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GAMMA GLOBULIN COMPLEXES IN SYNOVIAL FLUIDS OF PATIENTS WITH RHEUMATOID ARTHRITIS

PARTIAL CHARACTERIZATION AND RELATIONSHIP TO LOWERED COMPLEMENT LEVELS

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SUMMARY

Gamma globulin complexes were demonstrable in certain joint fluids from patients with rheumatoid arthritis by analytic and density gradient centrifugation. They form a continuum of high molecular weight components ranging from 7S to 30S and were dissociable primarily to 7S γG globulin. The larger complexes were also detectable by precipitation reactions with C1q and with γM rheumatoid factor. This permitted the isolation and partial characterization of the complexes. Non-immunoglobulin constituents were not detectable. Evidence was obtained that 7S γG globulin rheumatoid factors represented an important constituent of the complexes.

A relationship was encountered between the amount of γ globulin complex present in the joint fluids and diminution in total haemolytic complement activity. All fluids with abundant γ globulin complexes contained markedly lowered complement levels. A decrease in levels of C1q and β_{1A} was found to correlate with the amount of γG globulin complexes. Although patients who had diminished complement levels in their joint fluid have serum γM rheumatoid factor, its titre does not correlate well with the extent of complement depression.

Joint fluids with abundant γ globulin complexes manifested an anticomplementary effect. This activity was most apparent at 37°C when fresh rheumatoid serum was used as a source of complement. Evidence indicating the participation of γM rheumatoid factor in this anticomplementary effect was obtained. All fluids with significant anticomplementary activity formed precipitin bands with C1q on agarose plates. γM rheumatoid factor was not necessary for this reaction.

INTRODUCTION

The possibility that immune reactions are involved in the pathogenesis of rheumatoid arthritis has received considerable attention in recent years. In certain patients the documen-

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tation of diminished synovial fluids haemolytic complement, despite the presence of normal or elevated serum levels, suggested that localized immune processes were occurring within the joint. Furthermore, the pattern of depletion of the first four components of complement was consistent with fixation and activation of the complement system by immune complexes or aggregates (Pekin & Zvaifler, 1962; Hedberg, 1963; Pekin & Zvaifler, 1964; Fostiropoulos, Austin & Bloch, 1965; Ruddy *et al.*, 1969). Also evidence was advanced for the presence of breakdown products of C3 in joint fluids (Zvaifler, 1969).

Immunopathologic studies have revealed substantial deposition of γG globulin, often in association with some complement components or γM in the matrix of the synovium and within certain phagocytic cells (Kaplan & Vaughan, 1962; Fish *et al.*, 1966; Rodman *et al.*, 1967; Rawson, Abelson & Hollander, 1965). The appearance of complement components in the phagocytic cells has been correlated with their diminution in the synovial fluid (Ruddy *et al.*, 1969). However, the nature of immune complexes or their equivalent capable of initiating these reactions remained obscure.

A feature characteristic of rheumatoid arthritis sera and joint fluids is the presence of γ globulin complexes which can be visualized by ultracentrifugation analysis (Kunkel *et al.*, 1961). Evidence was obtained that they were composed primarily of γG globulin and consisted of γG-anti γG complexes. While these have been investigated primarily in the serum, recently renewed interest has centred on similar complexes in the joint fluids of rheumatoid arthritis patients (Hannestad, 1967). The complexes are demonstrable by a variety of means, either through their interaction with γM rheumatoid factor to form precipitates (Hannestad, 1967) or upon analytic ultracentrifugation (Winchester & Kunkel, 1968). Their concentration within the joint is appreciably greater than in the serum suggesting the possibility of their local production. The components precipitable by rheumatoid factor have been considered to be aggregates of γ globulin (Hannestad, 1967).

In the present study additional observations on the nature of the complexes in the joint fluid are described, demonstrating the presence in them of enriched concentrations of 7S γG rheumatoid factor. An association was encountered between the quantity of such complexes and a diminution in the level of total haemolytic complement as well as various individual components of the complement system (Winchester, Agnello & Kunkel, 1969a, b). Evidence was obtained that these same fluids interacted directly with the complement system. Direct precipitation with C1q and an anticomplementary effect were found in joint fluids with abundant γ globulin complexes.

MATERIALS AND METHODS

Synovial fluid samples were treated with hyaluronidase and stored at 4°C. Aliquots for complement studies were stored at 60°C. Density gradient and analytic ultracentrifugation were performed according to methods described previously (Kunkel, 1960). Serum and joint fluid were diluted with isotonic saline to equal total protein concentrations for these studies. Gel filtration was performed in a 100 × 2·5 cm column of G-200 Sephadex, 270 to 320 mesh, equilibrated with 0·1 M sodium acetate buffer, pH 4·1. The mean K_{AV} of purified 7S γG globulin was 0·166 (Laurent & Killander, 1964). Heat aggregation of γ globulin, and euglobulin fractionation of joint fluids were performed according to methods in use in this laboratory (Kunkel *et al.*, 1961). 0·001 M phosphate buffer pH 6·8 was used to obtain the euglobulin. Concentrations were carried out in 23/32 Visking dialysis tubing by pervaporation against Sephadex at 4°C.

Measurement of complexes

Quantitative precipitin curves with isolated 19S rheumatoid factor at 1 mg/ml were performed with increasing quantities of joint fluid at constant volume. Dilution controls were included for each point. The resulting maximum ordinate value was divided by the total protein to give milligrams of precipitate per gramme of joint fluid protein. Double diffusion studies by the Ouchterlony technique were performed in 0·5% agar–agar in pH 7·2 phosphate buffered saline for testing the reactivity of joint fluids with rheumatoid factor or aggregated γ globulin. Here purified γM rheumatoid factor was used at 1·5 mg/ml.

Precipitation experiments with joint fluids and C1q were performed as previously described (Agnello *et al.*, 1969). C1q was obtained from plasma by DNA precipitation and DNAase treatment of the precipitate. It was further purified by gel filtration and preparative electrophoresis according to the methods of Müller-Eberhard (1968). For preparative precipitation with C1q, the joint fluid was dialysed against 0·065 M pH 8·6 sodium barbital buffer, 0·01 M in EDTA. Sufficient C1q was added for complete precipitation of the complexes as determined by testing the supernate with γM rheumatoid factor. The precipitate was initially dissociated in 0·3 μ sodium phosphate buffer, pH 5·3, for partial characterization and thereafter in pH 4·1 acetate buffer for gel filtration.

The cells for haemagglutination inhibition were incubated with serum Ripley for 1 hr at 37°C and 24–48 hr at 4°C prior to washing. As agglutinator a rheumatiod serum, Dr, was used throughout. One drop of the sample to be tested was reduced for 3 hr, or longer if necessary to destroy γM rheumatoid factor activity. Alkylation was not found to be necessary. Dilutions of the inhibitor agglutinator and coat were added successively on flocculation trays and readings made at 15 and 30 min.

Measurement of rheumatoid factors

Haemagglutination with Ripley coated erythrocytes was performed by methods described previously (Osterland, Harboe & Kunkel, 1963). The FII latex agglutination test was according to Singer & Plotz (1956). Purified aggregated Fraction II was used on flocculation trays according to the method of Schrohenloher (1966), γG rheumatoid factor activity was assessed from the 7S fraction obtained on gel filtration of serum or joint fluid. The γG rheumatoid factors were also estimated by the method of Torrigiani & Roitt (1967) employing both rabbit or human cross-linked γ globulin. Standard curves were constructed with purified 7S γG rheumatoid factor as well as aggregate free Cohn Fraction II.

Gamma M rheumatoid factors were isolated by the method of Schrohenloher, Kunkel & Tomasi (1964). To isolate 7S γG rheumatoid factor the γG fraction obtained by these means was repetitively absorbed on and eluted from heat aggregated human γ globulin insolubilized by bisdiazotized benzidine (BDB). The absorption was begun at pH 4·0 for the first 30 min and then over the next 30 min the mixture was brought to neutral pH. All washing was carried out strictly at 4°C.

Complement determination

Haemolytic complement was determined by the method of Kent & Fife (1963). Percentage of expected complement activity was calculated according to Hedberg (1967). The level of complement was corrected by dividing by the total protein to give CH_{50} units per 10 mg of joint fluid protein.

Quantitation of C3 as β_{1A} was performed by radial immunodiffusion on 'Partigen' plates

purchased from Behringwerke. A stabilized normal serum batch 263 AV with the concentration of β_{1A} 0·55 mg/ml was employed as a standard. C1q levels were determined using absorbed specific rabbit anti C1q serum in single radial diffusion technique. The standard samples of C1q were quantitated by analytic ultracentrifugation and Kjeldahl determination. Both components of complement were corrected to milligrams per gramme of joint fluid protein.

The anticomplementary activity of joint fluids was assayed without prior heat inactivation of complement components to avoid denaturation of γ globulin. Joint fluids in general were aged at 4°C for 1–2 weeks. The fresh human sera employed as a source of complement came from normal subjects or patients with classical rheumatoid arthritis. Equal volumes of fresh human serum and joint fluid were mixed and incubated for 1 hr at 37°C or 18 hr at 4°C. Results were expressed as the difference in CH_{50} units between the value in the incubated mixture and the sum of values from similarly diluted, incubated, but unmixed controls. No correction was made for varying total protein contents.

Patient Lo

Considerable studies dealing with the characterization of the complexes were performed on joint fluid from patient Lo. This 56-year-old woman has had severe classic rheumatoid arthritis for 22 years. On five occasions, over a 9-month period, aspiration of joint fluid from the knees revealed absent haemolytic complement activity and abundant γ globulin complexes.

RESULTS

1. *Studies on γG globulin complexes*

Previous studies have indicated certain joint fluids contain γ globulin in a form precipitable by γM rheumatoid factor. In view of these findings several systems were employed which permitted some reproducible quantitation of these γ globulin complexes for the purpose of establishing comparisons. The complexes were measured as maximal precipitable protein or by the haemagglutination inhibition method and the results are indicated in Table 1.

The use of isolated γM rheumatoid factor instead of whole serum simplified the measurements on complexes in the precipitin system. However in certain fluids, such as Fe, the presence of marked spontaneous precipitation on dilution prevented quantitation by this precipitation system.

Density gradient ultra-centrifugation indicated that complexes larger than approximately 15S gave precipitin reactions while the inhibition system detected both complexes of this size and those in lighter fractions. However aggregate free 7S γG did not inhibit. The system was not inhibited by reduced γM rheumatoid factor.

In general the amount of γ globulin complex as measured by these means varied independently of the γM rheumatoid factor titre in either the serum or the joint fluid, as is apparent in Table 1. Marked elevations of rheumatoid factor were not encountered in the presence of large amounts of complexes.

Ultracentrifugation

Joint fluids containing complexes have a wide variety of unusual components detectable on analytic ultracentrifugation. Forty fluids were studied and, in all instances where joint

TABLE 1. Amounts of γ globulin complex measured by precipitation with rheumatoid factor and by inhibition of rheumatiod factor agglutination compared to rheumatiod factors titres, complement components and total complement in joint fluids of patients with rheumatoid arthritis and other diseases

Patient	Diagnosis	Precipitate (mg/g)	Inhibition (1/titre)	Total protein (g%)	Joint fluid rheumatoid factor (1/titre)	Serum rheumatoid factor (1/titre)	C1q (mg/g)	β_{1A} (mg/g)	Total complement CH_{50} units/10 mg
Ce	RA	18	16,000	4·0	1,000	1,000	0·9	2·8	0
Lo	Ra	15	16,000	5·7	0	64	0·7	1·9	0
Mu	RA	13	4,000	5·0	256	4,000	0·7	2·2	0
We	RA	12	4,000	6·1	0	0	1·6	2·5	2·5
Jo	RA	8·4	1,000	2·4	4,000	64,000	1·0	2·5	0
Ma	RA	8·0	4,000	4·4	256	16,000	2·5	8·2	3·4
In	RA	6·5	1,000	4·0	64		0·7	3·5	0
Ea	RA	6·4	16,000	6·0	256	1,000	1·8	1·8	0
Fe	RA	—	256	5·6	16,000	16,000	1·9	8·4	12
Co	RA	3·6	64	4·6	16		3·6	8·1	20
Ka	RA	3·3	256	4·9	256	1,000	2·9	8·8	13
Wt	RA	NT	256	3·9	1,000,000	1,000,000	2·6	8·3	15·5
Al	RA	1·6	64	4·4	1,000	1,000	2·3	3·6	16
Pa	RA	1·6	64	5·0	64,000	64,000	2·0	6·8	16
Be	RA	1·0	64	6·0	16,000	16,000	2·0	6·6	11
Re	RA	0·8	64	3·8	64	256	3·1	3·4	27
Gr	RA	0·7	16	4·2	256	1·000	2·5	11·1	31
Mo	RA	0	16	1·8	64	1,000	2·5	7·2	25
Zi	RA	0	16	5·2	4,000	4,000	3·1	5·8	19
Ha	Gout	0	16	5·4	0	0	2·8	11	22
He	OA	0	16	5·0	0	0	2·9	8·6	23
Ho	TA	0	16	3·0	64		3·5	4·7	19
La	TA	0	16	6·2	0	0	2·7	9·8	39
Ln	TA	0	64	6·3	0	0	2·3	7·3	33
Or	RF	0	16	5·8	0	4	2·9	5·9	15
Si	TA	0	16	3·0	0	0	2·1	7·3	27
Sw	MA	0	16	6·2	0	0	2·1	7·2	25
Ye	TA	0	16	3·8	0	0	4·5	7·9	24

fluids gave significant rheumatoid factor precipitation or inhibition titres of 1:256 or greater, heavy components were visualized. A general relationship existed between the quantity of complexes as determined by the inhibition system and the area measurements of complexes apparent upon ultracentrifugation. Since, in the course of investigation no essential differences other than size were detected among the complexes detected by the different systems, the term γ globulin complex was used to refer to them collectively. Fig. 1

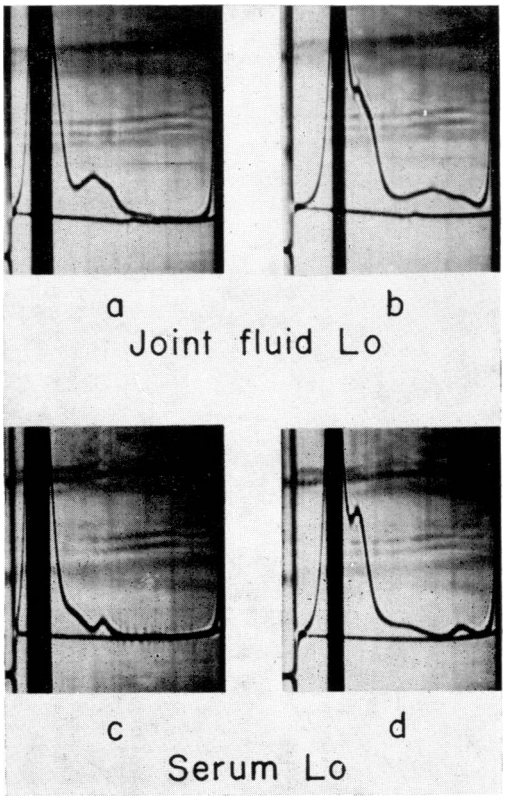

FIG. 1. Ultracentrifugal patterns of Lo joint fluid and serum showing the elevated concentration of complexes heavier than 7S in the joint fluid. Frames a and c were taken at 36 min, b and d at 80 min.

depicts a representative joint fluid, Lo, and its paired serum. Serum Lo contained a shoulder of complexes between 7S and 14S and a slight increase in complexes heavier than 19S. Joint fluid Lo contains a higher concentration of complexes in the 9 to 14S region and an additional broad poorly resolved double peak in the 15 to 18S region partially obscuring the small 19S components. There is a distinct zone of material sedimenting heavier than 19S. A clear '22S complex' was not observed in either Lo serum or joint fluid and only small quantities of γM rheumatoid factor were present.

The euglobulin precipitate of joint fluids with abundant γ globulin complexes contains large amounts of complexes visualized upon analytic ultracentrifugation. These are dissociable to 7S γG globulin in pH 4·1 buffer. Lo, a representative sample, is illustrated in Fig. 2(c) and (d). In neutral buffer the principal peak is broad and sediments at 8–9S without a

definite 7S peak. In acid buffer the preponderance of 7S γ globulin is apparent. The Lo joint fluid euglobulin preparation was subjected to gel filtration in pH 4·1 acetate buffer. The γG globulin complexes were fully dissociated and as depicted in Fig. 3, the 150,000 molecular weight fraction contained the rheumatoid factor activity. No rheumatoid factor was detectable in the excluded volume of the column. On analytic ultracentrifugation joint fluids with

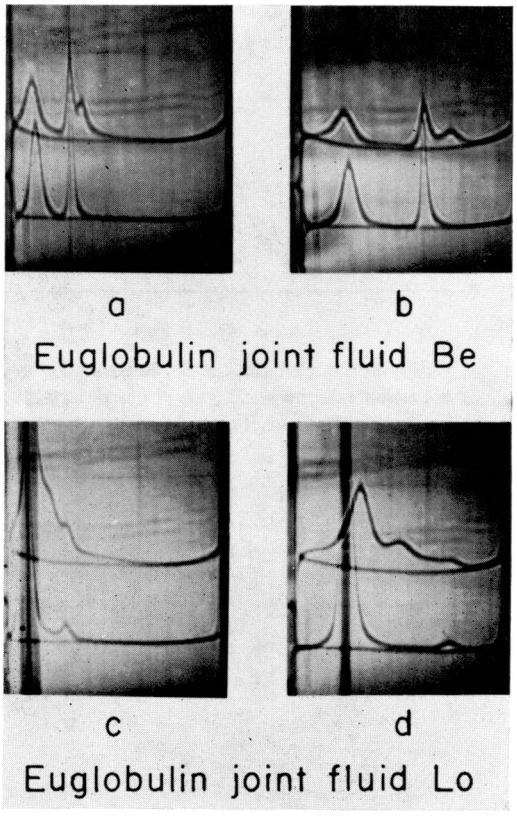

FIG. 2. Ultracentrifugal patterns of euglobulin fractions from two joint fluids. Lo, rich in γ globulin complexes and Be with a limited amount as detected by other methods described in the text. Double centrifuge cells are used to indicate the pattern in pH 7·2 as well as pH 4·1 buffers; these are respectively the upper and lower patterns in each frame. Various intermediate complexes are evident in Lo which are dissociable to 7S γG in pH 4·1. There is a marked preponderance of γG. Be lacks significant intermediate complexes and contains a dissociable 22S complex.

small amounts of γ globulin complexes by precipitation exhibit a different pattern with little or no intermediate complex and more equal amounts of 19S and 7S components. Fig. 2 (a) and (b) depict a representative fluid Be, of this type.

Upon the density gradient centrifugation of joint fluids which showed complexes by analytical ultracentrifugation, γG globulin was found distributed in fractions heavier than in their respective sera. Fig. 4 depicts Lo joint fluid in a pH 7·2 sucrose density gradient. The γG globulin is detectable in all fractions from 7S through heavier than 19S. The reac-

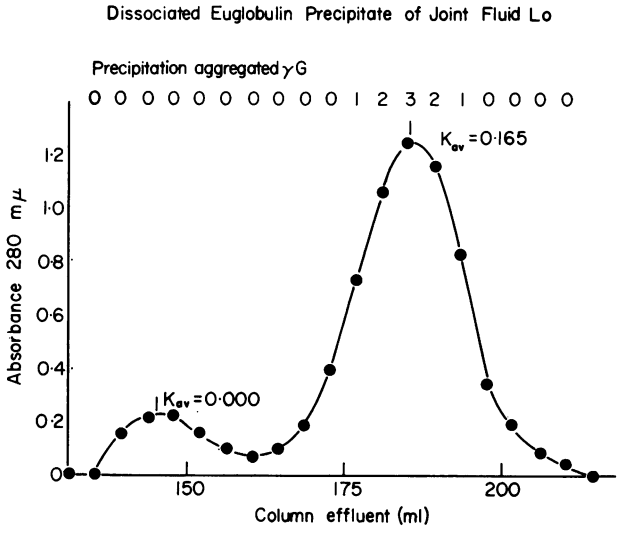

FIG. 3. Gel filtration of Lo joint fluid euglobulin on G-200 Sephadex in pH 4·1 buffer with a principal peak at the same elution volume as 72 γG. γM is detectable in the void volume. Rheumatoid factor is present in the 7S γG fraction.

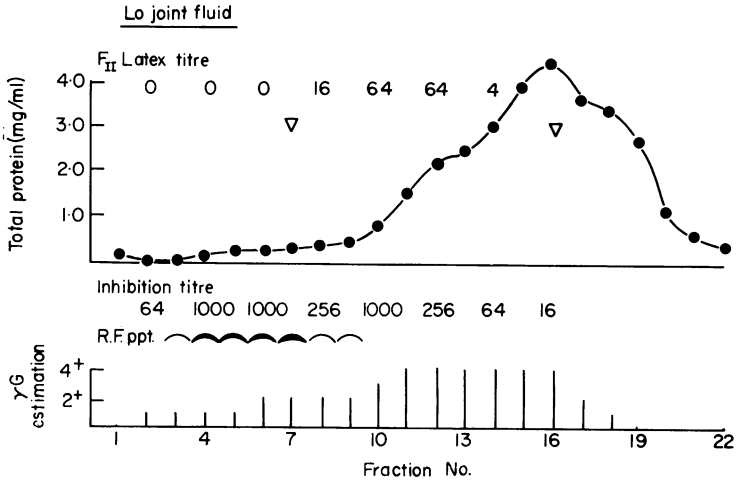

FIG. 4. Density gradient centrifugation of Lo joint fluid at pH 7·2. The 19S marker peak is in tube 7 and 6·5S marker is in tube 16. The lower portion of the figure depicts the occurrence of γG globulin from 7S through greater than 19S. The heavier fractions give precipitates with γM rheumatoid factor and both intermediate and heavier fractions react in the inhibition system. Fractions 4 through 7 formed precipitin lines with Clq. The upper portion of the figure contains the protein concentration of each fraction. Mercaptoethanol resistant rheumatoid factor activity was detectable in the intermediate fractions.

tivity of each fraction is indicated with γM rheumatoid factor, and in the inhibition system. Significant inhibition is evident in the lighter fractions that do not yield precipitates. Mercaptoethanol resistant rheumatoid factor activity was present up to the 19S fractions. Gel filtration in agarose or Sephadex at neutral pH revealed similar higher molecular weight complexes of γ globulin in these joint fluids, but the increased dissociating power of this technique made it less useful. With density gradient centrifugation of joint fluid Lo in pH 4·1 acetate buffer, γG globulin was not encountered in fractions heavier than 7S. All the mercaptoethanol resistant rheumatoid factor activity was contained in the 7S fractions. After neutralization, these fractions gave a precipitin reaction with γM rheumatoid factor while the heavier fractions did not. Treatment of 7S fractions from undissociated joint fluids did not induce the formation of components reacting with the γM rheumatoid factor.

One of the problems encountered in relying only on analytic ultracentrifugation for the identification of γ globulin complexes is the presence of fibrinogen aggregates in certain joint fluids. These were most frequently observed in intense acute synovitides with elevated non clottable fibrinogen and were usually suspected when the baseline was uniformly elevated without peaks in the 7 to 19S regions. Confirmation of the presence of fibrinogen aggregates required density gradient centrifugation and analyses of the fractions with anti-fibrinogen antisera. Careful studies in the case of Lo indicated that the components visualized on analytic ultracentrifugation were not related to fibrinogen aggregates.

Characterization of isolated γ globulin complexes

Two methods were utilized for the isolation of the complexes, one involving the precipitation with isolated γM rheumatoid factor, and the other, precipitation with isolated C1q. The C1q method did not require dilution with saline thus avoiding the problems of spontaneous precipitation. Following the addition of C1q, a precipitate quickly formed which was poorly soluble at neutral pH in 2 molar NaCl but readily dissolved between pH 5·3 and 4·1 in 0·1 molar buffer. The quantity of complex in the supernate detectable by γM rheumatoid factor was lowered thirty-two to sixty-four-fold. Upon analytic ultracentrifugation the complexes in the supernate heavier than 15–16S were markedly diminished while the 7S to 13S components were unchanged.

The precipitate was solubilized in pH 5·3 acetate buffer and examined with a number of antisera. In addition to C1q the principal constituent was γG globulin along with small quantities of γM. No γA was found. Several antisera made against whole joint fluid, their cryoprecipitates or fractions rich in the complexes were employed. These exhibited numerous specificities when tested against whole joint fluids, however, they did not reveal the presence of additional antigenic determinants in the C1q precipitate.

The precipitate was further acidified to pH 3·8 and subjected to gel filtration (Fig. 5). The γG globulin was limited to the 150,000 molecular weight region. The void volume contained C1q and small amounts of γM. Testing of the fractions with FII latex reagent and soluble γG aggregates revealed two peaks of agglutinating activity, the principal one which was resistant to reduction and 56°C inactivation was in the 150,000 molecular weight fraction. The strength and titres of agglutination were compared to the 150,000 molecular weight fraction of dissociated whole Lo joint fluid by gel filtration. The rheumatoid factor activity of the γG fraction isolated by C1q was five-to ten-fold greater than that of the γG fraction of whole joint fluid when adjusted to equivalent γG concentrations. Similar experiments were performed on joint fluids Cr, Mu and Ea employing γM rheumatoid factor as

the precipitating agent. The results were comparable with the exception that C1q was not found in the precipitate. Mercaptoethanol resistant rheumatoid factor of the 7S type was found concentrated in the precipitates.

An attempt was made to quantitate the amount of 7S γG rheumatoid factor in whole joint fluid using the method of Torrigiani & Roitt (1967). A number of problems were encountered with this procedure so that the absolute values obtained are not included in Table 1. It was apparent however that joint fluids with the largest amount of complexes had

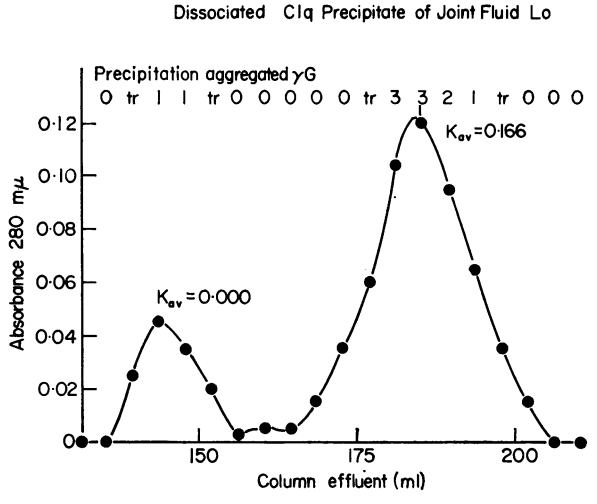

FIG. 5. Separation of dissociated Clq precipitate of Lo joint fluid by gel filtration in G-200 Sephadex equilibrated with pH 4·1 buffer. The γG globulin elutes as 7S γG forming the principal peak. The void volume peak contains Clq and γM. Fractions were tested with aggregated γG on flocculation trays. The precipitation in the void volume was diminished by heating at 56° C and abolished by mercaptoethanol reduction. Maximal rheumatoid factor activity was in the 7S γG fraction which resisted this treatment.

the most marked elevation. The levels of γG rheumatoid factor in these fluids were higher than in the respective sera. For example in Lo and Ce the ratio of joint fluid to serum in γG rheumatoid factor was 5·7 and 6·2 whereas the ratios of total protein were 0·81 and 0·58 respectively. Conversely, rheumatoid fluids without complexes had much diminished levels of γG rheumatoid factor detectable by these means. Control fluids had similar levels. The fluids with 1–6 mg of complex per ml in general had intermediate levels but here considerable variation was observed.

Absorption of joint fluids with insoluble BDB aggregated γ globulin lowered the amount of complexes precipitable with rheumatoid factor and C1q. In the case of Lo after two absorptions the complexes detectable with isolated 19S rheumatoid factor were more than five-fold diminished.

2. *Total haemolytic complement levels and quantity of γ globulin complexes*

In the course of measurements on the total haemolytic complement in joint fluids it became evident that the fluids with the most abundant γ globulin complexes had the most marked depression in haemolytic complement levels. These data are presented in Table 1

and summarized in Table 2. The eight fluids with markedly diminished complement levels of 3·4 CH_{50} units per 10 mg or lower had an inhibitory titre of 1000 or greater and upon the addition of rheumatoid factor gave a corrected precipitate of more than 6 mg/g. The titres of conventional γM rheumatoid factor in the sera of this group varied from four to 64,000.

Seven joint fluids from patients with rheumatoid arthritis had moderately diminished complement levels, with values between 25 and 70% of the expected level. CH_{50} units per 10 mg ranged from eleven to sixteen. In each, γG globulin complexes were present but in lesser amounts than in those fluids with markedly lowered complement levels.

In control joint fluids obtained from patients with gouty arthritis, subacute rheumatic fever, Reiter's syndrome and menisceal tears there were no γ globulin complexes and the complement levels were proportional to the total protein. The mean corrected haemolytic complement level per 10 mg was twenty-six and the range from fifteen to thirty-nine.

TABLE 2. Comparison of quantity of γ globulin complex with level of haemolytic complement and two components of complement

γ globulin complex (mg/g)	Complement level (CH_{50} units/10 mg)	Clq (mg/g)	βlA (mg/g)
>6 (8)*	0·5 (0–3·4)†	1·3 (0·7–2·5)†	3·2 (1·8–8·2)†
1–6 (5)	15 (11–20)	2·8 (2·0–3·6)	6·8 (3·6–8·8)
<1 (5)	23 (12–31)	2·8 (1·9–3·1)	7·2 (3·4-11·1)
Control fluids			
0 (8)	26 (15–39)	3·0 (2·1–4·5)	8·7 (4·7–11·0)

* Number of fluids in group. † Mean value and range.

Fluid Or with 15 CH_{50} units per 10 mg had 90% of the expected haemolytic complement content since the serum total complement was at low normal levels. Similarly in the synovial effusions of certain patients with rheumatoid arthritis the complement levels were not diminished below the expected range and there was little or no evidence of γ globulin complexes by centrifugation, euglobulin fraction or precipitation and inhibition tests.

Fig. 6 depicts the relationship between the quantity of γ globulin complex measured as milligrams of precipitate per gramme and haemolytic complement content in CH_{50} units per 100 mg. Considerable variation is evident in complement levels particularly for smaller quantities of γ globulin complexes, however, marked depression of complement was only encountered in fluids with over 6 mg/g of precipitate. The quantity of γM rheumatoid factor was not well correlated with the complement level in the joint fluids. In the eight joint fluids with complement levels below 4 CH_{50} units per 10 mg, serum titres varied from four to 64,000. The four joint fluids with the highest titres of rheumatoid factor had an average of 14 CH_{50} units per 10 mg.

3. *C1q and β_{1A} measurement*

Two constituents of the complement system C1q and C3 were measured in joint fluid by radial immunodiffusion. As is indicated in Table 2 the levels of these components were found to be lowered in proportion to the quantity of γ globulin complexes. However,

considerable variation was encountered and the relationship was not as conspicuous as that seen with total haemolytic complement.

Of the six joint fluids with abundant γ globulin complexes and no measurable haemolytic complement, five had less than 1 mg of C1q and 3·6 mg of β_{1A} per gramme. The sixth, Ea, had 1·8 mg of C1q and 1·8 mg of β_{1A}.

Fluid We was a notable exception in that the level of C1q was elevated above that found in other fluids with similar quantities of γ globulin complexes. It was otherwise atypical in that similar to patient Lo, no γM rheumatoid factor was detectable in the joint fluid and was present at very low levels in the serum. 7S γG rheumatoid factor was present in both.

Anticomplementary effect

An anticomplementary effect of joint fluids containing complexes was found in certain instances. The fluids exhibiting the greatest anticomplementary activity had abundant

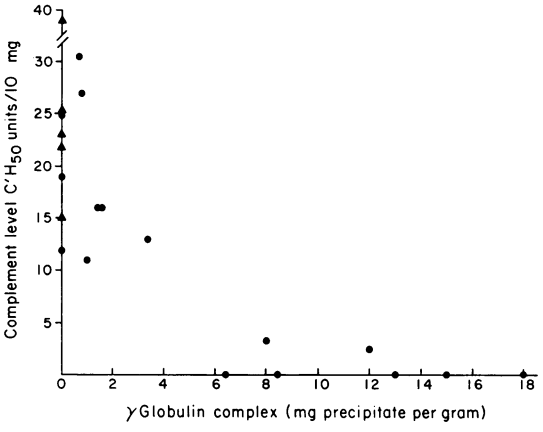

FIG. 6. The relationship in individual joint fluids between the quantity of γG globulin complex measured as milligrams of precipitate per gram and hemolytic complement in CH_{50} units per 10 mg is depicted. A marked depression in complement level is only encountered in fluids with abundant γG complexes.

γ globulin complexes and diminished haemolytic complement. This is indicated in Table 3. The anticomplementary effect of joint fluids varied considerably according to the methods of measurement. Three joint fluids exhibited anticomplementarity at 4°C with normal sera. These three fluids had the largest quantity of complexes as measured by milligrams of precipitate and the quantity of euglobulin. Upon analytic ultracentrifugation they all contained components sedimenting in greater than 19S regions at concentrations up to 1·9 mg/ml. There was no detectable C1q or measurable haemolytic complement. The anticomplementary effect in these fluids was greater at 37°C with either the serum from normal individuals or those with rheumatoid arthritis.

The anticomplementary effect of joint fluid from patients with rheumatoid arthritis was most pronounced when rheumatoid arthritis serum was employed at 37°C incubation temperature. Under these conditions all fluids with 3·3 mg/g of precipitate or more, had an anticomplementary effect of ten or more ΔCH_{50} units. All fluids with 1·8 mg or less of C1q per gramme were anticomplementary.

Among the joint fluids tested, no anticomplementary effect was found in the three samples with the highest titre of rheumatoid factor in the joint fluid. However, in fluids Ce, Lo and Mu, with abundant γ globulin complexes, increasing titres of rheumatoid factor were associated with a heightened anticomplementary effect at 37°C with normal serum. All patients exhibiting an anticomplementary effect in their joint fluid contained detectable

TABLE 3. Anticomplementary effect of synovial fluid with human serum as the source of complement* and direct precipitation with Clq

Patient	Precipitate mg ppte./g	Anticomplementary effect in ΔCH_{50} units: Normal serum incubation temperature 4°C	Normal serum incubation temperature 37°C	Rheumatoid serum incubation temperature 4°C	Rheumatoid serum incubation temperature 37°C	Precipitation reaction with Clq†
Ce	18	−12	− 93	−20	− 77	++
Lo	15	−26	− 32	− 8	− 84	++
Mu	13	−70	−139	−37	−113	++
We	12	0	41	−16	− 39	++
Jo	8·4	4	5	10	− 10	++
Ma	8·0	10	35	−11	− 30	++
Ea	6·4	13	− 32	−83	− 87	++
Fe	—	28	29	5	18	++
Ka	3·3	11	6	55	− 20	++
Al	1·6	22	22	35	35	0
Pa	1·6	24	35	53	20	0
Be	1·0	0	3	14	3	±
Re	0·8	11	19	21	13	0
Gr	0·7	35	37	10	15	±
Mo	0	4	22	53	20	0
Zi	0	45	49	50	− 1	0
Ha	0	22	22	49	26	0
He	0	20	47	30	7	0
La	0	10	10	24	20	0
Or	0	5	40	10	0	0
Ye	0	15	20	38	35	0

* The CH_{50} levels of unmixed but similarly diluted and incubated joint fluid and serum were subtracted from the observed CH_{50} level to give the anticomplementary effect expressed as ΔCH_{50} units.

† Distinct precipitin lines are scored as ++; diffuse haze reactions are indicated by ±.

rheumatoid factor in their serum although the amounts varied widely. The anticomplementary activity was similar in extent when three different sera from patients with rheumatoid arthritis containing high titre rheumatoid factor were employed as a source of complement and in the two instances tested the patient's own serum gave similar results. The data in Table 3 are based on the use of serum Jo.

C1q precipitation

A direct precipitation reaction with C1q is given by aggregated γ globulin and various immune complexes (Agnello *et al.*, 1969). Certain rheumatoid joint fluids give distinct precipitin lines with C1q that fuse with those given by aggregated γ globulin. These reactions

are depicted in Fig. 7. This direct precipitation reaction is not diminished and variably increased by heating joint fluids at 56°C for 30 min. In certain fluids such as Sw, interfering reactions due to DNA were encountered; these were eliminated by DNAase treatment.

Nine joint fluids from rheumatoid arthritis patients gave definite precipitates with C1q as indicated in Table 3. All fluids with abundant γ globulin complexes as determined by an inhibition titre of 1:256 or greater, gave a distinct precipitate. Eight of these nine fluids have

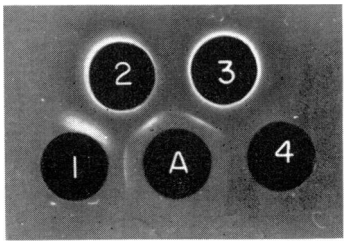

FIG. 7. An agarose gel diffusion experiment showing the precipitin reaction between Clq in well A and aggregated γ globulin in well 1. Wells 2 and 3 contain joint fluid Lo and Ea respectively which have γ globulin complexes demonstrable by other methods described in the text. Precipitin lines have formed with Clq. In addition a precipitation reaction between the 7S γG rheumatoid factor of Lo and aggregates is evident between wells 1 and 2. Well 4 contains joint fluid Mo which lacks complexes, and which does not form a precipitin reaction with Clq.

more than 3·3 mg/g of precipitable γG complex. All seven fluids tested with less than 25% of the expected haemolytic complement level reacted and the remaining two had between 25 and 50% of the expected total complement. The eight fluids with the lowest measurable quantities of C1q were among the nine fluids reacting with C1q. Forty additional joint fluids from patients with varying diseases were investigated for precipitation with C1q. Distinct precipitation was only encountered in those joint fluids that contained evidence for significantly increased γ globulin complexes by other methods. All of the fluids that gave a definite precipitate with C1q exhibited an anticomplementary effect except Fe.

DISCUSSION

Both analytical and density gradient ultracentrifugation have demonstrated complexes containing γG globulin in the synovial fluid of many patients with rheumatoid arthritis. These could be isolated by selective precipitation with purified 19S rheumatoid factor or by precipitation with purified C1q. The latter proved of particular utility and permitted isolation for detailed studies of the complexes. They were readily dissociable in acid or urea and were found to consist primarily if not entirely of γG globulin. Antisera made against the purified complexes failed to show the presence of additional antigens such as would be expected if they contained, for example, a foreign antigen. The accumulated evidence obtained in the present study indicated that the complexes consisted of γG globulin and anti γG globulin of the 7S type (7S rheumatoid factors) similar to those described previously in the sera of certain patients with rheumatoid arthritis (Kunkel *et al.*, 1961). However, the synovial fluid complexes appeared in higher concentration and contained a portion which sedimented more rapidly than those in the respective sera. It was this portion which reacted directly with C1q to form precipitates. Anti γ globulins of the 7S type were found concentrated in these precipitates as well as those obtained with isolated 19S rheumatoid factor

(Winchester, Agnello & Kunkel, 1969a). In addition absorption with insoluble BDB aggregated γ globulin markedly lowered the level of precipitable complexes. Finally an association was noted between the occurrence of these larger complexes in the synovial fluids and the presence of 7S anti γ globulins in the whole synovial fluid as well as the corresponding serum. In such fluids the level of γG rheumatoid factor was elevated over that found in the sera. The smaller complexes in the sera failed to precipitate with C1q but sometimes gave some precipitation with 19S rheumatoid factor which was always less than that of the synovial fluid.

At the present time there is not sufficient evidence available to conclude that the complexes are exclusively based on 7S anti γ globulins or rheumatoid factors. The possibility remains that there might be a 'core' of denatured γ globulin or perhaps another type of immune complex. There is, however, no direct evidence favouring the latter possibilities. Studies in progress on the ability of purified 7S γG rheumatoid factor to form large complexes indicates that the size of the complexes depends on the ratio of 7S γG to 7S γG rheumatoid factor and that 7S γG rheumatoid factor can form complexes large enough to precipitate with 19S rheumatoid factors (Winchester, Kunkel & Agnello, 1970).

A principal characteristic of the complexes is their dissociability. Both the complexes isolated by γM rheumatoid factor and those isolated by C1q are dissociable to 7S γG. Reformation of the complexes readily occurs from these fractions. Hannestad (1967) considered the complexes to represent aggregated γ globulin similar to that obtained from heat aggregation. However, the completeness of dissociation in the present experiments and subsequent reformation of the joint fluid complexes suggests that the complexes have the properties attributed to immune complexes and not those associated with non-specifically aggregated γG.

The direct precipitation reaction of the γ globulin complexes with C1q does not require the presence of γM rheumatoid factor. Reduction of the joint fluid followed by dialysis adequate to destroy 19S rheumatoid factor does not alter the strength of precipitation. Reduction with subsequent alkylation which prevents reformation of disulfide bonds in the γG molecules does abolish the precipitin reaction with C1q as is expected from previous studies on whole complement (Wiedermann, Miescher & Franklin, 1963). From density gradient experiments with joint fluid or with aggregated γ globulin (Agnello, Winchester & Kunkel, 1970), the size of the γ globulin complex precipitating with C1q has been shown to be approximately 19S or heavier. Upon examination of the supernate of whole joint fluid after precipitation by C1q all the components heavier than 14–15S are depleted with the pattern becoming more similar to that of the serum pair. This suggests that co-precipitation of complexes in the 15–19S region has occurred. It is of interest to note that the smaller γ globulin complexes previously described as occurring in the sera of idiopathic hyperglobulinemic purpura patients (Kunkel *et al.*, 1961) fail to react with C1q in agarose diffusion analysis in the same manner as the synovial fluid complexes do. In some instances precipitin reactions were given with γM rheumatoid factor (Winchester, Agnello & Kunkel, 1969a).

The occurrence of γG globulin complexes was related to both evidence of complement consumption and the ability to interact with the complement system. The interaction with the complement system was assessed by anticomplementary effect and by direct precipitation with C1q. The direct precipitation reaction with C1q does not require the presence of γM rheumatoid factor. This mode of complement activation may account for the deposits of γG and complement without γM rheumatoid factor observed by Rodman *et al.*,

1967. The anticomplementary effect appeared to be dependent on a number of different factors involving both the presence of γ globulin complexes as well as 19S rheumatoid factor. This may account for some of the variations in anticomplementarity encountered by various authors (Pekin & Zvaifler, 1964; Townes, Stewart & Marcus, 1966; Hedberg, 1967). In the present studies the size of the γ globulin complexes appeared to play a role since only the joint fluids with the heaviest and most abundant complexes were anticomplementary at 4°C with normal serum. Those with smaller complexes demonstrated reactivity under different conditions. In addition the following findings favour the participation of γM rheumatoid factor; (1) In fluids with approximately equal concentrations of complexes the anticomplementary effect increased with the quantity of detectable γM rheumatoid factor in the joint fluid. (2) The enhancement of the anticomplementary effect at 37°C over that at 4°C suggests that γM is involved. Such variation in the temperature optima for γM and γG antibodies has been described by Cuniff & Stoller (1968). (3) Fresh sera from patients with rheumatoid arthritis manifested enhanced sensitivity to the anticomplementary effect of joint fluid containing complexes. The accumulated evidence indicated that 19S rheumatoid factor in the serum reacted with γG globulin complexes and caused an increased consumption of complement. Because of anticomplementary effects it was not possible to use isolated γM rheumatoid factor for this purpose. In possibly related experiments employing phagocytosis as an indicator system, Hurd, LoSpalluto & Ziff (1969) observed that the addition of γM rheumatoid factor to certain joint fluids induced the formation of phagocytes staining for complement, γG and γM.

Taken together the evidence of complement consumption along with the findings of precipitation with C1q and anticomplementary effects indicates that the observed γ globulin complexes in joint fluid are the substances capable of fixing and activating the components of the complement system. Rheumatoid factors of the 19S type also interact and sometimes cause precipitation as well as potentiation of the effect on the complement system. It is possible that prior complete precipitation of the complexes in the joint by γM rheumatoid factor may diminish the ability of the complexes to cause subsequent complement fixation. The activation of complement may occur within the matrix of the synovium or in the synovial fluid and direct precipitation and secondary phagocytosis of the reaction products could result. The histamine release reported by Baumal & Broder (1968) probably results from the same complexes.

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