Precipitation of $^{125}$I-labelled IgG aggregates by factors in sera of healthy individuals and of patients with rheumatoid arthritis

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**SUMMARY** Several factors in human serum are capable of precipitating soluble $^{125}$I-labelled heat aggregated IgG (agg IgG*). A study of the nature of these factors resulted in the development of two new methods for the detection and assay of anti-IgG-immunoglobulins (rheumatoid factors) in serum. One method detected rheumatoid factors of the IgG and the IgA classes which are capable of binding and coprecipitating with Clq and agg IgG* in an EDTA milieu. In a second method the serum was first heat inactivated and the assay was then made in a polymeric milieu where rheumatoid factors of the IgM as well as the IgG and IgA classes could be detected. 67 sera from patients with rheumatoid arthritis were tested with this method and rheumatoid factors were detected in all seropositive (as assayed with conventional rheumatoid factor tests) sera and in $58\%$ of the seronegative sera. In the presence of certain anti-IgG-immunoglobulins or polymers, the precipitation of Clq and soluble agg IgG* is greatly enhanced, and we suggest that this can be used as a basis of a sensitive method for the assay of agg-IgG-binding activity of Clq.

When soluble $^{125}$I-labelled heat aggregated human IgG (agg IgG*) was added to fresh human sera diluted in phosphate buffer with EDTA, an almost complete precipitation of radioactive material was found with some sera at low dilutions. Sera of some patients could precipitate a substantial amount of the radioactive material even when diluted several thousand times. A low precipitating activity was found when the sera were diluted in the same buffer without EDTA present and the precipitating activity was completely lost after heat treatment. However, some sera which had a low precipitating activity in the buffer with or without EDTA had a strong precipitating activity even after heat treatment when a polymer like polyethylene glycol (PEG) was added to the buffer. We investigated which factors in serum were responsible for the remarkable precipitating activities and these studies resulted in the development of new methods for detection of different types of anti-IgG-immunoglobulins (rheumatoid factors) in serum.

**Materials and methods**

**ASSAY BUFFER**

500 ml 0.1 mol/l phosphate buffer pH 7.5, 500 ml 0.15 mol/l NaCl, 10 ml 5% (w/v) Na$_2$P$_2$O$_7$, 5 ml Tween–20, 5 ml 20% human serum albumin (Kabi, Sweden).

**ASSAY EDTA BUFFER**

This buffer consisted of assay buffer with the addition of EDTA to a final concentration of 10 mmol/l.

**POLYETHYLENE GLYCOL 6000 (PEG)**

Solutions of different concentrations were prepared by dissolving polyethylene glycol of molecular weight 6000 (Union Carbide Corp., USA) by heating and mixing in assay buffer.

**DIAMINOBUTANE**

Diaminobutane was obtained from Koch-Light Laboratories Ltd, England.

**AGGREGATED HUMAN IgG**

Human IgG (Cohn Fr. II) was obtained from pooled human sera and supplied by Kabi AB, Sweden. Heat aggregation was performed for 20 minutes.
at 60°C of a 2% IgG solution. Aggregated IgG (agg IgG) was separated from monomeric IgG by gel filtration on a 90 x 1.5 cm column of Sephadex G-200 (Pharmacia AB, Sweden) equilibrated with 0.1 mol/l TRIS-HCl buffer containing NaCl (0.5 mol/l). Concentrations of agg IgG were determined spectrophotometrically at 280 nm.

**C1q**
This was prepared from fresh human serum according to the method of Shelton et al. (1972). The degree of purity of the C1q preparation was shown by immunoelectrophoresis using rabbit antihuman whole serum and anti-C1q serum (Behringwerke AG, G. W. Germany). Concentrations of C1q were determined spectrophotometrically using the extinction coefficient of 6.8 at 278 nm (Yonematsu et al., 1971).

**LABELLING OF PROTEINS**
To 500 μCi 125I (Amersham, IMS-30) was added 40 μg agg IgG which was labelled by the Chloramine T method (Hunter and Greenwood, 1962) to a specific activity of about 10 μCi/μg agg IgG. The reaction mixture (75 μl) was separated by gel filtration on Sephadex G-200 (0.9 x 30 cm column). The eluted labelled agg IgG was centrifuged at 3500 g for 5 minutes to remove any spontaneously precipitable IgG. The labelled protein was diluted in assay-buffer to about 40 μg/l (40 000 cpm in 0.1 ml) and called the agg IgG* solution.

**GEL FILTRATION USING SEPHADEX G-200**
Serum (3-5 ml) was separated on a 90 x 1.5 cm column of Sephadex G-200 equilibrated with PBS pH 7.6 and the elution pattern was determined by optical density measurement at 280 nm.

**AFFINITY CHROMATOGRAPHY USING IgG-SEPHAROSE**
Human IgG was slightly heat aggregated at 60°C for 10 minutes and covalently coupled to CNBr-activated Sepharose (50 mg protein/2 ml CNBr-Sepharose, Pharmacia AB). A column was poured and equilibrated with 0.2 mol/l TRIS-HCl buffer pH 8, containing NaCl (0.5 mol/l). Bed dimensions were 0.9 x 5 cm. After the sample had been applied to the column, immunoadsorption was allowed to continue for 1 hour. The column was then washed with equilibration buffer until the absorbancy at 280 nm of the effluent was negligible. The column was then eluted with 0.2 mol/l glycine-HCl at pH 2.8 containing NaCl (0.5 mol/l). The eluate, after neutralization with 0.5 mol/l NaOH and concentration, was tested by immunodiffusion against rabbit antihuman serum, anti-IgA, -IgG, -IgM antiserum (Behringwerke).

**ION EXCHANGE CHROMATOGRAPHY**
This was performed using DEAE Sephadex A-50. The equilibration buffer was 0.05 mol/l TRIS-HCl buffer pH 7.4, and elution was obtained with a continuous salt gradient to 0.5 mol/l NaCl. Bed dimensions were 0.9 x 8 cm and the flow rate 0.4 ml/min.

**LATEX AGGLUTINATION TEST**
The slide test reagents for the detection of conventional rheumatoid factor was obtained from Hyland (Costa Mesa, Calif.). The IgG used to coat the polystyrene latex was of human origin.

**PRECIPITATION OF AGG IgG**
*Rheumatoid factor precipitating activity*
200 μl of the serum sample heat inactivated at 56°C for 30 minutes and diluted 1:20 in assay buffer + 200 μl of a 4% PEG solution and 100 μl of the agg IgG* solution were put into disposable plastic test tubes, which were sealed with a plastic cap, placed in racks attached to a Heto Rotamix, and rotated for 16 hours at 4°C. (This rotation procedure was used in all the experiments described. However, it was later found that a similar precipitation was obtained when the test tubes were standing in vertical position for 16 hours at room temperature (22°C)).

After incubation all tubes were removed from the rack and centrifuged at 3500 g for 3 minutes. The plastic plugs were carefully removed. One ml of saline solution containing 0.5% Tween-20 was added to each tube with an automatic pipetting machine. The tubes were again centrifuged at 3500 g for 3 minutes. The supernatants were removed by suction, using a modified syringe needle attached to a plastic plate to prevent the needle from removing more than the desired amount of supernatant. The washing cycle was repeated three times, the tubes sealed with plastic caps, and placed in the automatic gamma counter.

**C1q precipitating activity**
200 μl of the test solution containing C1q was incubated with 200 μl of a 4% PEG solution and 100 μl of the agg IgG* solution in plastic test tubes. The incubation, washing, and counting were performed as above.

**C1q influenced precipitating activity**
200 μl of the serum samples in different dilutions (assay buffer) were incubated with 200 μl of assay EDTA and 100 μl of the agg IgG* solution, and handled as above.

**Serum samples**
Serum samples were obtained from apparently normal human individuals of both sexes.
healthy blood donors at the Blood Centre, University Hospital, Uppsala. Specimens were also obtained from patients diagnosed as having seropositive rheumatoid arthritis (RA), seronegative RA, and systemic lupus erythematosus (SLE). Blood was drawn aseptically into plain tubes, allowed to clot at room temperature, and centrifuged at 3000 g. Sera were either stored at −70°C or used immediately.

Results

PREcipitation of AGG IgG\(^*\) BY SERA OF healthy individuals

The precipitating activity of agg IgG\(^*\) in sera of healthy individuals was tested in the following way. To 200 μl of serum diluted 1:2, 1:20, and 1:200 in assay buffer was added either 200 μl of assay EDTA buffer and then 100 μl of the agg IgG\(^*\) solution. The mixtures were incubated for 16 hours at 4°C during constant rotation. After washing and centrifugation, the radioactivity of the precipitate was assayed. Detergent and albumin were added to the incubation buffers to prevent the nonspecific attachment of the isotope to the plastic tubes. In this way the background values were reduced to less than 1% of total amount of added radioactivity. In Fig. 1 the precipitation activity of 45 sera of healthy individuals is shown. These results indicate that there was a wide variation in the precipitating ability of normal sera and that the precipitation of agg IgG\(^*\) was enhanced significantly in the presence of EDTA at 1/5 serum dilution.

PREcipitation of AGG IgG\(^*\) BY SERA OF healthy individuals after treatment at 56°C for various times

Pooled serum from healthy individuals was treated at 56°C for 5, 10, 15, and 25 minutes and diluted in the assay buffer 1:4, 1:16, and 1:32. 200 μl of the various dilutions of the serum pool and 200 μl of the assay EDTA buffer were incubated with 100 μl of the agg IgG\(^*\) solution. The incubation, washing, and centrifugation procedures were as described above. Fig. 2 shows that the precipitating activity of normal sera is lost after heat treatment at 56°C for 20 minutes, indicating the presence of a heat labile serum factor responsible at least in part for the precipitation found in normal sera.

Influence of DIaminobutane on PREcipitation of AGG IgG\(^*\) BY SERA OF healthy individuals

200 μl of the pooled serum from healthy individuals diluted 1:6 in the assay buffer was incubated with 200 μl of the assay EDTA buffer containing different amounts of diaminobutane for one hour at room

Fig. 1 Precipitation of agg IgG\(^*\) by sera of 45 healthy individuals and the influence of EDTA. The different dilutions of sera (at a final dilution of 1:5, 1:50, and 1:500) were incubated with soluble agg IgG\(^*\) (40 000 cpm) at 4°C during constant rotation for 16 hours. The mixture was then centrifuged at 3500 g for 3 minutes and the precipitate washed three times. Precipitating activity in the presence of EDTA (B), and in the absence of EDTA (A) is shown.
temperature. The final diaminobutane concentration ranged from 0.12 mol/l to 0.12 mmol/l. 100 μl of the agg IgG* solution was then added and the precipitating activity was assayed as described above. The results (Fig. 3) showed a distinct inhibition of the precipitation of agg IgG* by normal serum in the presence of diaminobutane at a concentration of 12 mmol/l.

**Fig. 2** Precipitation of agg IgG* by a pooled normal serum of healthy individuals and the influence of heat treatment. The pooled serum was treated at 56°C for various times and diluted in assay EDTA buffer. The final serum dilution ranged from 1:8 to 1:64. The duration of heat treatment of the serum with concomitant change of the precipitating activity is shown.

**Fig. 3** Influence of different concentrations of diaminobutane on the precipitation of agg IgG* by pooled normal serum in assay EDTA buffer. The final serum dilution was 1:16. The final diaminobutane concentrations are shown.

**Fig. 4** Precipitation of agg IgG* by serum from a healthy individual and the influence of PEG. The serum was diluted in assay EDTA buffer and incubated with agg IgG* (40 000 cpm) in the presence of different concentrations of PEG. The different serum dilutions and PEG concentrations are expressed as final in the figure.

**Influence of PEG on precipitation of agg IgG* by sera of healthy individuals**

A serum from a healthy individual with a moderate precipitating activity of agg IgG* in assay EDTA buffer was tested for its ability to precipitate agg IgG* in a polymeric milieu. 200 μl of different serum dilutions were incubated with 200 μl assay EDTA buffer containing different amounts of PEG. 100 μl of the agg IgG* solution were added and the subsequent handling was as described. The final serum dilutions were from 1:5 to 1:320 (twofold dilution steps) and the final PEG concentrations were from 0 to 2%. It is evident from Fig. 4 that the precipitating activity of the serum was greatly enhanced in a polymeric medium. After heat inactivation of the serum at 56°C for 20 minutes the precipitating activity was lost even at a final PEG concentration of 2%.

**Precipitation of agg IgG* by Clq**

To 200 μl of different concentrations of highly purified Clq diluted in assay buffer was added 200 μl of 4% PEG in assay EDTA buffer and then 100 μl of agg IgG* solution. The incubation and subsequent procedures were as before. The enhancement of the Clq precipitating activity in a polymeric medium is shown in Fig. 5. By this procedure Clq was detectable at a concentration of 0.5–1 mg/l test solution. After heat treatment at 56°C for 30 minutes and in the presence of diaminobutane at a concentration of 12 mmol/l the Clq precipitating activity was lost.
Precipitation of \textsuperscript{125}I-labelled IgG aggregates by factors in sera

Precipitation of agg IgG* in the presence of EDTA by a serum from a patient with seronegative RA (●—●) and from a patient with seropositive RA (○—○). The range of the precipitating activity of sera from healthy individuals is indicated by the shaded area. The final serum dilutions in assay-EDTA buffer are shown.

Precipitation of agg IgG* in the presence of EDTA by fresh sera from patients with RA and SLE

The precipitating activity of sera from a few patients with chronic inflammatory disease was compared with the activity of sera from healthy individuals. 200 \( \mu l \) of the serum samples at different dilutions (twofold dilution steps) were incubated with 200 \( \mu l \) assay EDTA buffer and 100 \( \mu l \) of the agg IgG* solution. The assay procedures were as described above. The precipitating activity of the sera studied at the different dilutions from 5 patients with seropositive RA was within the range for normal sera. However, sera from 2 of 3 patients with seronegative RA and serum from 1 of 4 with SLE precipitated agg IgG* far above the normal range even at very high serum dilutions (Fig. 6). The extraordinary precipitation of agg IgG* by these pathological sera was further studied.

The serum from the SLE patient which precipitated agg IgG* in the presence of EDTA at a final serum concentration of 1:2024 was separated by gel chromatography on a Sephadex G-200 column. Each fraction was tested for the ability to precipitate agg IgG* in the presence of EDTA and in a polymeric medium. The incubations were performed as follows. To 200 \( \mu l \) of the different fractions was added 200 \( \mu l \) assay EDTA buffer or 200 \( \mu l \) 4% PEG in assay-EDTA buffer and 100 \( \mu l \) of the agg IgG* solution. The assay procedure was as previously described.

As shown in Fig. 7, there was a precipitating activity corresponding to the first and second peak of the chromatogram when tested in the presence of PEG. The precipitating activity of the first peak was lost after heat inactivation at 56°C for 20 minutes. The activity of the second peak was, however, intact after heat treatment. Without the presence of PEG there was no detectable precipitation of agg IgG* in any serum fraction. However, after the addition of Clq to each fraction, at a final concentration of 10 mg/l the precipitating activity was again located at the second peak of the chromatogram (Fig. 7). The pooled concentrated second peak (corresponding to the fraction volumes 160 ml–210 ml, Fig. 7) was further fractionated on a DEAE-Sephadex A-50 column. The precipitating activity was located in the IgG fractions of the chromatogram as shown by immune electrophoresis. Using a single affinity step on aggregated IgG-coupled Sepharose the material eluted in the acidic solution was identified as IgG by immunodiffusion against anti-IgG and antitotal sera. These findings suggest that the high serum precipitating activity of serum from this patient with SLE in the presence of EDTA was due to a coprecipitation between agg IgG*, Clq, and an anti-immunoglobulin of IgG nature. In the absence of Clq this antoglobulin still precipitated agg IgG* in the presence of certain polymers.

The serum from one of the patients with a seronegative RA and a high, whole serum precipitating activity in EDTA milieu (agg IgG* positive precipitation at a serum dilution of 1:1012) was also
chromatographed on a Sephadex G-200 column. Each fraction was analysed for the precipitation of agg IgG* in the presence of (1) EDTA buffer, (2) PEG, and (3) Clq, as in the previous case. Fig. 8 shows that in the presence of Clq or in a PEG milieu a precipitating activity is located in the fractions situated between the first and second peak of the chromatogram. These fractions were pooled and run through a column of agg IgG-Sepharose. After elution in the acidic solution a preparation was obtained which precipitated against anti-IgA in gel diffusion. This indicated the presence of an anti-immunoglobulin of IgA type, which evidently had the property of coprecipitating with Clq-agg IgG* complexes and also of precipitating agg IgG* in a polymeric medium.

Fig. 7 Fractionation pattern of serum from an SLE patient separated on a Sephadex G-200 column with optical density (OD) measurement at 280 nm (solid line). Each fraction was assayed for the precipitation of agg IgG* in assay EDTA buffer (●—●), and in a final 1-6% PEG milieu (x—x). By addition of Clq (10 mg/l) to each fraction the precipitating activity (o—o) was obtained.

Fig. 8 Fractionation pattern of a serum from a patient with seronegative RA separated on a Sephadex G-200 column. OD measurements are indicated by a solid line. Each fraction was assayed for the precipitating activity of agg IgG* in assay EDTA buffer (●—●), in a final 1-6% PEG medium (x—x), and in assay EDTA buffer with Clq (10 mg/l) present (o—o).
Precipitation of $^{125}$I-labelled IgG aggregates by factors in sera

Precipitation of Agg IgG* by heat-treated sera in PEG milieu: comparison between sera of healthy individuals and patients with RA

Patients with RA and rheumatoid factor activity assayed according to a conventional latex agglutination test (seropositive) and patients with a rheumatoid factor negative RA (seronegative) were compared with a control group of healthy individuals for the precipitating activity of agg IgG* by heat inactivated sera in a polymeric milieu. Sera, after treatment at 56°C for 20 minutes, were diluted 1:20 in assay buffer. 200 µl of the serum dilution and 200 µl of 4% PEG in assay buffer were incubated with 100 µl of the agglutination of IgG-coated sheep red blood cells or polystyrene latex particles. Sera from 80% of patients with RA are positive when tested by this method. There is evidence that these agglutination tests mainly reflect the amount of IgM-type rheumatoid factors. Recently, however, raised levels of anti-IgG-immunoglobulins of the IgG and IgA classes have been reported to occur in seronegative as well as seropositive (tested with agglutination methods) RA sera (Torrigiani et al., 1969, 1970).

These anti-IgG-immunoglobulins have been measured by binding them to insoluble cross-linked IgG as immunosorbent with subsequent elution of the specific antigen and quantitation by radial immunodiffusion (Torrigiani and Roitt, 1967). By using different techniques, increased levels of circulating immune complexes in sera of patients with RA have been found (Kunkel et al., 1961; Norberg, 1974; Hällgren and Wide, 1976). The nature of these complexes is still obscure, but there is evidence that the dominant constituents are IgG-rheumatoid factor complexes (Schrohenloher, 1966). Studies by Kunkel et al. (1961) and Winchester et al. (1969) have implicated anti-IgG-immunoglobulins of IgM as well as IgG types as factors in the pathogenesis of RA. The need for a simple and specific method of detecting all types of anti-IgG-immunoglobulins is apparent.

We have described a new technique for the detection and quantitation of anti-IgG-immunoglobulins which not only detects IgM antiglobulins but also antiglobulins belonging to the IgG and IgA classes, without defining the class specificity of the antigen activity in a given serum. The method is based on the principle that after anti-IgG-immunoglobulins have reacted with soluble IgG* a precipitation of the radioactive complexes will take place. Studies on sera from healthy individuals showed the presence of precipitins in these sera which influenced the specificity of the method for detecting rheumatoid factors. When the nature of the precipitins in normal serum was considered, it was found that there was an enhancement of IgG* precipitation in the presence of EDTA and polymers, and an inhibition of the precipitating activity after heat treatment or in the presence of diaminobutane.

The precipitating activity of soluble IgG aggregates in normal serum strongly suggests that it is mediated mainly by C1q due to similarities in reaction pattern. C1q, part of the first component of human complement, binds to the Fc-region of

Discussion

A serological feature of RA is the presence of rheumatoid factors that bind firmly to IgG which has undergone alteration of the Fc fragment during the formation of immune complexes or aggregation (Edelman et al., 1958). The conventional tests for rheumatoid factor activity are based on the agglutination of IgG-coated sheep red blood cells or polystyrene latex particles. Sera from 80% of patients with RA are positive when tested by this method. There is evidence that these agglutination tests mainly reflect the amount of IgM-type rheumatoid factors. Recently, however, raised levels of anti-IgG-immunoglobulins of the IgG and IgA classes have been reported to occur in seronegative as well as seropositive (tested with agglutination methods) RA sera (Torrigiani et al., 1969, 1970).

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The precipitating activity of soluble IgG aggregates in normal serum strongly suggests that it is mediated mainly by C1q due to similarities in reaction pattern. C1q, part of the first component of human complement, binds to the Fc-region of
IgG and IgM (Augener et al., 1971) and is thermo-labile (Müller-Eberhard and Kunkel, 1961). Diaminobutane interacts directly with Clq and inhibits its binding to immunoglobulins (Sledge and Bing, 1973). EDTA is known to enhance the interaction between Clq in normal serum and aggregated IgG in other systems (Agnello et al., 1970). The precipitating activity of isolated Clq was also found to be greatly enhanced in a polymeric milieu consisting of polyethylene glycol. This procedure, due to its precision and sensitivity enabling detection of 0.25–0.5 mg Clq/l test solution, may be used in experimental studies of Clq interactions with IgG.

The enhancement of the precipitation condition of Clq–IgG aggregates in a medium containing polymers could have a similar explanation as that suggested by Helsing (1966) for immune complexes: a decreased solubility because of steric exclusion.

Sera from several patients with seropositive RA had in a polymer-free medium less precipitating activity of agg IgG* than certain normal sera. This might be explained as a blockage of fixation of Clq to IgG by rheumatoid factors of the IgM type. There is evidence from other studies that the anti-IgG-immunoglobulins of the IgM class, probably by steric hindrance, prevent complement fixation to the IgG aggregate or complex (Schmid and Roitt, 1965; Zvaifler and Schur, 1968).

On the other hand, some sera from patients with seronegative RA and SLE had in the presence of EDTA a very high precipitating activity, well above normal. These sera contained anti-IgG-immunoglobulins of the IgA and IgG types and our results indicated that coprecipitation could occur between these anti-IgG-immunoglobulins, isolated Clq, and aggregated IgG. Using this technique for detection of Clq influenced precipitating activity, it seems possible to detect raised levels of complement binding rheumatoid factors. Further work will be needed to evaluate the clinical importance of such information.

The enhancement by dextran of the rheumatoid factor-IgG interaction was shown earlier both by agglutination of IgG-coated latex particles by sera containing rheumatoid factor and the formation of immune precipitates (Finkelstein et al., 1973). Polyethylene glycol in a final concentration of 1–6% was also found to increase the precipitation reaction between anti-immunoglobulins and soluble agg IgG*. These observations, explained by the mechanism of steric exclusion, were the basis for quantitation of anti-IgM-immunoglobulins only.

In the final design chosen for the clinical method for detecting of rheumatoid factor activity, the serum samples were heat inactivated to minimize the influence of Clq. In order to discriminate between different rheumatoid factor activities the sera were diluted to a final concentration of 5% before being assayed. In higher serum concentrations a considerable number of normal heat-treated sera also had the property to precipitate soluble IgG aggregates in a polymeric milieu. This is consistent with other investigations and the finding of IgG and IgA anti-IgG-immunoglobulins at low levels in sera from normal individuals (Torrigiani and Roitt, 1967). When the adopted method was then tested on sera with definite and classical RA it was found that all patients with seropositive and 58% of patients with seronegative RA had raised concentrations of anti-IgG-immunoglobulins. This is not surprising, as our test is more sensitive than agglutination procedures. Furthermore, the precipitation of agg IgG* by heat inactivated sera in a polyethylene glycol medium evidently reflects the presence of anti-IgG-immunoglobulins of the IgG or the IgA, as well as the IgM classes.

The method is sensitive and highly reproducible and in laboratories which have radioactive counting equipment it can be used to analyse many samples simultaneously. In those cases where the clinical diagnosis of RA is less obvious and when conventional rheumatoid factor tests are negative the precipitating activity in a polymeric milieu might be helpful in establishing the proper diagnosis.

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Precipitation of $^{125}$I-labelled IgG aggregates by factors in sera

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