming to 37°C, and the process of cryoprecipitation and washing repeated. The cryoglobulin was then redissolved in 0·1M acetic acid and applied to a Sephadex G-200 (90 × 2·5 cm) column equilibrated in 0·1M acetic acid, pH 4. Two peaks were eluted following chromatography; an initial peak in the void volume containing monoclonal IgM and a later peak containing polyclonal IgG. The fractions containing IgM were immediately pooled, neutralized and then dialysed against PBS. It was then concentrated to its original volume using an Amicon ultrafiltration apparatus. The protein concentration was determined by spectrophotometry assuming an extinction coefficient for IgM of 1·24 for a 1 mg/ml solution in a 1 cm cuvette. Homogeneity of the mRF preparations was confirmed by SDS polyacrylamide electrophoresis while immunoelectrophoresis confirmed monoclonality. The mRF from one patient was designated $mRF_{(A)}$ and for the second $mRF_{(D)}$.

Radiolabelling in mRF. The $mRF_{(A)}$ reagent was radiolabelled with I^{125} by the chloramine-T method using 100 μg protein in 1 ml PBS and 1 mCi ^{125}I (Hunter & Greenwood, 1962).

HAGG. Commonwealth Serum Laboratories IgG (20 mg/ml in PBS) was heated for 7 min or 30 min at 63°C in a water bath. The HAGG was immediately aliquotted into small volumes and stored at $-70°C$.

Nephelometric mRF assay. This assay was used as previously described (Roberts-Thomson & Bradley 1979); briefly 5 μl of mRF (= 22·5 μg) was added to 500 μl of column fractions or serum diluted 1:25 in PBS. The solution was incubated at room temperature for 60 min and the degree of light scatter in mV was then measured in the laser nephelometer. Blanks, i.e. column fractions or

serum diluted 1:25 in PBS without mRF, were subtracted from the solution containing mRF. All estimations were the mean of duplicates.

Radiolabelled mRF precipitation assay. The assay used was as described by Barrett & Naish (1979) with minor modifications. Test serum (100 μl) or synovial fluid was added to 400 μl of PBS/0·13 M EDTA pH 7·4. To this solution was added 10 μl of radiolabelled mRF^{125}I followed by 500 μl 5% polyethylene glycol (PEG) in PBS pH 7·4. The final mixture in glass tubes was gently mixed and allowed to stand at room temperature for 120 min. It was then centrifuged at 1,000 *g* for 20 min at room temperature. Half the supernatant (S) was aspirated off into a separate tube. Both supernatant and precipitate fractions were counted in a gamma counter. The percentage precipitation of mRF^{125}I was calculated from the formula $(P-S)/(P+S) \times 100\% \times T$ where T was the percentage precipitation of mRF^{125}I in 10% trichloroacetic acid (usually around 90%). All estimations were the mean of duplicates. Following column chromatography 100 μl of foetal calf serum (FCS) was added to 400 μl of column fractions, and the percentage precipitation of mRF^{125}I for each fraction determined as described above.

Sucrose gradient ultracentrifugation. Sucrose gradient ultracentrifugation was performed at 4°C in the Beckman L5/50 ultracentrifuge using a 5 ml 10–40% (w/v) continuous sucrose gradient in PBS or 0·1M glycine/HCl pH 3·0. Serum (0·5 ml) was applied to the top of the gradient at a final dilution of 1:4 and centrifuged for 18 hr at 285,000 *g* and five drop aliquots were collected from the bottom of a gradient. Acid fractions were immediately neutralized with 1·0M Tris buffer pH 8·2. To each fraction 1 ml PBS was then added and the optical density at 280 nm determined. The amounts of immunoglobulins and reactivity with mRF were then determined in each fraction.

Gel filtration chromatography. Filtration chromatography was performed with Sepharose 6B and Sephadex G-200 (Pharmacia, Uppsala, Sweden) columns (90 × 2·5 cm) equilibrated in PBS and 4 ml fractions were collected at the rate of 10–20 ml/hr. In some experiments filtration over Sephadex G-200 was also performed in the presence of 0·1M acetic acid buffer and the eluted fractions immediately neutralized with 1·0M Tris buffer. The location of IgM or IgG was determined by nephelometry using antisera obtained from Dako-Immunoglobulins (Copenhagen, Denmark) as previously described (Roberts-Thomson & Bradley, 1979).

Statistical analysis. Comparison between two groups was calculated by the Wilcoxon sum of ranks method while comparison between two variables was calculated by linear regression.

RESULTS

Reactivity of mRF with HAGG

The degree of light scatter and percentage precipitation of mRF$_{(A)}$ obtained with HAGG and native uncomplexed IgG in human serum is shown in Fig. 1. It is seen that the radiolabelled precipitation assay is 100-fold more sensitive in detecting HAGG than the nephelometric assay while mRF$_{(A)}$ reacts with native IgG in both assays at a similar threshold concentration of approximately 1 mg/ml. Both assays also reacted with Cohn fraction II isolated IgG—the curve being displaced a little to the left of that obtained for native IgG, suggesting the presence of aggregates in this preparation (data not shown).

Reactivity of mRF with pathological and control sera

Figure 2 demonstrates the degree of light scatter and percentage precipitation of mRF$_{(A)}$ obtained for sera and synovial fluid from patients and controls. Both assays could significantly distinguish pathological sera from control sera but it appeared that the nephelometric assay was more discriminating despite the enhanced sensitivity of the precipitation assay for HAGG.

Comparison of the nephelometric mRF assay and the radiolabelled mRF precipitation assay

The nephelometric mRF assay and the radiolabelled mRF precipitation assay were compared using the same 25 pathological sera. A highly significant correlation ($r=0{\cdot}74$, $P<0{\cdot}001$) was observed between the two assays (Fig. 3).

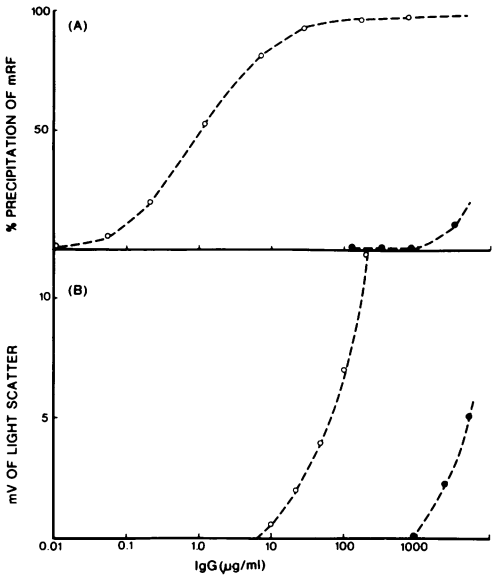

Fig. 1. The percentage precipitation in 2·5% PEG (panel A) and the degree of light scatter in millivolts (panel B) obtained between $mRF_{(A)}$ and HAGG O– – –O and native uncomplexed IgG ●– – –●.

Comparison of two different mRFs

HAGG (20 mg/ml heated at 63°C for 7 min) was fractionated on Sepharose 6B and the eluted fraction measured for protein (OD_{280}) and nephelometric reactivity using $mRF_{(A)}$ and $mRF_{(D)}$. Resulting profiles are shown in Fig. 4. Both mRFs reacted in a similar fashion with aggregated IgG in the size 10^6 daltons but $mRF_{(A)}$ showed a greater reactivity with smaller aggregates and with unaggregated monomeric IgG. The degree of light scatter obtained for 26 pathological sera using

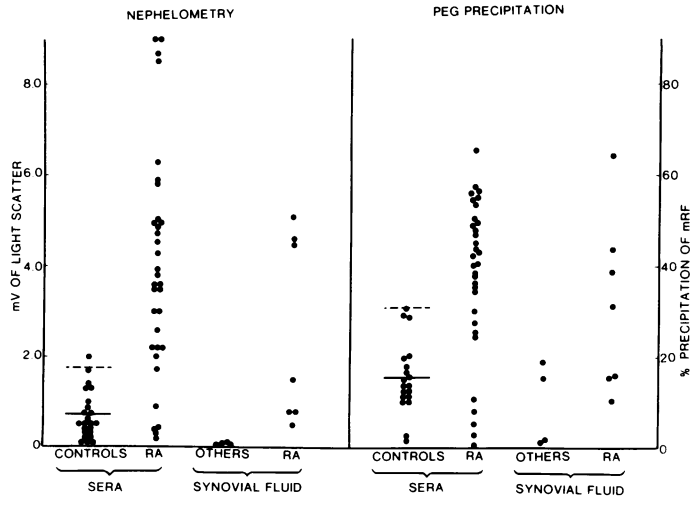

Fig. 2. The degree of light scatter and the percentage precipitation obtained between $mRF_{(A)}$ and sera from patients with rheumatoid arthritis and healthy controls and synovial fluid from patients with RA and other rheumatic diseases (2 gout, 1 Reiters, 1 psoriasis).

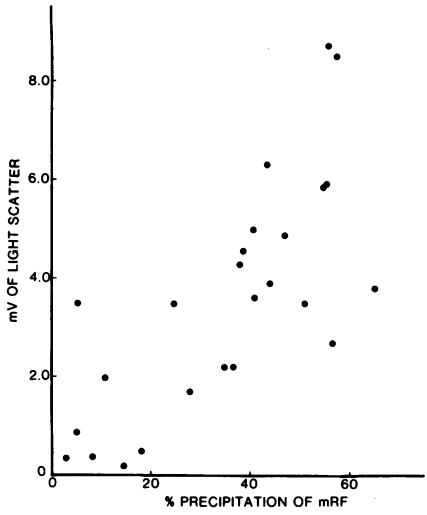

Fig. 3. Scattergram showing the significant correlation between the nephelometric mRF assay and the PEG mRF precipitation assay ($P < 0{\cdot}001$).

both mRFs was also compared. A significant correlation was observed ($r = 0{\cdot}45$, $P = 0{\cdot}02$) between these two monoclonal reagents. However, $mRF_{(A)}$ gave higher levels of light scatter than $mRF_{(D)}$ and this finding, together with the chromatographic studies with HAGG, suggested that $mRF_{(A)}$ was reacting additionally with smaller IgG aggregates and uncomplexed IgG in many of the sera.

Size of Material Reacting with mRF

Nephelometric mRF Assay. Normal and pathological sera were separated by sucrose gradient ultracentrifugation in neutral and acid buffers. Fractions were collected (neutralized if necessary), and the protein (OD_{280}), IgM, IgG, and reactivity against $mRF_{(A)}$ and $mRF_{(D)}$ determined. All sera were processed fresh (within 5 hr of collection). Examples are shown in Fig. 5. Gradient fractions from normal serum in neutral or acidic buffers showed no reactivity with both mRFs (panel 1, Fig. 5). However, in pathological sera the mRFs reacted preferentially with IgG sedimenting in the monomeric IgG position in neutral buffer. When centrifugation was repeated in acid buffer and the

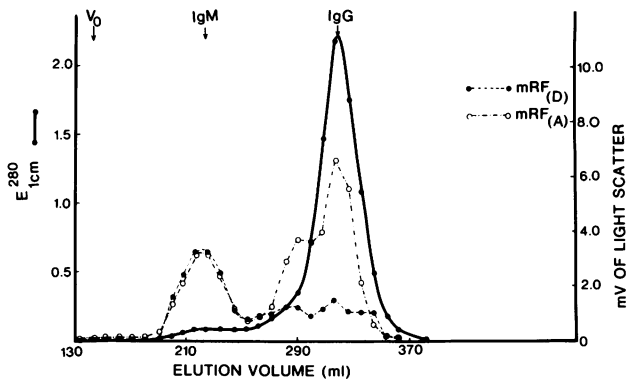

Fig. 4. The optical density profile of HAGG ●——● after Sepharose 6B chromatography. Fractions were assayed nephelometrically for reactivity with $mRF_{(A)}$ O– – –O and $mRF_{(D)}$ ●– – –●. The eluting positions of monomeric IgG, IgM and void volume (V_o) are indicated.

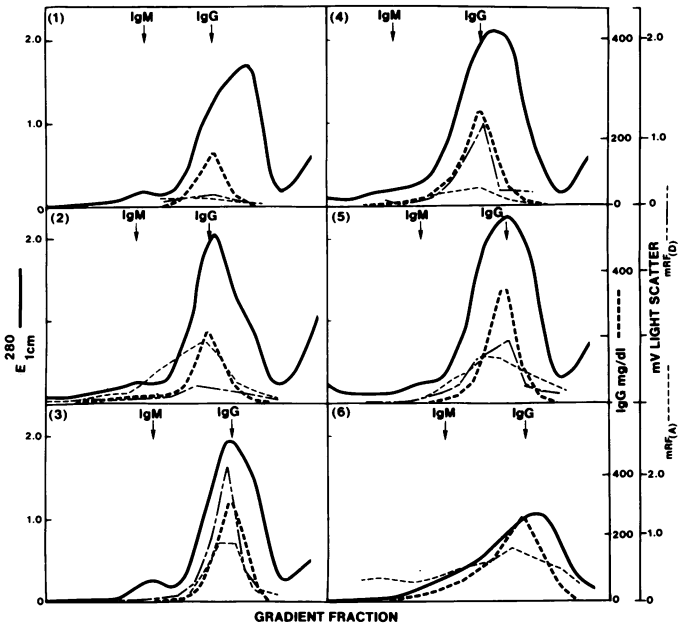

Fig. 5. Sucrose gradient ultracentrifugation profiles for normal and pathological sera. The sedimenting positions of monomeric IgG and IgM are indicated. In panel 1 the ultracentrifugation profiles for normal serum in neutral buffer are shown. In panel 2 (neutral buffer) and panel 3 (acidic buffer) the profiles obtained with a serum obtained from a patient with SLE are shown. In panel 4 (neutral buffer) and panel 5 (acidic buffer) the profiles obtained with a serum obtained from a patient with RA are shown while panel 6 demonstrates the profiles in neutral buffer obtained from a patient with rheumatoid hyperviscosity syndrome.

fractions neutralized, both mRFs continued to react with this uncomplexed monomeric IgG. In addition in one sera (panel 2, Fig. 5), some reactivity with heavier sedimenting IgG was also apparent in neutral buffer (particularly with $RF_{(A)}$). This reactivity together with the heavier sedimenting IgG disappeared after centrifugation in acid buffer (panel 3, Fig. 5) suggesting the presence of small immune complexes. However, even in this serum in both neutral and acid buffers, maximal reactivity with mRF was observed in the monomeric IgG position. Both mRFs failed to react with fractions containing large amounts of IgG–RF intermediate complexes (panel 6, Fig. 5). Serum containing these complexes (stored at $-70°C$) was obtained from a patient with the rheumatoid hyperviscosity syndrome and the clinical and laboratory features of this patient will be described elsewhere.

Radiolabelled mRF precipitation assay. Normal and pathological sera were separated on Sepharose 6B and the fractions collected and assayed for protein (OD_{280}), IgG, IgM and percentage precipitation with $mRF_{(A)}$. All sera were fresh and studied within 5 hr of collection. Synovial fluid had been frozen at $-70°C$ and stored for periods up to 2 months. Six illustrative chromatographic profiles of sixteen sera and five synovial fluids studied are shown in Fig. 6. The percentage precipitation of $mRF_{(A)}$ profile coincided with monomeric IgG profile in both normal and pathological sera in the majority of sera studied. In pathological sera higher levels of precipitation were generally seen and in one serum (panel 3, Fig. 6) some precipitation occurred in the intermediate range coinciding with the shelf of higher-sized IgG. Varying amounts of precipitation was also observed in high molecular regions in two additional rheumatoid vasculitic sera which had been frozen at $-20°C$ for periods greater than 3 months, but no such high molecular reactivity was seen in any of the fresh serum specimens studied including specimens obtained from patients with florid rheumatoid vasculitis and active SLE. Fractionated synovial fluid showed mRF reactivity in both high and low molecular weight regions (panels 2 and 4, Fig. 6). Fresh synovial fluid was not

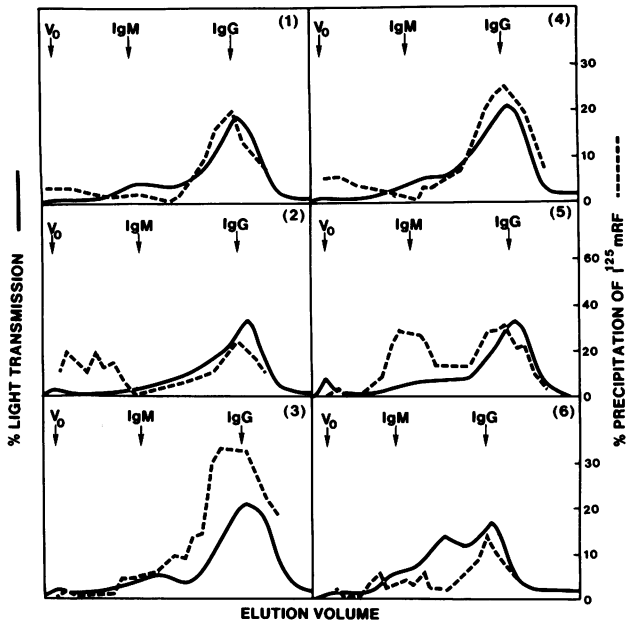

Fig. 6. Sepharose 6B chromatographic profiles obtained for normal and pathological sera. Normal serum is shown in panel 1, RA synovial fluid in panels 2 and 5, RA serum in panel 4, SLE serum in panel 3, and panel 6 demonstrates the findings for a serum obtained from a patient with the rheumatoid hyperviscosity syndrome. The eluting position of IgG, IgM and void volume (V_o) is indicated.

studied. As with the mRF nephelometric assay the $mRF_{(A)}$ radiolabelled precipitation assay also failed to precipitate with fractions containing large quantities of IgG–RF intermediate complexes (panel 6, Fig. 6).

DISCUSSION

These results show that two different mRFs used in both a radiolabelled precipitation assay and a nephelometric assay are not specific in detecting IgG containing immune complexes but also react with unaggregated or monomeric IgG. This latter form of reactivity is particularly prominent in RA sera (Roberts-Thomson & Bradley (1979) and is not eliminated after separation of the sera in low pH sucrose gradients (in which small IgG complexes would disassociate). However, the nature of this reactive monomeric or uncomplexed IgG is unknown. As previously suggested (Roberts-Thomson & Bradley (1979) it is unlikely to be a particular IgG allotype since the preferential reactivity is not seen in a large study of normal sera and the mRF reagents shows a broad reactivity with a variety of other mammalian IgG. Monoclonal RF will react with monomeric IgG in normal human sera if present in sufficiently high concentration but this explanation is unlikely to explain the selective reactivity with monomeric IgG in RA serum since IgG levels are not particularly elevated in this disease (Zawadzki, 1971). Our previous studies have shown only a weak correlation between mRF reactivity and IgG levels (Roberts-Thomson & Bradley 1979). A possible explanation is that mRF is reacting with monomeric IgG which is conformationally altered. This form of IgG has been described in RA sera and steric changes are thought to occur in the hinge region (Johnson & Watkins, 1974). The antigen determinants for different mRFs include both the second and third domain of IgG more particularly the second domain, but the precise locations are unknown (Johnson & Faulk 1976). It is possible therefore that certain mRFs could react with this form of sterically altered IgG.

A possible corollory to the explanation given above is that mRF might react with monomeric IgG that contains small fragments of C3 (Pereira, Theofilopoulos & Dixon, 1980). This form of IgG has been found in the majority of patients with RA, particularly in those with less severe forms of the disease, and is also found in other rheumatic diseases. It does not disappear after sucrose gradient ultracentrifugation in acid buffers (compare mRF reactivity) and also reacts with conglutinin and antisera to C3 demonstrating the presence of bound fragments of C3. It is presently not known whether this form of IgG is also reactive with C1q, rheumatoid factors or cell Fc receptors. From our previous studies (Roberts-Thomson & Bradley 1979; Roberts-Thomson *et al.*, 1980) mRF reactivity does not correlate with other indices of severe or active disease and its persistence after sucrose gradient ultracentrifugation in acid buffers shown in the present study suggest that mRFs could be reacting with the same monomeric IgG identified by Pereira *et al.* (1980).

In comparing the radiolabelled precipitation assay with the nephelometric assay it was observed that although the former assay was more sensitive in detecting HAGG, the latter assay was more discriminating when studying RA sera. A possible explanation for this apparent enigma may involve the use of PEG in the radiolabelled precipitation assay. We, and others (Polseon *et al.* 1964; Creighton, Lambert & Miescher, 1973), have found that the percentage precipitation of mRF or other radiolabelled ligand varied according to the concentration of protein present and the degree of non-specific protein precipitation induced by 2·5% PEG. Non-specific protein precipitation is dependent on both size and quantity of protein present and these may vary considerably in both control and pathological sera. Thus with certain sera, elevated levels of total protein or the presence of high molecular weight protein such as IgM or haptoglobins of the phenotype 2-1 or 2-2, would result in enhanced precipitation.

Using either the radiolabelled or nephelometric mRF assay we could not detect high molecular weight complexed IgG ($> 10^6$ daltons) when more than 30 fresh RA and SLE sera were examined. Such complexes were, however, detected in rheumatoid sera which had been stored at $-20°C$ for long periods of time. Possibly these large complexes of IgG were artificially created in certain sera, perhaps by the prolonged storage and by freezing and thawing. This effect therefore behoves investigators in immune complex diseases to study carefully the effects of serum freezing and storage before equating the detection of such high molecular IgG complexes with the presence of these complexes *in vivo*.

These studies have shown that the mRF assay is not specific for circulating immune complexes. This finding may partly explain the large variance found between this assay and other assays for immune complexes such as the C1q binding assay (Roberts-Thomson *et al.*, 1980). However, the nature of the reactive monomeric IgG detected in the mRF assay is unclear. The possibility that this material has important biological activities is interesting and requires further clarification.

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