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LOW-MOLECULAR-WEIGHT RHEUMATOID FACTOR *

BY W. B. CHODIRKER † AND T. B. TOMASI, JR. ‡

(From the Division of Experimental Medicine, University of Vermont College of Medicine,
Burlington, Vt.)

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The interaction of rheumatoid sera with gamma globulin has been known for many years. This reaction, detectable by a variety of serological tests (sensitized sheep cell agglutination, latex fixation, FII precipitin, etc.), has been found, in recent years, to be mediated by a macroglobulin commonly called the rheumatoid factor (2). Similar if not identical macroglobulins have been detected in the sera obtained from patients with a variety of chronic granulomatous and inflammatory diseases (tuberculosis, sarcoidosis, kala-azar, syphilis, etc.) (3-5). Although considerable clinical and experimental work has been done in this area, the significance of these factors remains uncertain. Widely held at the present time is the concept that the rheumatoid factor represents an antibody specific for gamma globulin in an altered form (6). In support of this concept are *a*) the striking physicochemical and immunological similarities between the rheumatoid factor and certain well-established macroglobulin antibodies (typhoid-O agglutinins, antipneumococcal antibody, isoagglutinins, etc.) (7); *b*) the plasma cell origin of the rheumatoid factors (8); and *c*) the marked specificity shown by the closely related anti-Gm factors for the genetically determined gamma globulins (9). Very little is known, however, of the antigenic stimulus and the immunizing events, deficiencies which have retarded acceptance of the rheumatoid factor as an antibody in the classic sense.

Recently, complexes of gamma globulin with sedimentation coefficients between 7 and 19 S have

been found in the sera of patients with rheumatoid arthritis (10), Sjögren's syndrome, and idiopathic pulmonary fibrosis (11). Kunkel, Müller-Eberhard, Fudenberg, and Tomasi (10) found these complexes in the euglobulin fraction of the serum and showed that they were dissociated to 7 S subunits by acid buffers and 4 to 6 M urea, but not by sulfhydryl reagents. Since many of the properties of these complexes were similar to those of the 22 S complexes frequently seen in rheumatoid sera, it was suggested that the intermediate complexes represented the *in vivo* interaction of gamma globulin with 7 S rheumatoid factor.

The existence of a low-molecular-weight rheumatoid factor analogous to the 7 S counterparts of the classical macroglobulin antibodies would constitute further presumptive evidence for the antibody nature of the rheumatoid factor.

The results of this investigation indicate the existence of two varieties of rheumatoid factor in some rheumatoid sera having intermediate complexes. One is the sulfhydryl-sensitive 19 S rheumatoid factor, the other a low-molecular-weight factor that is stable in the presence of sulfhydryl reagents.

MATERIALS AND METHODS

Twelve serum samples obtained from eight patients were studied. The sera, obtained from several sources,¹ are listed in Table I. All had high titers of rheumatoid factor in the latex fixation test. Six of the sera were obtained from patients with rheumatoid arthritis, three with intermediate complexes (Em, Do, and Sn), and three with no complexes (Db, Dn, and Bc). Three of the rheumatoid sera were obtained from cases complicated by Felty's syndrome (Em), Sjögren's syndrome (Sn), and pulmonary fibrosis (Bc). None of the rheu-

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† Fellow, Canadian Arthritis and Rheumatism Society.

‡ Senior Investigator, Arthritis and Rheumatism Foundation.

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TABLE I

Summary of clinical and laboratory data of patients studied

Patient	Age	Diagnosis	Serum date	Int. comp.	Total protein	Gamma globulin	Latex fixation titer (reciprocal)
	years			0 to 4+	g/100 ml	g/100 ml	
Em	61	Rheumatoid arthritis	July '61	4	8.1	3.57	81,920
		Leukopenia	Aug. '61	3	6.8	2.90	20,480
		Hepatomegaly	Jan. '62	1	6.1	1.56	1,280
		Splenomegaly	June '62	3	7.3	3.12	20,480
Do	28	Rheumatoid arthritis		3	7.6	2.45	640,000
Sn	58	Rheumatoid arthritis	Aug. '61	2	5.8	1.63	1,300,000
		Sjögren's syndrome	Oct. '61	1	7.5	1.79	20,480
Db	35	Rheumatoid arthritis		0	7.3	1.32	20,480
Dn	56	Rheumatoid arthritis		0	6.8	1.51	10,240
Bc	71	Rheumatoid arthritis					
		Pulmonary fibrosis		0	7.5	3.0	20,480
Yd	65	Hepatic cirrhosis		0	9.4	5.26	10,240
Be	59	Waldenström's macroglobulinemia		0	7.4	3.63	10,240*

* Rheumatoid factor activity determined by latex fixation test performed at pH 7.0.

matoid sera demonstrated a cryoglobulin precipitate, or contained complement-fixing antibodies to human lung, liver, or kidney antigens, or to calf thymus nuclei.

Also included in this study were serum Yd from a patient with hepatic cirrhosis and serum Be, previously studied by Kritzman, Kunkel, McCarthy, and Mellors (12), from a patient with Waldenström's macroglobulinemia. Both were hypergammaglobulinemic with high latex fixation titers and cryoglobulin precipitates.

Analytical ultracentrifugation was performed with the Spinco model E ultracentrifuge as described previously (2). Centrifugation was at 52,640 rpm and 20.0° C. A 1 to 4+ scale was used to semiquantitate the amounts of intermediate complexes present.

Total serum proteins were determined from measurements of refractive indexes with the temperature-com-

pensated American Optical T.S. meter. Protein determinations were carried out according to the modified Folin-Ciocalteu method (13). Gamma globulin was quantitated from the total serum protein concentration and the percentage of gamma globulin on the paper electrophoretograms of whole serum.

Density gradient ultracentrifugation was carried out as described by Kunkel (14) in a Spinco model L ultracentrifuge with a swinging bucket rotor and a gradient of 10 to 40% sucrose. Centrifugation was allowed to proceed at 35,000 rpm for 16 to 18 hours. The gradient was collected in a series of 25 to 30 tubes through a small needle puncture in the bottom of the tube. The tubes pooled into 4 to 8 fractions were dialyzed exhaustively against phosphate buffer of pH 7.3, ionic strength 0.15.

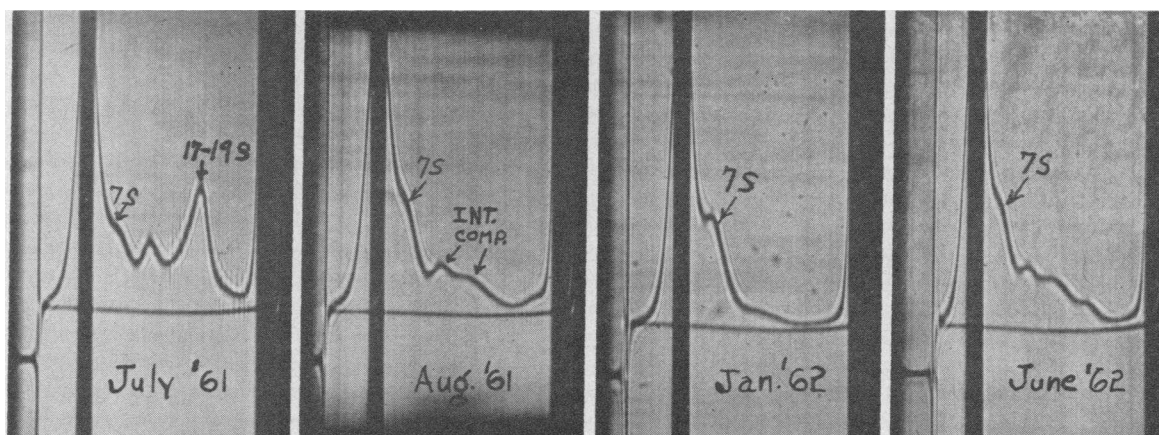


FIG. 1. ULTRACENTRIFUGAL PATTERNS OF FOUR SERUM SAMPLES OBTAINED FROM EM DURING ONE YEAR. Centrifugation proceeds from left to right.

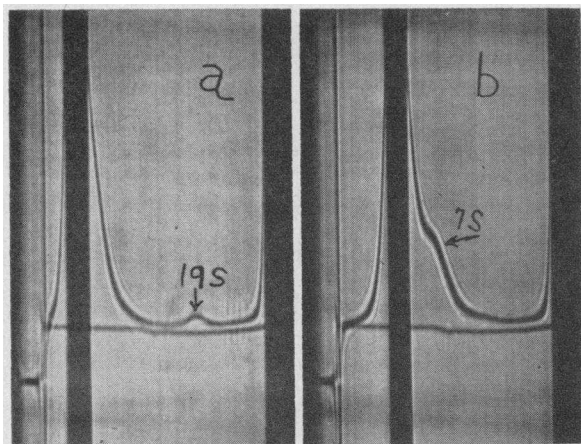


FIG. 2. ULTRACENTRIFUGAL PATTERN OF SERUM DB AT (a) 48 MINUTES AND (b) 80 MINUTES OF CENTRIFUGATION. Centrifugation proceeds from left to right.

Column chromatography was carried out as described by Fahey (15). One ml of whole serum dialyzed against the initial buffer was applied to columns (0.90 cm i.d.) containing 3 g of DEAE cellulose² packed under 5-pound pressure to a height of 31 cm. In two instances (columns Do and Be), 75 and 25 mg, respectively, of a euglobulin preparation were chromatographed. A continuous gradient of decreasing pH and increasing ionic strength with an initial 0.03 M phosphate buffer of pH 7.3, and a final 0.4 M buffer of pH 4.8 was employed.

Disulfide bonds were reduced by allowing samples of serum, made 0.1 M with respect to 2-mercaptoethanol, to stand at room temperature in tightly stoppered tubes for 24 hours. After prolonged dialysis against 0.02 M iodo-

²DEAE cellulose was purchased from Brown Inc., Berlin, N. H.

acetamide in 0.85% saline, rheumatoid factor activity was determined. Control sera were treated as above except that 0.85% saline was added instead of 2-mercaptoethanol.

The latex fixation test of Singer and Plotz (16) was used to determine rheumatoid factor activity in the density gradient and chromatographic fractions and in the disulfide reduction experiments.

Double diffusion in 1% agar gel was performed as described previously (17) with rabbit antisera to localize the albumin and 7 S and 19 S gamma globulins in the density gradient and chromatographic experiments.

RESULTS

Analytical ultracentrifugation. A considerable variation in ultracentrifuge patterns, protein concentrations, and latex fixation titers of the four serum samples obtained from Em over a period of one year is seen in Figure 1 and Table I. A broad distribution of proteins with sedimentation constants greater than 7 are noted in the serum of July, 1961. The sharp peak of 17 to 19 S in this sample was not present in any of the other samples studied. After a prostatectomy complicated by an *E. coli* septicemia, the serum of January, 1962, showed a marked diminution of intermediate complexes coincident with a near-normal gamma globulin (1.56 g per 100 ml) and a much lower latex fixation titer (1/1,280). The sera of August, 1961, and June, 1962, had very similar ultracentrifuge patterns, protein concentrations, and rheumatoid factor activities. Serum Sn also showed considerable variability in the amount of

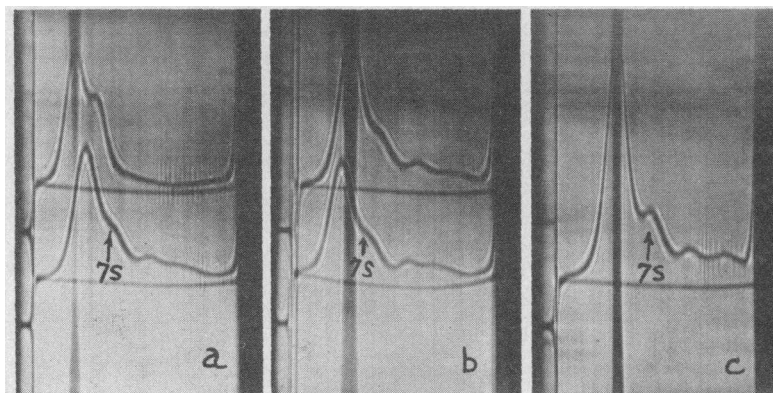


FIG. 3. EFFECT OF ACID AND 2-MERCAPTOETHANOL ON THE ULTRACENTRIFUGE PATTERN OF SERUM EM. a) Untreated (bottom), after dialysis against glycine-saline buffer of pH 3.0 (top). b) Untreated (top), after dialysis of acidified serum against phosphate buffer of pH 7.3 (bottom). c) Serum made 0.1 M with respect to 2-mercaptoethanol. Centrifugation proceeds from left to right.

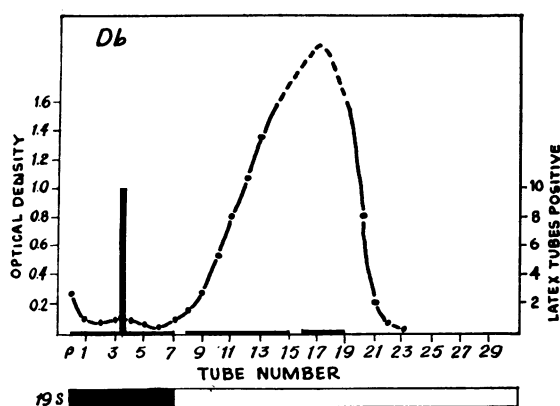


FIG. 4. DENSITY GRADIENT PATTERN OF SERUM DB. Horizontal bars at base line indicate the pooled tubes. Vertical bars indicate tubes positive in latex fixation test. Location of immunologically detected 19 S gamma globulin indicated below.

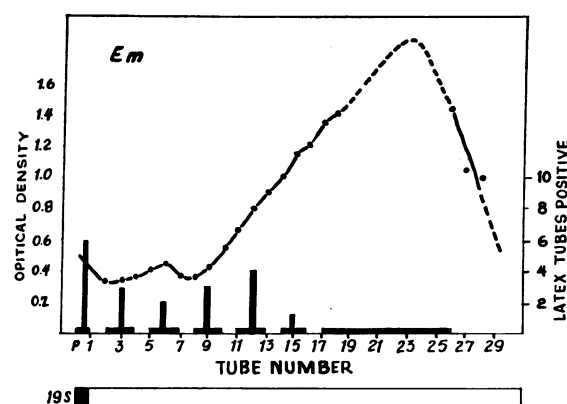


FIG. 5. DENSITY GRADIENT PATTERN OF SERUM EM. Horizontal bars at base line indicate the pooled tubes. Vertical bars indicate tubes positive in latex fixation test. Location of immunologically detected 19 S gamma globulin indicated below.

intermediate complexes and rheumatoid factor activity in different serum samples. The ultracentrifuge pattern of serum Db, seen in Figure 2, contained no intermediate complexes. Sera Dn and Bc were similar to Db except that Bc had a 22 S complex. Serum Yd showed an elevation of 7 S proteins, but no intermediate complexes. Serum Be showed a high concentration of a homogeneous protein representing the abnormal macroglobulin (18.5 S). No intermediate complexes were present.

Effect of acid, alkali, and 2-mercaptoethanol. The effect of acid and 2-mercaptoethanol on the protein pattern of serum Em is seen in Figure 3. After dialysis against 0.1 M glycine-saline buffer

of pH 3.0, a marked decrease in the amount of intermediate complexes and a corresponding increase in 7 S gamma globulin was noted (Figure 3a). Dialysis of the acidified serum against phosphate buffer of pH 7.3 resulted in the reformation of the complexes (Figure 3b); these were unchanged at pH 9.6, but completely dissociated at pH 10.6. No apparent change in pattern or amount of intermediate complexes was noted in serum made 0.1 M with respect to 2-mercaptoethanol (Figure 3c).

Density gradient ultracentrifugation. The distribution of rheumatoid factor activity in all experiments fell into one of two groups exemplified by the sera Db (Figure 4) and Em (Figure 5).

TABLE II
Results of density gradient, chromatographic, and sulfhydryl experiments

Latex fixation tubes positive											
Serum	Intermediate complexes	Density gradient				Chromatography				Disulfide reduction	
		19 S	Int.	7 S	Alb.	Fall-through	Pre-albumin	Albumin	Post-albumin	2-Mercaptoethanol	Control
	0 to 4+										
Em	3	6	2	4	0	4	0	9	7	6	10
Do	3	10	5	3	0	0	5	6	7	7	13
Sn	1	6	3	3	0	5			10	4	10
Db	0	10	0	0	0					0	10
Dn	0	8	0	0	0	0	0	0	7	0	9
Bc	0	8	0	0	0					0	10
Yd	0	4	0	0	0					0	10
Be	0	5	0	0	0	0*			+	0†	10

* Rheumatoid factor activity determined by FII precipitin test and recorded as present (+) or not present (0).

† Rheumatoid factor activity determined by latex fixation test performed at pH 7.0.

The rheumatoid factor activity of serum Db, which contained no intermediate complexes, was present at the bottom of the gradient where 19 S gamma globulin was concentrated. Serum Em, on the other hand, with 3+ intermediate complexes, contained rheumatoid factor activity well into the 7 S region of the gradient (tubes 9 to 20, approximately) where no 19 S gamma globulin was detected immunologically. The bimodal distribution of activity with peaks in both 7 S and 19 S regions is indicative of the presence of two sizes of molecules with rheumatoid factor activity.

The results of the density gradient experiments are tabulated in Table II. Experiments were repeated on several occasions with identical results. All sera with intermediate complexes (Em, Do, and Sn) contained rheumatoid factor activity in both 7 S and 19 S regions of the gradient. In sera with no intermediate complexes, the rheumatoid factor activity was present in the 19 S regions only.

Column chromatography. The chromatographic experiments are summarized in Table II. Two patterns of distribution of rheumatoid factor activity were noted. In serum Dn (Figure 6), the activity was found in the postalbumin eluate of the chromatograph (80 to 140 ml) where 19 S gamma globulin was present. In serum Em (Figure 7), however, a bimodal distribution of activity with peak activity in the initial protein eluate (fall-through, 20 to 42 ml), albumin (86 to 105 ml), and postalbumin regions is seen. 19 S

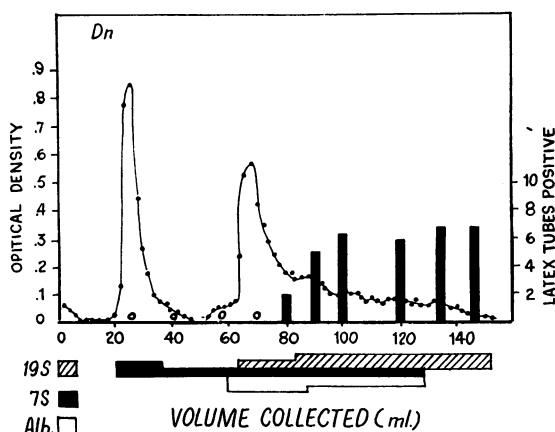


FIG. 6. CHROMATOGRAPHIC PATTERN OF SERUM Dn. Location of immunologically detected 7 and 19 S gamma globulin and albumin indicated below.

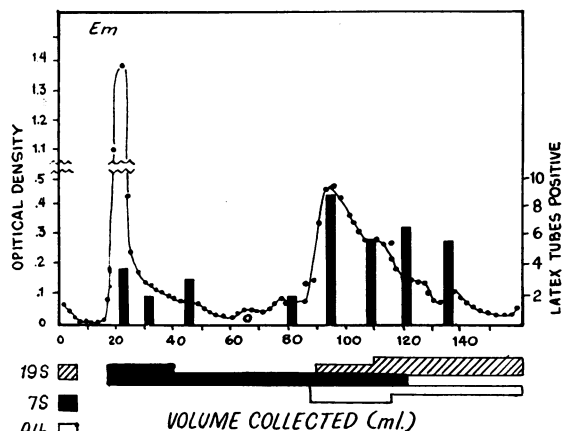


FIG. 7. CHROMATOGRAPHIC PATTERN OF SERUM Em. Location of immunologically detected 7 and 19 S gamma globulin and albumin indicated below.

Gamma globulin was not detected in any of the prealbumin fractions.

All active prealbumin fractions contained variable amounts of acid-dissociable intermediate complexes. The fall-through peak of serum Em (20 to 42 ml, approximately; Figure 8), contained 67% 7 S and 33% 10 S gamma globulin. The 10 S complexes dissociated completely into 7 S gamma globulin in acid buffers (pH 3.0).

Some variability of the chromatographs obtained from sera Em and Do was noted. The active complexes were eluted in either the fall-through peak, or prealbumin region, or in both

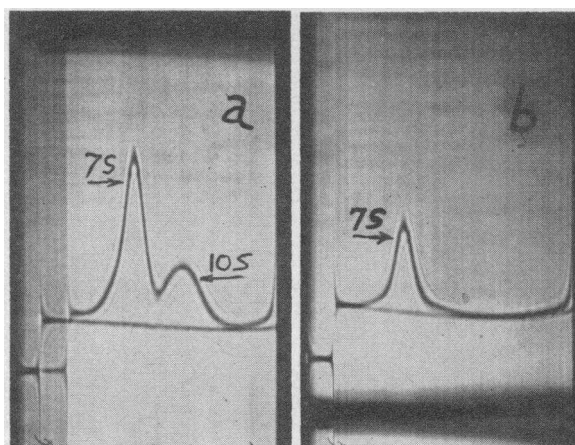


FIG. 8. ULTRACENTRIFUGE PATTERN OF THE FALL-THROUGH PEAK OF EM CHROMATOGRAM. a) Untreated and b) at lower concentration after dialysis against glycine-saline buffer of pH 3.0. Centrifugation proceeds from left to right.

of these fractions. The reason for this variability is not understood.

Effect of 2-mercaptoethanol on rheumatoid factor activity. In sera with no intermediate complexes, rheumatoid factor activity was completely abolished when made 0.1 M with respect to 2-mercaptoethanol. Considerable reactivity remained, however, in sera with intermediate complexes when similarly treated (Table II).

DISCUSSION

Three interpretations of the interaction of rheumatoid factor with gamma globulin have been proposed: *a*) nonspecific, *b*) complement-like, and *c*) antigen-antibody. It seems unlikely that the interaction is nonspecific in view of the apparent specificity of the rheumatoid factors for human, or animal gamma globulins, or both (18), and the recently demonstrated specificity of the anti-Gm factors for native gamma globulin of different Gm specificities (9). The affinity of rheumatoid factor for antigen-antibody complexes has suggested a complement-like action (19). The nonidentity of rheumatoid factor with any of the main components of complement, or with conglutinin, or properdin makes this hypothesis unlikely (20), although it does not exclude the possibility that the rheumatoid factor is a complement-like substance of a type not previously described.

Most widely held at the present time is the concept that the rheumatoid factor is an antibody against gamma globulin. This hypothesis is supported by the many similarities between rheumatoid factor and the high-molecular-weight antibodies (7). Both are gamma₁ globulins with approximately 10% carbohydrate and a molecular weight of about 1,000,000. Both are dissociable into 7 S subunits with sulfhydryl reagents, have antigenic determinants in common, and are synthesized by cells of the lymphocyte-plasma cell series (8).

Although the natural antigen is unknown, the increased reactivity of rheumatoid factor with aggregated gamma globulin suggests that the antigenic stimulus may be gamma globulin in an altered form. Antigen-antibody complexes with biological effects similar to those of aggregated gamma globulin (21) have been suggested as natural antigens (6). This concept is supported

by the findings of Milgrom and Dubiski (22) of rheumatoid factor-like substances in rabbits immunized with isologous antigen-antibody complexes. Rheumatoid factor-like substances have also been demonstrated in rabbits immunized with pure ovalbumin (23) and with *E. coli* grown on a synthetic medium (24). The immune complexes (ovalbumin-antiovalbumin, *E. coli*-anti-*E. coli*) produced in these animals presumably stimulated the production of rheumatoid-like factors. More difficult to understand, however, is the occurrence of anti-Gm factors in some rheumatoid sera that are specific for Gm types not present in those sera (25).

As a result of uncertainties such as these and the lack of more direct evidence concerning the natural antigen and immunizing events, the antibody nature of the rheumatoid factors can be considered suggestive, but not firmly established at the present time.

The finding of intermediate complexes in some rheumatoid sera by Kunkel and co-workers (10) led to a new approach to this problem. These workers suggested that the intermediate complexes may represent a low-molecular-weight rheumatoid factor, although they were unable to demonstrate any interaction between the isolated complexes and aggregated gamma globulin in the FII precipitin test, or in the Rh rheumatoid test. However, alterations in the pattern of the complexes produced by adding normal gamma globulin to the serum were reminiscent of antigen-antibody complexes formed in antigen excess.

The report by Meltzer and Franklin (26) of a single serum with acid dissociable intermediate complexes whose rheumatoid factor and cryoglobulin activity resided in the 7 S portion of the serum is consistent with this hypothesis. Also pertinent are the studies of Osterland, Harboe, and Kunkel (27) which demonstrated the agglutination of red blood cells sensitized with a pepsin-treated, incomplete, anti-Rh antibody by a 7 S component of rheumatoid sera. 19 S Rheumatoid factor agglutinated only red blood cells sensitized with untreated, incomplete, anti-Rh antibody.

In this study, a factor that interacts with human gamma globulin in the latex fixation reaction, but is stable in the presence of sulfhydryl reagents has been demonstrated in sera containing intermediate complexes. Hypergammaglobulinemia per

se was not related to the presence of low-molecular-weight rheumatoid factor, since these factors could not be demonstrated in serum Yd (elevated 7 S) or in serum Be (elevated 19 S), although both contained rheumatoid factor in high titer. The factor sediments in the 7 S and intermediate regions of a density gradient and in prealbumin chromatographic fractions. The intermediate complexes, readily dissociable in moderately acidic media, may consist of normal gamma globulins complexed with other gamma globulins containing the specific rheumatoid-factor combining sites. Low-molecular-weight rheumatoid factor activity, however, was demonstrable only in those chromatographic fractions containing intermediate complexes (10 to 14 S), and not in fractions consisting entirely of 7 S gamma globulin. The possibility exists, therefore, that the complexes and not their acid-dissociable products are the smallest molecules containing the specific rheumatoid-factor combining sites.

It may be postulated that molecules of gamma globulin capable of complexing with other gamma globulins are synthesized in some rheumatoid patients by processes that may or may not be immunologic in nature. Since the complexes are dissociable in moderate acid or alkali, but stable in the presence of sulfhydryl reagents, neither peptide nor disulfide bonds are involved in the complexing process. The interaction, therefore, probably involves electrostatic and possibly other forces, and results in both complex formation and perhaps the creation of the unique secondary and tertiary structure that contains the specific rheumatoid-factor combining sites.

A mechanism such as this is not unreasonable in view of recent findings concerning the relationship of structure to activity of the enzyme ribonuclease. Vithayathil and Richards (28) fractionated ribonuclease by the hydrolysis of a single peptide bond into a twenty-carbon peptide and a residual protein fragment. These products, by themselves inactive, interact and regain full activity when mixed together. In the presence of moderate acidity, however, the complex is dissociated with complete loss of activity, indicating the participation of noncovalent bonds in the determination of a structure appropriate to the formation of an active enzyme.

The stable titer of rheumatoid factor in the

sera of most cases of rheumatoid arthritis contrasts with the marked fluctuations in titer noted in sera obtained from several of our patients with intermediate complexes. That these fluctuations are due to changing amounts of 7 S rheumatoid factor is indicated by the parallel changes in the amounts of intermediate complexes. A rheumatoid factor titer that closely follows the disease activity is seen in a variety of infectious diseases (e.g., tuberculosis and subacute bacterial endocarditis) (3, 29). It seems possible, therefore, that changes in the activity of the rheumatoid process in those patients whose sera contained intermediate complexes may be reflected by the titer of rheumatoid factor. The marked decrease in intermediate complexes and latex fixation titer noted in serum Em in January, 1962 occurred after a transurethral prostatectomy and an *E. coli* septicemia. There was, however, no change in corticosteroid or other specific antirheumatoid therapy. It is possible that the interaction of *E. coli*-anti-*E. coli* complexes formed in this serum with the rheumatoid factors resulted in their elimination from the serum, and hence the marked decrease in intermediate complexes and rheumatoid factor found in the serum of January, 1962. The variation in rheumatoid factor found in serum Sn, however, was not attended by any change in therapy, or by any apparent change in clinical status. Clearly, a larger series is necessary before any relationship between disease activity and 7 S rheumatoid factor can be established.

Low-molecular-weight rheumatoid factor has been detected only in those sera containing intermediate complexes. It seems likely, however, that complexes are present in many rheumatoid sera in amounts too small to be demonstrable by analytical ultracentrifugation. In retrospect, small amounts of intermediate complexes have been found in many rheumatoid sera. It is difficult, however, to identify them with certainty with the complexes described here. An analogous situation is known to exist with respect to 19 S rheumatoid factor, which forms 22 S complexes in sufficient amount to be demonstrable by analytical ultracentrifugation only in those rheumatoid sera with high titers in the latex fixation reaction (2).

Attempts to isolate 7 S gamma globulins with rheumatoid factor activity from sera containing

intermediate complexes are currently in progress. Determination of the specificity or lack of specificity of the low-molecular-weight rheumatoid factors for antigen-antibody complexes, animal gamma globulins, and gamma globulins of different Gm specificities would also be of considerable interest. Preliminary data suggests that the 7 S rheumatoid factors are not reactive with rabbit gamma globulin in the sensitized sheep-cell agglutination reaction.

The existence of 7 and 19 S forms of rheumatoid factor, analogous to the low- and high-molecular-weight forms of many other antibodies, supports the concept of the antibody nature of these factors. It must be emphasized, however, that these findings constitute circumstantial, and not direct, evidence for this theory.

SUMMARY

The distribution of rheumatoid factor activity in sera with and without acid-dissociable intermediate complexes has been studied by means of density gradient and analytical ultracentrifugation, column chromatography, and disulfide bond reduction experiments.

In sera with intermediate complexes, a bimodal distribution of activity noted in density gradient and chromatographic experiments indicates the presence of a low-molecular-weight rheumatoid factor in addition to the classical 19 S type. Treatment with 2-mercaptoethanol completely inactivated control rheumatoid and hypergamma-globulinemic sera, but considerable activity remained in similarly treated sera with intermediate complexes. Considerable variability in the amount of intermediate complex and in the titer of rheumatoid factor was demonstrated in several sera drawn at different times.

The possible significance of these findings in terms of the antibody nature of the rheumatoid factors is discussed.

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