

# Detection of IgG rheumatoid factor by concanavalin A treatment and complement fixation with IgG rheumatoid factor

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**Tanimoto, K., Moritoh, T., Azuma, T., and Horiuchi, Y. (1976).** *Annals of the Rheumatic Diseases*, 35, 240–245. **Detection of IgG rheumatoid factor by concanavalin A treatment and complement fixation with IgG rheumatoid factor.** Concanavalin A (Con A) forms precipitates with carbohydrate-rich protein such as IgM, IgD, IgE, and IgA. Since IgG contains little carbohydrate and does not react with Con A, the activity of IgG-rheumatoid factor (RF) can be measured in the supernate of the Con A-treated serum. When the latex fixation test (LFT) and the sensitized sheep cell agglutination test (SSCA) were performed in the supernate for the detection of IgG-RF, LFT was positive in 32.1% of sera, out of 137 sera originally positive for LFT, and SSCA was positive in 18.5% of sera, out of 119 sera originally positive for SSCA.

IgG-RF exhibited lower complement fixing ability than IgM-RF and correlated with agglutination titres of IgG-RF, while the  $CH_{50}$  of the original serum did not correlate with haemolytic activities of either IgM-RF or IgG-RF.

The immunoglobulin class of most rheumatoid factors (RF) is IgM, but recent reports have shown that RF also belongs to IgG and IgA classes (Chodirker and Tomasi, 1963; Heimer and Levin, 1966; Torrigiani and Roitt, 1967). Since the usual agglutination tests for RF, such as the latex fixation test (LFT) or the sensitized sheep cell agglutination test (SSCA), are based on the agglutinating activity of IgM-RF, they are not suitable to detect IgG-RF or IgA-RF because neither has as strong an agglutinating activity as IgM-RF. Therefore, another method or procedure to eliminate IgM-RF from serum is required to detect IgG-RF or IgA-RF.

A new method for the detection of IgG-RF using concanavalin A (Con A) is described. Con A reacts with carbohydrate-rich protein and forms precipitates (Leon, 1967; Goldstein and others, 1969; Weinstein, Givol, and Strausbauch, 1972). Since IgM, IgD, IgE, and IgA contain relatively high amounts of carbohydrate (>8%), they form precipitates with Con A, while IgG contains only 2.9% carbohydrate and does not form a precipitate with Con A (Leon, 1967; Goldstein and others, 1969). Therefore when rheumatoid sera are treated with Con A and precipitates are removed, IgG-RF is easily detectable

in the supernatants without contamination of RFs belonging to other immunoglobulins.

Complement fixing ability of IgG-RF can also be studied with Con A-treated sera, and the results are discussed from the point of view of the role of complement in rheumatoid inflammation.

## Materials and methods

### RHEUMATOID SERA

137 LFT positive sera from patients with classical or definite rheumatoid arthritis (RA) in our clinic were used. The sera were heat-inactivated at 56°C for 30 minutes and natural antibodies to sheep red cells were absorbed by incubation with washed packed sheep red cells at 37°C for 90 minutes.

### CON A TREATMENT

Jack bean meal (Sigma Chemical Co., St. Louis, U.S.A.) was dissolved in 0.15 mol/l NaCl and centrifuged at 10000 r.p.m. for 30 minutes. The supernates were applied on the Sephadex G 50 column and Con A in the supernates were adsorbed to the Sephadex beads (Pharmacia Fine Chemicals, Uppsala, Sweden). Con A was eluted from the adsorbed Sephadex beads in 0.3 mol/l methyl- $\alpha$ -D-glucoside and dialysed against 0.15 mol/l NaCl. Optimal

concentration of Con A for the experiment was determined as follows. 0.2 ml heat inactivated and absorbed rheumatoid sera were added to 0.8 ml of solutions containing various amounts of Con A and incubated at 37°C for 60 minutes. After centrifugation at 3000 r.p.m. for 10 minutes, precipitates were removed and 1.0 ml of 0.3 mol/l methyl- $\alpha$ -D-glucoside was added to each supernatant to neutralize the activity of Con A. Neutralization was confirmed by the disappearance of agglutinating activity of Con A against both sheep and human red cells.

Levels of IgG, IgA, and IgM in Con A-treated sera were measured by single radial immunodiffusion as described by Mancini, Carbonara, and Heremans (1965) using Partigen Immunoplates (Behring Institute, Germany). Optimal concentration of Con A was chosen at the point of maximal decreased IgM level and minimal decreased IgG level.

MEASUREMENT OF RF ACTIVITY

After Con A treatment with the optimal concentration, RF activity of each serum was measured with LFT (RA-test, Hyland, Calif.) and with SSCA. For SSCA, sheep red cells (SRC) were sensitized with four times dilution of minimal agglutination dose of IgG rabbit anti-SRC (IgG haemolysin) and haemagglutination procedure was performed with Micro-Titer (Cooke Engineering Co., Va.). 7S fractions of rabbit anti-SRC serum were separated by Sephadex G 200 gel filtration and used as IgG haemolysin. Gelatin veronal buffer (GVB, pH 7.4) containing 0.15 mol/l methyl- $\alpha$ -D-glucoside was used as the reaction buffer.

MEASUREMENT OF COMPLEMENT FIXING ABILITY OF RF

Complement-fixing ability of IgM- and IgG-RF was evaluated in the direct haemolytic test as described previously (Tanimoto and others, 1975). Rabbit IgG haemolysin was reduced with 0.01 mol/l dithiothreitol at room temperature for 30 minutes and alkylated with 0.015 mol/l iodacetamide at 0°C for 120 minutes. Reduced and alkylated IgG haemolysin loses complement fixing ability while retaining antigen binding activity and reactivity to RF (Tanimoto and others, 1975). SRC sensitized with the reduced and alkylated IgG haemolysin were prepared in the same way as the nonreduced IgG haemolysin described above. The sensitized SRC were incubated with Con A treated or nontreated rheumatoid sera at 37°C for 60 minutes and at 0°C for 180 minutes. The reaction between the sensitized SRC and RF was macroscopically observed as haemagglutination.

SRC treated with the above method were washed three times with 0.15 mol/l gelatin veronal buffer pH 7.4, containing optimal amounts of Ca<sup>++</sup> and Mg<sup>++</sup> (GVB<sup>++</sup>). Excess Con A and methyl- $\alpha$ -D-glucoside were removed during this procedure. The cells suspended in GVB<sup>++</sup> were added to diluted human complement in which natural antibody to SRC had been absorbed in advance.

Haemolysis of the sensitized SRC by rheumatoid factor occurred after incubation at 37°C for 60 minutes with human complement. Complement fixing ability of RF was estimated by the measurement of haemoglobin concentration in the supernatant at 412 m $\mu$  on Hitachi's spectrophotometer as per cent haemolysis.

MEASUREMENT OF SERUM COMPLEMENT LEVELS

Serum complement levels were determined by Mayer's 50% haemolytic method (CH<sub>50</sub>) (Mayer, 1961) using SRC sensitized with rabbit IgM haemolysin.

Results

As shown in Fig. 1, treatment with 10 mg/ml Con A resulted in maximal reduction of IgM to undetectable level and IgA to about 40% of original serum, while the IgG level remained almost unchanged. At this concentration of Con A, 93 of 137 LFT positive sera (67.9%) became negative after the treatment (Fig. 2, below the horizontal line). 44 cases (32.1%)

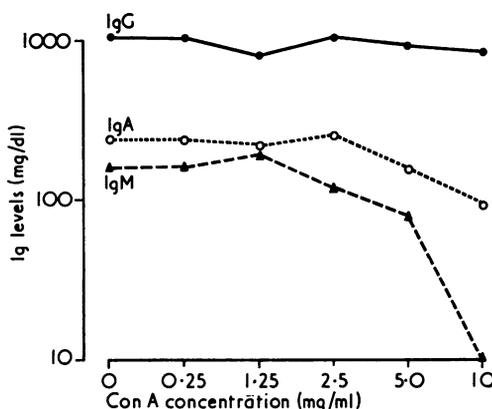


FIG. 1 Effect of concanavalin A (Con A) treatment on IgG, IgA, and IgM levels. Rheumatoid serum was incubated at 37°C for 60 minutes with various amounts of Con A. After removal of precipitates, levels of IgG, IgA, and IgM were measured by single radial immunodiffusion

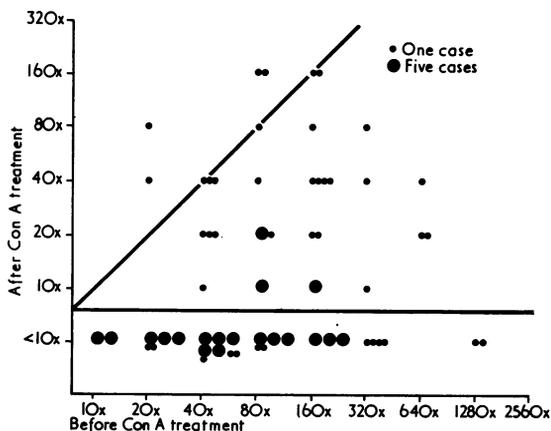


FIG. 2 Relation of latex fixation test (LFT) titres of rheumatoid sera before and after Con A treatment. Negative LFT cases after Con A treatment are shown below the horizontal line. Cases with increased LFT titres after Con A treatment are seen over the diagonal line.

remained positive, although 34 of them showed reduction in titres. On the other hand, 4 showed raised titres (over the diagonal line) and 6 cases were unchanged.

Similarly, 97 of 119 SSCA positive rheumatoid sera (81.5%) lost agglutinating activities after Con A treatment (Fig. 3). Only 22 cases (18.5%) remained positive, and 20 of them showed some reduction of their titres. A single case gave a raised titre and another one no change.

In neither LFT nor SSCA was a significant correlation found between agglutination titres before and after Con A treatment, that is, IgG-RF activity did not depend on agglutinating activity of IgM-RF in original serum.

When 85 cases of normal sera and 20 seronegative rheumatoid sera were tested in the same manner as above, none of them showed RF activity with Con A treatment. (Data were not included in the figures.)

The results of complement fixing ability of rheumatoid sera before and after Con A treatment are summarized in Figs. 4 and 5. Before Con A treatment the rheumatoid sera showed relatively high haemolytic activities correlated with agglutinating activities of RF assessed by LFT or SSCA. After Con A treatment, haemolytic activities were markedly reduced, although they still maintained higher levels than control and showed positive correlations with LFT or SSCA activities. The cases which showed raised RF activities after Con A treatment produced slightly increased haemolysis.

Haemolytic activities of the rheumatoid sera either before or after Con A treatment did not correlate with  $CH_{50}$  of the original sera as shown in Fig. 6.

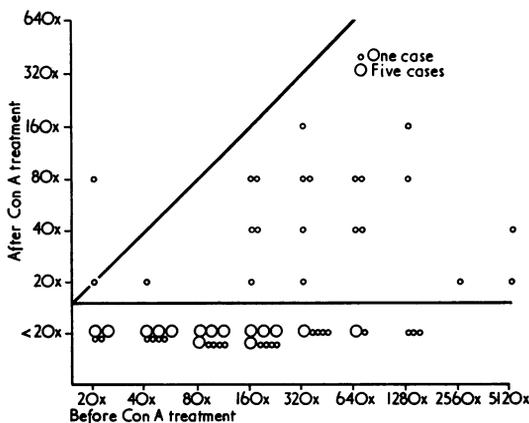


FIG. 3 Relation of sensitized sheep cell agglutination (SSCA) titres before and after Con A treatment of rheumatoid sera. Negative SSCA cases after Con A treatment are given below the horizontal line, and a case with increased SSCA titres is seen over the diagonal line

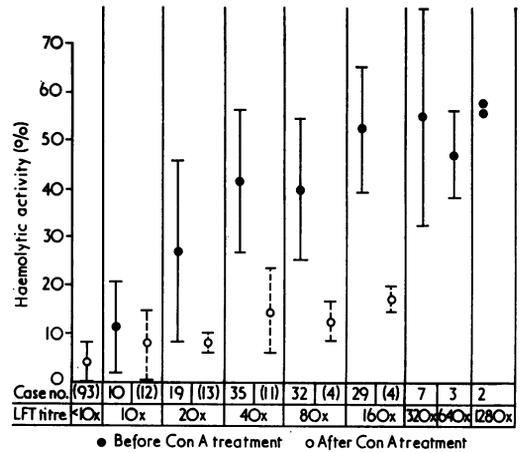


FIG. 4 Relation between LFT titre and complement fixing ability before and after Con A treatment of rheumatoid sera. Haemolytic activities before Con A treatment (●) and after Con A treatment (○) are given as vertical bars. Cases were divided into 9 groups according to their LFT titres, and case numbers are shown

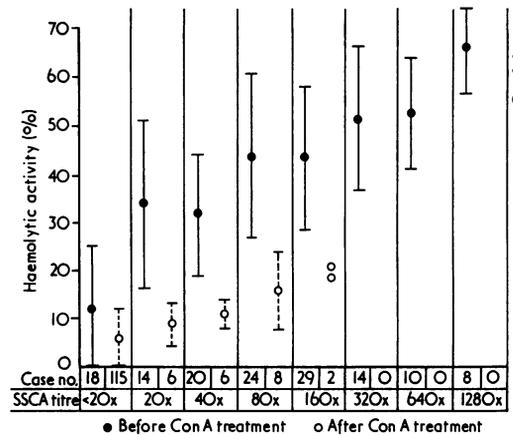


FIG. 5 Relation between SSCA test titres and haemolytic activities before (●) and after (○) Con A treatment of rheumatoid sera, shown by vertical bars. Cases were divided into 8 groups according to their SSCA titres, and case numbers are shown

**Discussion**

The method for the detection of IgG-RF described has several advantages over reported methods, which are summarized as follows.

(1) Immunoabsorbent method (Torrighiani and Roitt, 1967; Panush, Bianco, and Schur, 1971; Itler and Turner, 1973; Abruzzo and Heimer, 1974). RFs are first adsorbed on to the insoluble aggregated IgG and dissociated with acid or high ionic strength

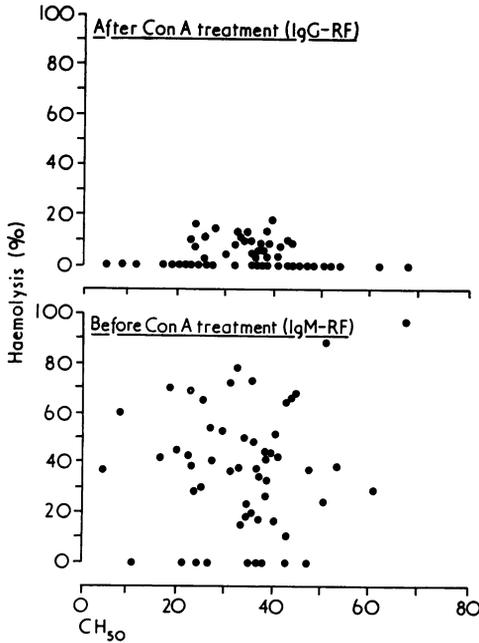


FIG. 6 Comparison of  $CH_{50}$  of original rheumatoid sera with haemolytic activities of IgG- and IgM-RF.  $CH_{50}$  (unit/ml) measured with Mayer's method were plotted against haemolytic activities (% haemolysis) before Con A treatment and after Con A treatment

solutions. Dissociated RF levels are determined quantitatively as immunoglobulin class.

(2) Augmentation technique (Adachi and others, 1969). IgG-RF can be evaluated by the augmentation of haemagglutination, when rabbit antihuman IgG serum is added to the test system using SRC sensitized with equine IgG.

(3) Pepsin digestion of IgM (Theofilopoulos and others, 1974). Since pepsin-treated serum loses agglutinating activity of IgM-RF, weak agglutinating activity of IgG-RF in the treated serum can be found with the usual agglutination tests.

(4) Immunofluorescent method (Estes, Atra, and Peltier, 1973). Fluorescein isothiocyanate (FITC) labelled rabbit antihuman IgG serum stains IgG-RF bound to the human red cells sensitized with rabbit antihuman red cell serum.

In the immunoabsorbent method RF dissociated from insoluble aggregated IgG in acid may lose antibody activity since acid has the ability to destroy antibody molecule. When the levels of IgG and IgA were measured with single radial immunodiffusion after dissociation, the reported values were considerably higher than expected (Panush and others, 1971; Bianco and others, 1971). Therefore, the possibilities of nonspecific adsorption of immunoglobulins other than RF to aggregated IgG might be taken into consideration in this procedure.

Similarly there remains some doubt whether in the immunofluorescent method (Estes and others, 1973) positive staining on the human red cells sensitized with rabbit antibody really represents the specific RF activity. In order to exclude these nonspecific adsorptions, Theofilopoulos and others (1974) reported the detection of IgG-RF with pepsin digestion of rheumatoid serum. However, the procedure has further problems. Firstly, since IgA molecules might remain undigested even after pepsin digestion, the values of their IgG-RF might contain a considerable amount of IgA-RF. Secondly, it is well known that pepsin digestion destroys the Fc portion of the IgG molecule and produces  $F(ab')_2$ . For this reason this procedure is not appropriate for the estimation of biological activities of IgG-RF such as complement fixation.

Detection of IgG-RF with Con A treatment does not have any effect on the Fc portion of the IgG molecule, since Con A reacts only with carbohydrate-rich proteins such as IgM, IgD, IgE, and IgA. Therefore this method is suitable for the study of complement fixation. In addition the loss of IgG is almost negligible.

Published reports have indicated that Con A treatment removed not only IgM but also IgA (Leon, 1967; Goldstein and others, 1969; Weinstein and others, 1972), while in our study 60% of IgA was removed. Such contamination of IgA also occurred in pepsin digestion of serum for the detection of IgG-RF (Theofilopoulos and others, 1974). However, the influence of contaminated IgA will be negligible for the estimation of complement fixation because IgA antibody cannot activate complement via the classical pathway or haemolyse the red cells.

For the detection of IgG-RF, methods other than LFT or SSCA should be considered since agglutinating activities of IgG-RF are not so strong as those of IgM-RF. Authors tried to use reversed immunodiffusion in agarose containing aggregated human IgG as described by Theofilopoulos and others (1974) for the detection of IgG-RF after Con A treatment. However, sharp precipitin rings did not appear in the plate, and we have considered that this method would not be appropriate for the quantitation of IgG-RF. When LFT was compared with SSCA for sensitivity in the detection of IgG-RF, LFT showed a higher incidence of positive results than SSCA. This result confirmed the report by Theofilopoulos and others (1974).

A few cases exhibited increased RF activities after Con A treatment, the exact cause of which is not clear. One explanation is the removal of some unknown inhibitor containing Con A reactive carbohydrate, although this mechanism was not proved. These unusual cases did not have any special clinical features and their values of  $CH_{50}$  were within normal

limits. There are several reports indicating that even seronegative cases contain considerable amounts of IgG-RF (Panush and others, 1971; Bianco and others, 1971), and sera with high titre RF sometimes showed prozone phenomenon (Harboe, 1960). These findings may have some bearing on our results. Further investigations are in progress in order to clarify the mechanism of the raised RF activity after Con A treatment.

The results of the present study indicate that IgG-RF has weak complement-fixing ability which correlated with its agglutination activity. It was already proved that the direct haemolytic method used in this study is sensitive for the estimation of complement fixation with IgM-RF (Tanimoto and others, 1975). It is conceivable that this method is also sensitive with IgG-RF.

The finding that IgG-RF is able to fix complement suggests that IgG-RF could play a role in the inflammatory process in RA through complement activation. In fact, some synovial fluids of patients with RA show anticomplementary activities (Marcus and Townes, 1971; Townes and Marcus, 1972) and in these cases immune complexes mainly consist of IgG-RF rather than IgM-RF (Winchester, Agnello, and Kunkel, 1969, 1970). These findings also support the concept that some IgG-RFs have the ability to fix complement.

Finally, there was no correlation between the level

of serum CH<sub>50</sub> and haemolytic activity of either IgM-RF or IgG-RF. Although it has been reported that some groups of RA patients with systemic vasculitis showed high RF titres and low serum complement levels (Mongan and others, 1969; Franco and Schur, 1971), we could not confirm this because the number of such specific type of RA was so small in our study. It is more likely that immune complexes or aggregated IgG, which have strong anticomplementary activity, will directly affect the reduction of the serum complement level in RA rather than RF in either Ig class. In fact, several reports indicate that immune complexes were detected in hypocomplementaemic synovial fluids in RA (Hannestad, 1967; Winchester and others, 1969; Marcus and Townes, 1971; Munthe and Natvig, 1971; Mellbye and Natvig, 1971; Townes and Marcus, 1972). Although in this study IgG-RF positive cases did not exhibit any special clinical features, further analysis will be required for the elucidation of a pathogenetic role and clinical significance of IgG-RF.

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