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# PRESENCE OF AGGREGATED γG-GLOBULIN IN CERTAIN RHEUMATOID SYNOVIAL EFFUSIONS

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SUMMARY

A precipitin reaction occurred between rheumatoid arthritis sera with high titre rheumatoid factor (RF) and certain rheumatoid synovial effusions. The precipitating factor in the sera was shown to be γM-type RF, reacting mainly with human γG-globulin. The precipitating component of synovial effusions was structurally altered, γG-globulin sedimenting much faster than native γG-globulin by density gradient ultracentrifugation. Following exposure to increasing hydrogen ion concentrations, an increasing amount of the RF-precipitating γG-globulin attained a slower sedimentation rate. Evidence for the *in vivo* presence of this altered γG-globulin was given, indicating that it may represent a stimulus for RF production.

## INTRODUCTION

The hypothesis that production of rheumatoid factors results from stimulation by autologous γG-globulin altered by immune-reactions or other processes rests on several lines of evidence; isolation and characterization of such structurally altered γG-globulin from tissues and native biological fluids of patients with rheumatoid arthritis have not yet been reported.

The presence in the inflamed synovial membrane of altered γG-globulin was suggested by the observation that rheumatoid sera enhanced the immunofluorescence staining reaction for γ-globulin-containing sites in synovial sections (Kaplan & Vaughan, 1959). Indirectly the demonstration of RF in plasma cells in the rheumatoid synovial membrane (Mellors *et al.*, 1959) also indicated the presence of an antigenic stimulus for the production of RF in the joint. This view was further strengthened by studies of the inclusions of granulocytes from inflammatory synovial fluids (Hollander *et al.*, 1965; Rawson, Abelson & Hollander, 1965; Zucker-Franklin, 1966; Barnett, Bienenstock & Bloch, 1966).

In a previous paper it was postulated that the low synovial fluid to serum ratio of RF of some patients with rheumatoid arthritis was due to local consumption or inhibition by

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some form of aggregated γG-globulin (Hannestad & Mellbye, 1967). The present study gives direct evidence for the presence of altered γG-globulin in certain rheumatoid synovial fluids and presents some characteristics of this protein.

## MATERIALS AND METHODS

*Rheumatoid sera* were selected from three patients (Br., Ma. and He.) with classical rheumatoid arthritis because of their high titres of rheumatoid factor. Serum Br. was mainly used since it had the highest precipitating ability with aggregated γG-globulin. All rheumatoid sera were inactivated at 56°C for 30 min before use.

*Antisera.* Rabbit antisera against pepsin-split γG-globulin (anti-Fab) and whole human serum, and antisera reacting specifically with γG-globulin (anti-γ) and γM-globulin (anti-μ) were prepared and tested for specificity by Dr M. Harboe according to methods described previously (Harboe, Rau & Aho, 1965). For Gm(4)* typing, SNagg serum A.J. (kindly provided by Dr L. Mårtensson) was used with anti-D Ha. Anti-Rh Ripley was kindly provided by Dr M. Waller. Rabbit antisera against human red cells were obtained from Institut Pasteur, Paris, France.

*Joint fluids.* They were obtained from patients with the following diagnoses: adult and juvenile rheumatoid arthritis (possible, probable, definite and classical), adult and juvenile monarthritis, ankylosing spondylitis, Reiter's disease, psoriatic arthropathy, arthropathy of ulcerative colitis (one case), acute gouty arthritis with urate crystals (one case) and osteoarthritis. The joint fluids were handled as described earlier (Hannestad & Mellbye, 1967) with one modification: Only 25 i.u. of hyaluronidase per millilitre of joint fluid were added, as this was sufficient to reduce viscosity. The supernatants were tested against RF serum not later than 2 weeks following the aspiration. Two fluids, from the left and right knee joint of patient Er., were tested on usual media, including media for acid fast bacilli, and inoculated on guinea-pigs. No infective organism was revealed. These were the only fluids cultured.

*γ-Globulin preparations.* Isolated γG-globulin was obtained commercially (Gammaglobulinløsning 'Kabi' 12%). Aggregation was effected by diluting this preparation to 10 mg/ml and heating in a waterbath at 63°C for 10 min. Isolation of Gm(−4) γG-globulin was performed by adding ammonium sulphate to the serum to a final concentration of 1·33 M. The precipitate was dissolved in a small volume of distilled water and dialysed for 2 days against several changes of saline. It was then aggregated as described. Rheumatoid factor of serum Br. was isolated from precipitates formed with heat aggregated γG-globulin in the region of maximum precipitation (Edelman, Kunkel & Franklin, 1958), and recovered in the fraction labelled 'P' in Fig. 5(b). Most preparations had a protein concentration around 1 mg/ml. RF negative Waldenstrøm type γM-globulin was isolated by density gradient ultracentrifugation of whole serum.

*Buffers.* Buffered saline was made by adding 1 volume of a 0·1 M-phosphate buffer of pH 7·4 to 9 volume of saline. Glycine–HCl buffers were prepared by mixing 0·1 M-glycine in 0·1 M-NaCl with 0·1 M-HCl, giving a pH of 2·4, 3·1 and 3·6. Citric acid–phosphate buffer was prepared by mixing 0·1 M-citric acid with 0·2 M-disodium phosphate to a pH of 4·6.

* This nomenclature corresponds to the notation for genetic factors of human immunoglobulins (*Bull. Wld Hlth Org.* 1965, **33**, No. 5); Gm(4) equals Gm(f).

*Protein concentrations* were determined by a modified Folin technique (Lowry *et al.*, 1951) using isolated γG-globulin as standard.

*Preparative ultracentrifugation.* Sucrose density gradients were prepared by layering 0·5 ml of 40% and 1·3 ml of 30, 20 and 10% sucrose solutions in saline, or one of the buffers, in cellulose nitrate tubes. The gradients were prepared 6–8 hr before use. Joint fluid, 0·1–0·2 ml, diluted 1:2 in saline or one of the acid buffers, was layered on top of the gradient. When the effect of acid pH was studied the joint fluid pH was equilibrated by dialysis against the buffer for several hours, and insoluble material removed by centrifugation. Ultracentrifugation was carried out in a Spinco L-50 preparative ultracentrifuge equipped with a SW 39 L head, at 4°C for 17 hr. The speed, unless otherwise stated, was 35,000 rev/min giving a maximal *g* of 134,211. Sometimes a speed of 27,500 rev/min was used giving a maximal *g* of 82,860.

Isolation of the fractions from the gradient was carried out by collecting drops from a pinhole in the bottom of the tube. In gel diffusion experiments the fractions were used without prior dialysis, since the agarose buffer easily neutralized the small volumes of weak acid buffers. The fractions were dialysed for 2 days before all haemagglutination experiments to remove sucrose and to adjust pH.

*Reduction of disulphide bonds* of rheumatoid sera was done as described previously (Hannestad & Mellbye, 1967). The preparations were used without alkylation or dialysis.

*Double diffusion in gel* was performed on 5 × 5 cm glass slides covered with 5 ml 1% agarose in 0·12 M-barbital buffer, pH 8·6. Sodium azide, in a final concentration of 0·1%, was originally added to the agarose because it prevents precipitate formation due to non-specific protein–protein interaction (Anderson *et al.*, 1962). No difference was observed in plates with or without sodium azide, and this step was, therefore, later omitted. The well diameters were 3 mm. When rheumatoid serum or isolated rheumatoid factor were used, the centre-to-centre distance was 7 mm. With anti-μ serum, this distance was either 7 or 9 mm, and with anti-γ serum 9 mm. When whole joint fluids were tested, the wells were usually filled only once. The slides were kept at room temperature in a moist chamber, and were photographed without prior staining.

*Quantitation of γG-globulin* was done as described before (Hannestad & Mellbye, 1967).

*Immunoelectrophoresis.* Glass plates of 8 × 8 cm were covered with 10 ml of a mixture containing equal volumes of 2% agar and 0·12 M-veronal buffer of pH 8·6. The subsequent procedure was according to Wadsworth & Hanson (1960).

*Quantitative precipitin reactions.* Two parallel reactions were set up. In one set, a constant volume of 0·1 ml rheumatoid serum was mixed with increasing amounts of whole joint fluid (Fig. 3). To the control set, 0·1-ml portions of normal serum negative in the RF tests were mixed with the same amounts of joint fluid. Buffered saline was added to a final volume of 2·1 ml. After 30 min at 37°C and overnight incubation at 4°C the tubes were centrifuged at 1000 *g* for 20 min at 4°C; the supernatants were decanted and the precipitates washed twice in cold buffered saline. A small amount of 0·1 M-NaOH was added to dissolve the precipitates and the amount of protein determined. The amount of specific precipitate between rheumatoid serum and joint fluid was calculated by subtraction of the protein content in the control tubes from the content in the tubes with rheumatoid serum. The protein content of the spontaneous precipitates in tubes containing diluted rheumatoid serum alone was 25–35 μg. When precipitin reactions between rheumatoid sera and heat

aggregated γG-globulin were measured, a similar procedure was employed except that 0·2 ml rheumatoid serum was used.

*Effect of pH on precipitates.* A large amount of precipitate from the zone of maximal precipitation was collected, washed twice in saline, dissolved in 0·2–0·4 ml glycine buffer of pH 3·1, and halved. One was layered on a pH 3·1 gradient. To the other was added an equal volume of citric acid–phosphate buffer, pH 4·7, which gave a final pH of about 4·5. This was layered on a saline density gradient and centrifuged at 34,000 rev/min.

*Tests for RF activity.* The Waaler–Rose and sensitized human cell (Ripley) tests for rheumatoid factor activities were done as previously described (Hannestad & Mellbye, 1967), except that buffered saline, pH 7·4, was used as the third drop instead of a dilution of normal serum. Haemagglutination inhibition in these test systems was performed using either a constant dilution of the rheumatoid serum (the highest dilution giving a 3+ agglutination) and serial doubling dilutions of the material to be tested for inhibition, or a fixed dilution of this material and serial doubling dilutions of the rheumatoid serum (Harboe 1959).

## RESULTS

### *Characterization of rheumatoid sera and isolated rheumatoid factor*

The three sera used (Br., He. and Ma.) had slide haemagglutination titres of 1000–4000 in the Ripley test and 500–1000 in the Waaler–Rose test. Their precipitating ability with

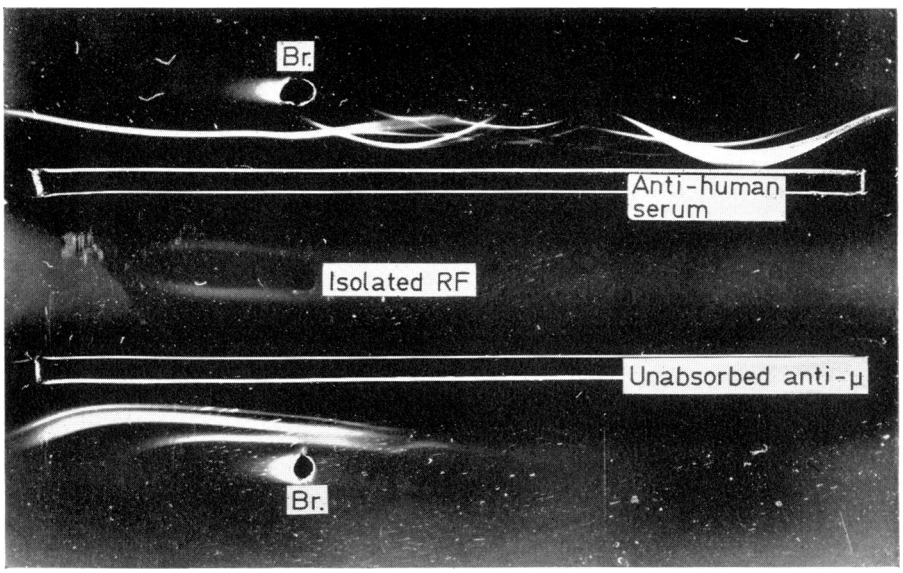


FIG. 1. Immunoelectrophoresis to test purity of RF isolated from serum Br.

heat aggregated γG-globulin is shown in the left part of Fig. 4. The isolated rheumatoid factor preparation contined pure γM-globulin as judged by immunoelectrophoresis; anti-whole human serum and unabsorbed anti-γM-globulin both gave a single line in the γ-region (Fig. 1). In agarose a strong precipitin line formed between isolated rheumatoid

factor and heat aggregated γG-globulin, demonstrating that it had a high reactivity with human γG-globulin.

Serum Br. gave a weakly positive LE cell test, while serum He. and Ma. were LE negative. A test tube with 3 ml of serum Br. was immersed in iced water for 19 hr followed by centrifugation at 1000 *g* for 20 min. A small amount of precipitate (0·47 mg protein) formed which did not dissolve in saline when incubated at 37°C for 1 hr with occasional shaking, showing that it was not a cryoprotein.

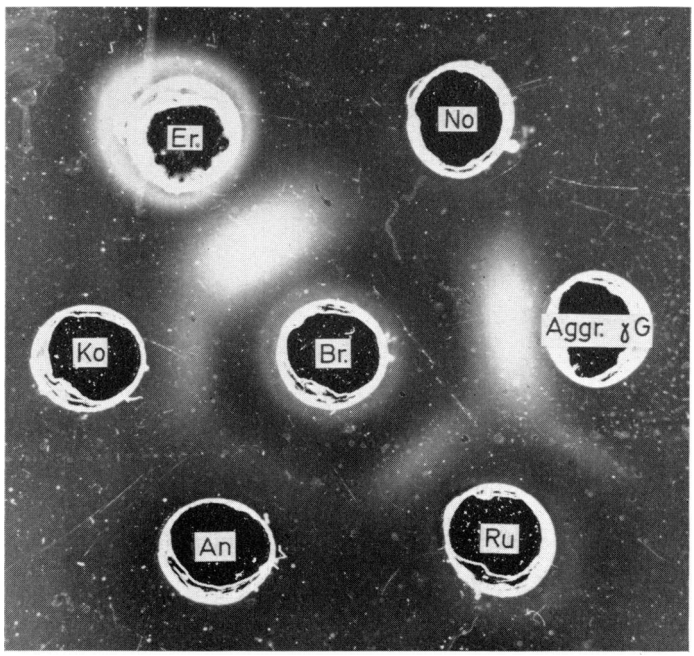


FIG. 2. Rheumatoid serum Br. (centre), five native joint effusions (Ru., An., Ko., Er. and No.) and heat aggregated γG-globulin (10 mg/ml) demonstrating no precipitation (No.) and precipitates of varying strength. The precipitate between Ru. and aggregated γG is due to the RF content of the joint fluid.

*Properties of precipitating joint fluids*

The γG-globulin content varied between 8·7 and 20 mg/ml, the cell count between 2200 and 41,000 per $mm^3$ except for one fluid (Ma.) with 600 cells/$mm^3$. The cells of this fluid were predominantly polymorphonuclear, and the fluid contained numerous cholesterol crystals which may have made the counting of cells difficult. The RF titre varied between less than 2 and 4000.

*Nature of disease in joints with precipitating joint fluids*

All the patients with joint fluids precipitating with rheumatoid serum Br. were adults with symmetrical polyarticular disease; no patients with an established diagnosis other than

adult rheumatoid arthritis exhibited the phenomenon. RF activity was detected in the serum in the majority but not all of the patients with precipitating joint fluids, in titres ranging from 16 to 8000. The disease duration in the joints ranged from ½ to 22 years. More detailed analysis of the clinical and laboratory features of the disease in patients with precipitating joint fluids will be presented when a larger number has been studied.

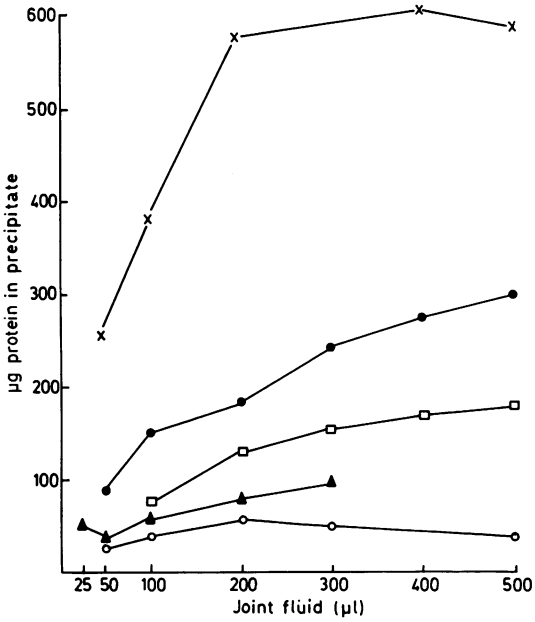


FIG. 3. Quantitative precipitin curves between serum Br. and different joint fluids to demonstrate range of protein in precipitates. ×, Er. (left); ●, Er. (right); □, Ma.; ▲, Ha.; ○, Tr.

*Double diffusion in agarose*

Fluids from seventy-five joints of seventy-two patients were tested against serum Br. Twenty-one joint fluids from eighteen patients gave a precipitate. It usually developed after 1–3 days, rarely after longer incubation. The precipitin lines were broad and diffuse, and sometimes quite weak (Fig. 2). Once formed, the precipitates did not disappear. When agar gel was used instead of agarose, spontaneous circular precipitates around the wells were very common, making this medium useless for the purpose of the present study. This was not a problem with agarose. When serum He. and Ma. were used instead of serum Br., the system became less sensitive—only the joint fluids that gave the strongest precipitates with serum Br. also precipitated with these two sera. Such strongly precipitating joint fluids also precipitated in double diffusion against *joint fluids* with high titres of rheumatoid factor. A few joint fluids were tested with and without added hyaluronidase; no difference was observed in precipitate formation.

*Effect of different factors on precipitate formation*

Rheumatoid serum Br. precipitated varying amounts of protein from different arthritic joints fluids, including fluids obtained from two different joints of the same patient (Fig. 3).

Three different taps over a 6 months' interval from the left knee of patient Er. gave almost identical amounts of precipitate with serum Br. There was a definite correlation between the strength of the precipitin lines in agarose and the amount of protein in the fluid precipitate. Three different rheumatoid sera (Br., Ma., He.) precipitated varying amounts of protein from one joint fluid (Er. left); a similar difference was observed in the same sera's ability to precipitate heat aggregated γG-globulin (Fig. 4). This explains why serum He. and

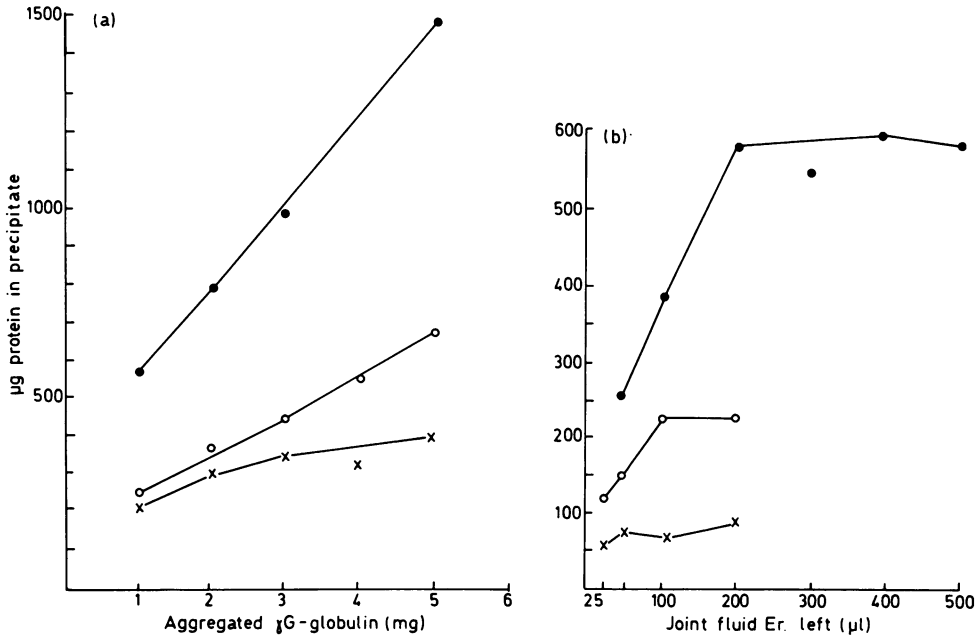


FIG. 4. Quantitative precipitin curves between three different rheumatoid sera (●, Br.; ○, Ma.; ×, He) and heat aggregated γG-globulin (a) and native joint fluid Er. (b).

Ma., which precipitated considerably less protein than serum Br. from heat aggregated γG-globulin, only precipitated with the strongest reacting joint fluids in the gel diffusion tests. Thus, the higher the precipitating ability of rheumatoid serum with aggregated γG-globulin, the more sensitive the system becomes in reactions with joint fluids.

About 40% less protein was precipitated at 37°C than at 4°C when serum Br. reacted with joint fluid Er. (left). A similar reduction occurred when the serum and joint fluid were mixed without diluting with saline; such volume effect was also observed in precipitin reactions of rheumatoid factor with unheated but not with heated preparations of Fraction II (Vaughan, Ellis & Marshall, 1958). No precipitate formed after reduction of serum Br. with mercaptoethanol. No effect on precipitate formation was observed when either serum or joint fluids were heated to 56°C for 30 min, after storage of joint fluid Er. at 4°C for 2 months, or at −20°C for 6 months, or after joint fluid Er. (left) had been centrifuged at 11,000 *g* (maximum) for 45 min which removed the major part of the lipids present in this fluid. Nine joint fluids that did not precipitate with serum Br. in double diffusion, were tested against the same serum 1–7 months later after storage at 4°C. None became precipitating

during storage. Four of the twenty-one precipitating fluids were examined on the day of the tap.

The precipitate formed between serum Br. and joint fluid Er. was moderately mucoid. It did not dissolve when incubated in saline at 37°C for 1 hr, and thus was not a cryo-precipitate.

TABLE 1. Decrease of rheumatoid factor activity in rheumatoid serum Br. after precipitation with joint fluid Er.

| Test | Material added to 0·1 ml serum Br. (diluted 1:20) | Reciprocal of dilutions of supernatant | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 |
| SHC (Ripley) | 0·2 ml normal serum | 3+ | 3+ | 3+ | 3+ | 3+ | 2+ | 2+ | 1+ | 0 |
| | 0·2 ml joint fluid Er. | 2+ | 2+ | 1+ | 1+ | 0 | 0 | 0 | 0 | 0 |
| Waaler–Rose | 0·2 ml normal serum | 3+ | 3+ | 3+ | 3+ | 3+ | 2+ | 1+ | 0 | 0 |
| | 0·2 ml joint fluid Er. | 3+ | 3+ | 3+ | 3+ | 2+ | 1+ | 1+ | 0 | 0 |

3, 2,1, Degree of agglutination; 0, no agglutination.

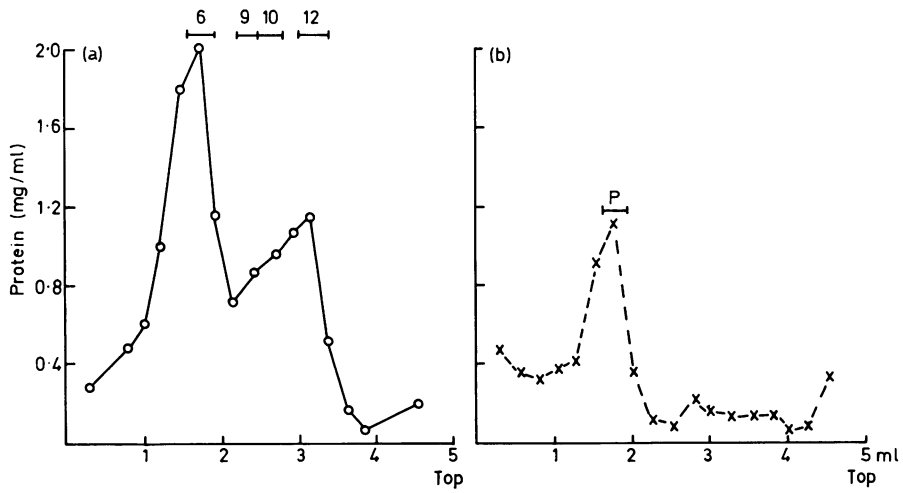


FIG. 5. Density gradient ultracentrifugation of pH 3·1 dissolved precipitate formed between serum Br. and joint fluid Er. (a), and serum Br. and heat aggregated γG-globulin (b).

*Characterization of the precipitating factor in serum*

The precipitate formation with joint fluids was initially noted with the high titred rheumatoid serum Br. Other sera with high titre rheumatoid factor had the same property, whereas normal or hypergammaglobulinaemic rheumatoid factor negative sera gave no precipitate with the joint fluids. It was, therefore, tested whether rheumatoid factor was the active serum component in the precipitin reaction.

After treatment of rheumatoid sera with mercaptoethanol, no precipitates formed. All joint fluids that precipitated with serum Br., also precipitated with isolated rheumatoid factor from this serum. No precipitates formed when the strongest reacting joint fluids were

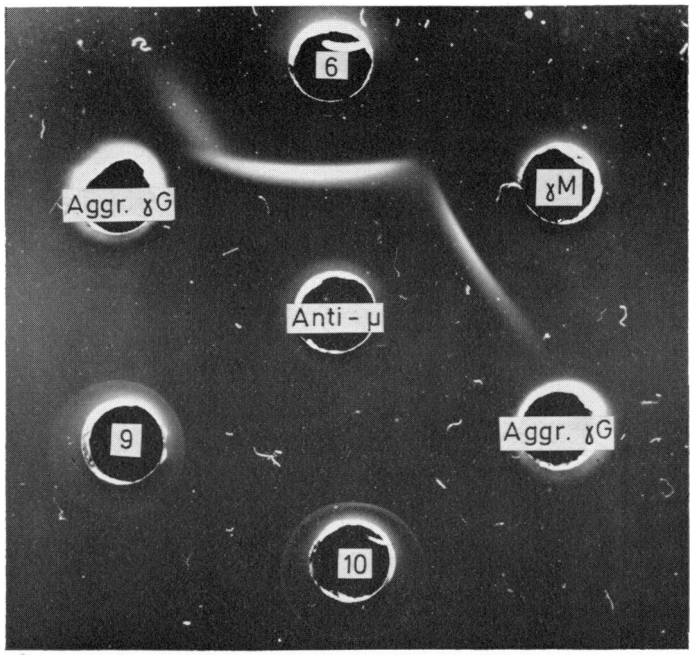


FIG. 6. Reaction between specific anti-γM-globulin (anti-μ), aggregated γG-globulin, different density gradient fractions (6, 9, 10, Fig. 5) and an isolated Waldenström γM-globulin (γM).

TABLE 2. Rheumatoid factor activity of a rheumatoid serum (Br.), and of γM RF isolated from this serum by precipitation with joint fluid Er. (fraction '6')

| Test | Material | Reciprocal of dilutions of material | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 |
| SHC (Ripley) | Serum Br. | 3+ | 3+ | 3+ | 3+ | 3+ | 3+ | 2+ | 2+ | 1+ | 0 |
| | Fraction '6' | 3+ | 3+ | 3+ | 3+ | 3+ | 2+ | 1+ | 0 | 0 | 0 |
| Waaler–Rose | Serum Br. | 3+ | 3+ | 3+ | 3+ | 2+ | 2+ | 1+ | 0 | 0 | 0 |
| | Fraction '6' | 2+ | 1+ | 1+ | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

tested against γM-globulin without rheumatoid factor activity isolated from macroglobulinaemic sera.

Further experiments were limited to the interaction between serum Br. and joint fluid Er. (right and left) because of the ease of working with a large quantity of precipitate. The

supernatant from a tube precipitin reaction showed a definite reduction of rheumatoid factor activity in the Ripley test (Table 1). When the precipitates were dissolved in a pH 3·1 glycine–HCl buffer and subjected to density gradient ultracentrifugation, the rapidly sedimenting protein fraction designed '6' (Fig. 5) contained γM-globulin with strong rheumatoid factor activity as evidenced by precipitation with aggregated γG-globulin (Fig. 6), and agglutination in high dilutions in the SHC (Ripley) test (Table 2). Since joint fluid Er. was barely positive in the RF agglutination tests (Waaler–Rose and Ripley titre of 8), it was concluded that the rheumatoid factor activity recovered from the precipitate originated from serum Br. Fraction '6' also precipitated strongly with whole joint fluid Er. in gel diffusion, demonstrating that this isolated rheumatoid factor was the serum component that originally reacted with the joint fluid.

These experiments showed that the γM rheumatoid factor caused a precipitin reaction with several rheumatoid joint fluids.

*Characterization of the precipitating component in joint fluids*

The identification of rheumatoid factor as the precipitating protein in serum suggested the presence of some form of aggregated γG-globulin in joint fluids.

*Immunoelectrophoresis*

By immunoelectrophoresis of native joint fluid Er. and Li. against serum Br. and isolated RF, a short, broad precipitin arc developed in the fast γ-region (Fig. 7). Its mobility was much more restricted than that of native γG-globulin and slightly slower than heat aggregated γG-globulin. When large amounts of precipitate from the interaction of joint fluid Er. and serum Br. were dissolved at pH 3·6 and subjected to density gradient ultracentrifugation, the bottom fraction precipitated in double diffusion against serum Br. Immunoelectrophoresis of this fraction against anti-whole human serum and specific anti-γ gave a precipitin arc with identical range and electrophoretic mobility as the arc of whole joint fluid with rheumatoid factor. That this characteristic was not due to low γG-globulin concentration was shown by diluting the fraction corresponding to the peak 7S γG-globulin in the gradient (labelled '12' in Fig. 5) to the same concentration as the bottom fraction: in this case a long precipitin arc with the same mobility as native γG-globulin developed. It is concluded that a narrow and relatively fast electrophoretic mobility in the γ-region is characteristic of the joint fluid precipitating component.

*Density gradient ultracentrifugation of native joint fluids*

To define the size of the precipitating component, two joint fluids (Er. left and Li.) which gave the strongest precipitates with serum Br. and one joint fluid (No.) that did not precipitate, were studied by density gradient ultracentrifugation. The γG-globulin content of the joint fluids was 20·0, 9·0 and 27·0 mg/ml, respectively. Fractions after density gradient ultracentrifugation of native joint fluid were tested by the Ouchterlony technique against serum Br., specific anti-μ and specific anti-γ. The results of this experiment are shown in Table 3.

While some overlapping occurred, the rheumatoid factor precipitating component sedimented faster than γM-globulin. The deep gradient fractions which precipitated with serum

Br. contained γG-globulin, as shown by a reaction of identity with isolated γG-globulin using specific anti-γ. The precipitin lines that developed between the bottom fractions of

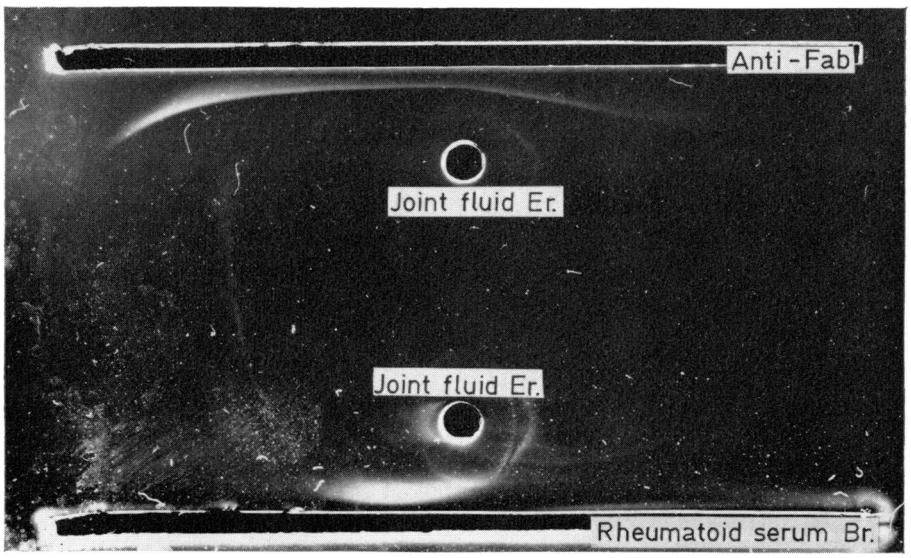


FIG. 7. Immunoelectrophoresis showing the migration of joint fluid RF precipitating component (below) and native γG-globulin of joint fluid Er.

TABLE 3. Density gradient sedimentation of γG-, and γM-globulin, and RF precipitating component of three joint fluids (Er., Li. and No.)

| Fraction | Precipitate with anti-μ | | | Precipitate with anti-γ | | | Precipitate with serum Br. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Er. | Li. | No. | Er. | Li. | No. | Er. | Li. | No. |
| Bottom 1 | 0 | 0 | 0 | 2+ | (+) | 0 | 2+ | 0 | 0 |
| 2 | 0 | 0 | 0 | 2+ | 1+ | 0 | 1+ | (+) | 0 |
| 3 | 0 | 0 | 0 | 2+ | 1+ | 0 | 1+ | (+) | 0 |
| 4 | 2+ | 1+ | 1+ | 2+ | 2+ | 0 | 1+ | (+) | 0 |
| 5 | 2+ | 1+ | 1+ | 2+ | 2+ | 0 | (+) | 0 | 0 |
| 6 | 1+ | 1+ | 0 | 2+ | 2+ | 1+ | 0 | 0 | 0 |
| Top 7–15 | 0 | 0 | 0 | 3–1+ | 3–1+ | 3–1+ | 0 | 0 | 0 |

3, 2, 1, Relative intensity of precipitin lines; 0, no precipitation.

Er. and Li. and specific anti-γ were distinctly curved like precipitin lines with other large and slowly diffusing antigen molecules, and different from native 7S γG-globulin (Fig. 8).

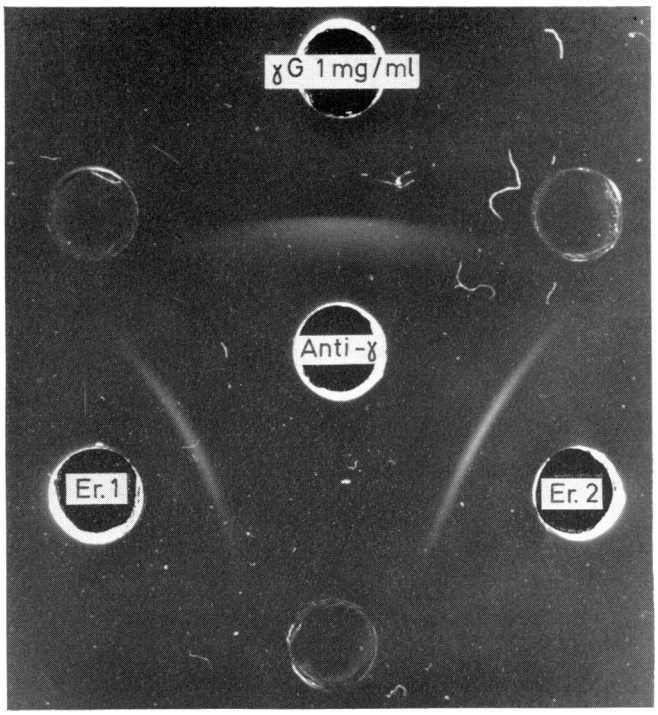


FIG. 8. Reaction between specific anti-γG-globulin (anti-γ), isolated γG-globulin (γG-1 mg/ml), and the two deepest density gradient fractions (Er. 1 and Er. 2) of native joint fluid Er. (left).

TABLE 4. Agglutination of Ripley sensitized red cells by diluted serum Br. after addition of density gradient fractions of joint fluid Er.

| Density gradient fractions (pools) | Protein concentration (mg/ml) | Main components of pools | Protein concentration of dilution (mg/ml) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 3·0 | 1·0 | 0·5 | 0·25 | 0·12 | 0·06 |
| Bottom 1–6 (0–1·5 ml) | 1·0 | γM+ heavy γG-aggregates | | | 0 | 0 | 0 | 3+ |
| 7–10 (1·8–2·5 ml) | 2·0 | 10–15S sedimenting proteins | | 0 | 0 | 3+ | 3+ | 3+ |
| 11–14 (2·8–3·5 ml) | 6·0 | Peak 7S γG +albumin | 1+ | 2+ | 3+ | 3+ | 3+ | 3+ |

By contrast, joint fluid No. contained only γG-globulin that sedimented at the expected slower rate than γM-globulin. The six deepest gradient fractions of joint fluid Er. were pooled and shown to inhibit the SHC (Ripley) agglutinations of a rheumatoid serum at very low protein concentrations (Table 4). These findings demonstrated that the joint fluid precipitating component behaved as γG-globulin aggregates sedimenting slightly faster than γM-globulin. When joint fluid Er. was centrifuged at 27,000 rev/min, the deepest fraction precipitating with serum Br. was number three from the bottom. At this speed, heat aggregated γG-globulin was still found in the bottom fraction, demonstrating that the major portion of the heat aggregates sedimented faster than those found in the joint fluid.

It was necessary to refill the wells twice with the gradient fractions of joint fluid Li. to obtain precipitates with serum Br. No refills were required to obtain precipitates with the same fractions against specific anti-γ. This demonstrated that the latter antibody formed precipitates with smaller amounts of antigen than the strongest rheumatoid serum available.

*Density gradient ultracentrifugation of dissolved precipitates*

The composition of the precipitate resulting from interaction between serum Br. and joint fluid Er. was studied by density gradient ultracentrifugation at pH 3·1. The protein peaks from such gradients had different proportions but similar sedimentation properties as dissolved precipitates formed between rheumatoid serum and heat aggregated γG-globulin (Fig. 5). The slowly sedimenting peak (designated '10' in Fig. 5) was located at the expected level of native 7S γG-globulin and contained this protein as shown by a reaction of identity with isolated γG-globulin using specific anti-γ.

To prove that this γG-globulin originated from joint fluid Er. and not from the rheumatoid serum, advantage was taken of the fact that serum Br. was Gm(−4) and serum Er. Gm(4). Peak '10' (Fig. 5) inhibited completely the agglutinations in the Gm(4) test system down to a protein concentration of 0·1 mg/ml, demonstrating that a major part of the protein in this fraction was γG-globulin of the Gm(4) type. To exclude that Gm(−4) γG-globulin is changed to Gm(4) after aggregation, the following control experiment was performed: γ-globulin from a Gm(−4) person was isolated and heat aggregated. This preparation precipitated strongly with serum Br. The Gm-type was still found to be Gm(−4). From these studies it is concluded that the major part of the γG-globulin in the precipitate is derived from the joint fluid.

When the different density gradient fractions of the dissolved precipitate formed between serum Br. and joint fluid Er. were subjected to immunoelectrophoresis against anti-human serum, precipitin lines developed exclusively in the γ-region. This indicated that the main serum protein components of this precipitate were γ-globulins. The fractions were not tested with specific antisera against serum complement components.

*Specificity of reacting rheumatoid factor*

Since haemagglutination systems indicate the existence of some RF molecules reacting exclusively with either human or rabbit γG-globulin (Milgrom *et al.*, 1962), it was of interest to see if the joint fluid γG-globulin aggregates reacted with RF of mainly one or more than one specificities. Inhibition experiments with joint fluid Er. diluted to a γG-globulin concentration of 0·5 mg/ml demonstrated a definitely stronger inhibition than by joint fluid No. which did not precipitate with high titred rheumatoid serum (Table 5). This inhibition,

which could also be demonstrated with other joint fluids reacting strongly with rheumatoid sera, was only found in the SHC (Ripley) test and not in the Waaler–Rose test. When the supernatant from a tube precipitin reaction between serum Br. and joint fluid Er. was tested for RF activity, a marked decline was found in the activity in the SHC (Ripley) test, but not the Waaler–Rose test (Table 1). The activity of isolated RF from precipitates between serum Br. and joint fluid Er. (fraction '6' in Fig. 5a) was high in the SHC (Ripley) test and low in the Waaler–Rose test (Table 2). These results indicate that the joint fluid γG-globulin aggregates mainly interact with rheumatoid factor of *human* γG-globulin specificity. This was also suggested by the results of the RF agglutination tests of paired joint fluids and sera where a decrease of the synovial fluid titre in the SHC (Ripley) test, but not the Waaler–Rose test, was frequent.

TABLE 5. Inhibition of RF activity of rheumatoid serum He. in the SHC (Ripley) test by two joint fluids diluted to a γG-globulin concentration of 0·5 mg/ml

| Joint fluid | Reciprocal of dilutions of serum He. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 16 | 32 | 64 | 128 | 256 | 512 | 1024 |
| No. (not precipitating) | 3+ | 3+ | 3+ | 3+ | 2+ | 1+. | 0 |
| Er. (precipitating) | 3+ | 2+ | 1+ | 1+ | 1+ | 0 | 0 |

*Effect of pH*

Since immune-complexes are dissociated by acid pH, in contrast to γG-globulin aggregates induced by physicochemical procedures (Christian, 1960a), the effect of pH was studied. Native joint fluid Er. and Li. were subjected to density gradient ultracentrifugation at varying pH, and the fractions tested in gel diffusion against specific anti-μ, anti-γ and rheumatoid serum Br. There was no change in the sedimentation of γM-globulin. At pH 4·6 γG-globulin was still found in the bottom fraction, and the line was curved, but weaker. Refill of the well was necessary to demonstrate precipitation of the three bottom fractions with serum Br. at this pH. At pH 3·6 a further reduction in the sedimentation of γG-globulin was seen, and precipitation against serum Br. was not found in any of the fractions (Table 6). No definite reduction in the sedimentation rate of the aggregates present in commercial preparations of γG-globulin or induced by heating was found at pH 3·6, as judged by strong precipitation of the bottom fractions with RF sera. Studies at pH 2·4 did not provide additional information, since this induced denaturation of normal γG-globulin which then precipitated with high titred rheumatoid sera.

The effect of pH on the joint fluid γG-globulin aggregates was further examined by density gradient ultracentrifugation of precipitates dissolved at pH 4·5 and 3·1. Two joint fluids, Er. (right) and Er. (left), were studied. At pH 4·5 the protein content in the deep fractions was higher and that in the 7S γG-globulin peak lower than at pH 3·1 (Fig. 9). In both instances, the bottom fractions contained γG-globulin as shown by immunoelectrophoresis and double diffusion in gel experiments. Double diffusion experiments with serum Br. revealed that the deepest fractions from the pH 4·6 gradient precipitated with this serum

without refill of the well, while the same fractions from the pH 3·1 gradient did not precipitate. Several fractions located *higher* in the pH 3·1 gradient precipitated *weakly* with serum Br. (Fig. 9); this was either due to a slight denaturation of normal γG-globulin at

TABLE 6. Sedimentation of γG- and γM-globulin, and RF precipitating component of joint fluid Er. in sucrose gradients dissolved in saline (left part) and pH 3·6 glycine–HCl buffer (right part)

| Fraction | Protein concentration (mg/ml) | Precipitate with | | | Protein concentration (mg/ml) | Precipitate with | | |
|---|---|---|---|---|---|---|---|---|
| | | Anti-μ | Anti-γ | Serum Br. | | Anti-μ | Anti-γ | Serum Br. |
| Bottom 1 | 0·36 | 0 | 2+ | 2+ | 0·00 | 0 | 0 | 0 |
| 2 | 0·68 | 0 | 2+ | 1+ | 0·24 | 0 | 0 | 0 |
| 3 | 1·00 | 0 | 2+ | 1+ | 0·24 | 0 | 0 | 0 |
| 4 | 1·20 | 2+ | 2+ | 1+ | 0·36 | 2+ | 2+ | 0 |
| 5 | 1·12 | 2+ | 2+ | (+) | 0·36 | 2+ | 2+ | 0 |
| 6 | 0·80 | 1+ | 3+ | 0 | 0·28 | 1+ | 3+ | 0 |
| 7 | 1·32 | 0 | 3+ | 0 | 0·56 | 0 | 3+ | 0 |
| 8 | 3·12 | 0 | 3+ | 0 | 1·84 | 0 | 3+ | 0 |
| Top 9–15 | — | 0 | 3–1+ | 0 | — | 0 | 3–1+ | 0 |

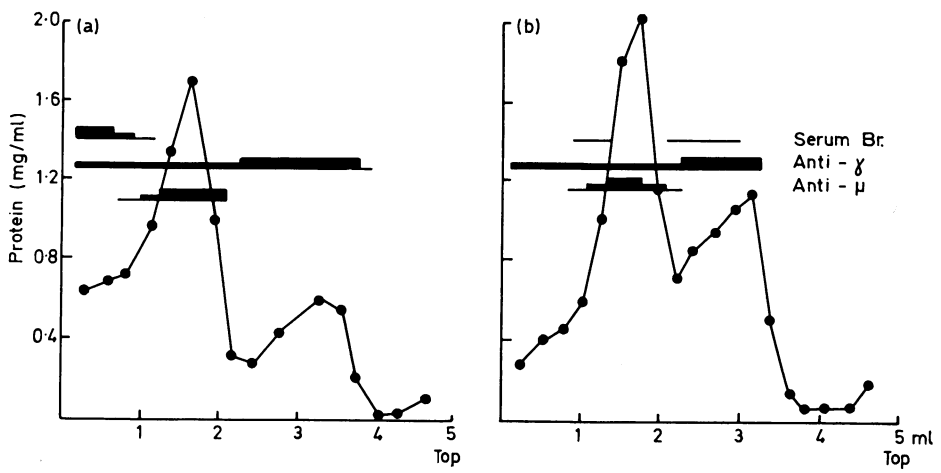


FIG. 9. Density gradient ultracentrifugation of dissolved precipitates between serum Br. and joint fluid Er. to show the relative sedimentation of γM-globulin (anti-μ), γG-globulin (anti-γ), and joint fluid RF precipitating component (serum Br.) at (a) pH 4·5 and (b) pH 3·1.

pH 3·1 or to reassociation of the dissociated aggregates in the wells of pH 8·6 buffered agarose. These experiments showed that a decrease of the pH from 4·5 to 3·1 caused an increasing, but not complete, dissociation of the γG-aggregates in the joint fluid.

The difference in the effect of pH 3·1 on the joint fluid γG-aggregates and heat-induced aggregates was clearly seen when the respective dissolved precipitates with the same rheumatoid serum were studied in the ultracentrifuge (Fig. 5). The precipitates obtained with joint fluid Er. gave a very marked peak (labelled '10' in Fig. 5) sedimenting like a 7S γG-globulin, in contrast to a very small similar peak with the precipitate containing heat-aggregated γG-globulin.

## DISCUSSION

The essential finding in the present study was that some rheumatoid joint effusions contained γG-globulin capable of precipitating with rheumatoid factor. This reactive joint fluid γG-globulin differed from native γG-globulin in several respects: it was electrophoretically more homogeneous and restricted to the fast γ-region; it sedimented faster than 19S γM-globulin in density gradient ultracentrifugation. These properties resemble the properties of γG-globulin aggregated by physico-chemical procedures (Edelman *et al.*, 1958; Christian, 1958; Christian, 1960a). In addition, the altered joint fluid γ-G-globulin formed curved precipitin lines against specific anti-γ antisera, and thus resembled antigen molecules of considerably larger size than native 7S γG-globulin.

The precipitate formation between the joint fluid γG-aggregates and rheumatoid sera was characterized by diffuse precipitin lines, a mucoid and opalescent appearance of the precipitates, a protracted plateau of the quantitative precipitin curves, and an effect of dilution on the amount of protein precipitated. Similar characteristics have been found previously for the precipitin reaction between artificially aggregated γG-globulin and rheumatoid sera (Epstein, Engleman & Ross, 1957; Vaughan *et al.*, 1958; Butler & Vaughan, 1965).

The joint fluid altered γG-globulin differed from the bulk of heat aggregated γG-globulin in that the electrophoretic mobility and sedimentation rate were slower; the sedimentation rate was decreased by acidification; it reacted with rheumatoid factor of more strict *human* γG-globulin specificity. The explanation for these differences is probably that the joint fluid γG-aggregates are denatured to a smaller extent and present in lower concentrations than in the heated preparations.

Besides the precipitin reaction between rheumatoid sera and Cohn Fraction II (Epstein, Johnson & Ragan, 1956) or soluble immune-complexes (Edelman *et al.*, 1958), several other precipitin reactions have been described where rheumatoid factor, native γG-globulin and aggregated γG-globulin are engaged. None of these reactions could explain the presently observed phenomenon. Spontaneous cold precipitates (Epstein *et al.*, 1957; Christian, 1959) and mixed cryo-precipitates between rheumatoid factor and γG-globulin (Meltzer & Franklin, 1966) were excluded by the minimal amount of precipitated protein in the tube containing rheumatoid factor serum alone, by precipitate formation at 37°C, and by lack of dissolution of precipitates at this temperature. The reaction between aggregated γG-globulin and a component of serum complement (Müller-Eberhard & Kunkel, 1961) was excluded by using heat inactivated rheumatoid sera. Precipitating autoantibodies against saline extracts of various organs (Anderson *et al.*, 1962) could be ruled out by identifying the reacting components in serum and joint fluid. Precipitin reactions between haemoglobin and $\alpha_2$-globulin (Peetoom *et al.*, 1960) and albumin (Metzgar & Grace, 1961) are found

in agar but not in liquid, and involve other components than those demonstrated in the present study. Sedimentation of aggregated γG-globulin on chylomicrons following lowering of the ionic strength of the serum (Quie & Hirsch, 1963) did not occur when the sera were diluted in physiological saline, which shows that it could not account for the precipitates with joint fluids.

The precipitin reaction with RF was only observed with certain of a large number of identically treated inflammatory synovial effusions. Joint fluids which originally did not precipitate RF sera still failed to do so after prolonged storage at 4°C. On the other hand, precipitation occurred with some joint fluids tested on the day of aspiration. These results favour the assumption that the altered γG-globulin is present in the joint *in vivo* and is not due to changes *in vitro*. It is thus probable that it represents an antigenic stimulus for RF production.

The altered γG-globulin of rheumatoid joint fluids may be the result of physico-chemical processes related to inflammation, or of immune-reactions where the γG-globulin exists in combination with one or several antigens as immune-complexes. It is difficult to distinguish between these two types of aggregates: Both fix complement with ensuing inflammatory changes in the skin (Ishizaka & Ishizaka, 1959; Ishizaka, Ishizaka & Campbell, 1959; Christian, 1960b); have a high sedimentation rate, and interact with rheumatoid factor (Edelman *et al.*, 1958; Christian, 1958), and exhibit changes in optical rotatory dispersion (Ishizaka & Campbell, 1959; Henney & Stanworth, 1965). The density gradient evidence of dissociation of the joint fluid γG-globulin aggregates by lowering of pH may be in favour of aggregates existing as immune-complexes since this effect has not been found with heat-induced aggregates in this or other (Christian, 1960a) studies.

Experiments with sera containing anti-antibodies of Milgrom type (Milgrom, Dubiski & Wozniczko, 1956) and antibodies against pepsin split incomplete anti-Rh (Osterland, Harboe & Kunkel, 1963) have demonstrated that these agglutinators are not inhibited by physico-chemically aggregated γG-globulin (Milgrom *et al.*, 1956; Milgrom, 1962; Harboe *et al.*, 1965) or non-precipitating antibody–hapten mixtures (Fudenberg, Goodman & Milgrom, 1964). Antibody of γG-globulin class in immune-precipitates (Milgrom, 1962; Fudenberg *et al.*, 1964; Harboe *et al.*, 1965) or on particulate antigens (Milgrom, 1962) has been shown to absorb anti-antibody. These serological systems may thus distinguish between the two types of γG-globulin aggregates. Further experiments will, therefore, be made on the effect of soluble immune-complexes containing human antibody on these serological systems for eventual comparison with joint fluids.

The composition of the joint γG-aggregates appears important both for a better understanding of the pathogenesis of joint symptoms in rheumatoid arthritis, and for the interpretation of immunofluorescence evidence for fixation of γG-globulin and complement in tissues, which may result both from physico-chemical aggregation of γG-globulin or from deposition of immune-complexes. The methods described in this study permit selection of joint fluids, and possibly other pathological body fluids, with altered γG-globulin in amounts suitable for further study.

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