Modified Anti-C3 Immune Complex Assay Which Avoids Interference by Anti-F(ab')2 Antibodies

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FUKUDA, K., SEINO, J., KINOSHITA, Y., SUDO, K., HORIGOME, I., FURUYAMA, T. and YOSHINAGA, K. Modified Anti-C3 Immune Complex Assay Which Avoids the Interference by Anti-F(ab')2 Antibodies. Tohoku J. exp. Med., 1985, 146 (3) 337-347 — The present authors and Olds et al. reported that the anti-F(ab')2 antibodies (Abs) in serum interfere with the solid phase (SP) anti-C3 immune complex assay. The anti-F(ab')2 Abs in human sera bind solid phase F(ab')2 anti-C3 of rabbit or goat, and were measured erroneously as C3 bearing circulating immune complexes (CIC). Gel filtration analysis of SP anti-C3 assay revealed that C3 bearing CIC is detected only in heavy fractions and 7S CIC-like activity is not CIC but anti-F(ab')2 activity. As the molecular weight of such CIC is heavy enough to be precipitated by 5% polyethylene glycol (PEG) and IgG anti-F(ab')2 Abs and free C3 are not included in 5% PEG precipitates, 5% PEG precipitates of the test sera were used for SP anti-C3 (Modified SP anti-C3). CIC measured by modified SP anti-C3 were positive in 14/16 at active stage of SLE and positive only in 2/16 at inactive stage. CIC by this test were also correlated well to serum complement activity, and were thought to be clinically reliable and useful.

Circulating immune complexes (CIC) have many complex biological activities and their assay has been difficult, though many different methods have been described.

Complement activating CIC are thought to bear C3 fragments of complement on their molecular surface. The solid phase (SP) anti-C3 assay (Pereira et al. 1980) is now widely used to detect such C3 bearing CIC, because the method is comparatively easy and also useful for detecting not only IgG-CIC but also IgA or IgM-CIC. The principle of SP anti-C3 assay is depicted in Fig. 1-a. For the detection of C3 binding CIC, F(ab')2 or Fab fragments of anti-C3 must be used in such sandwich assay in order to avoid the interference by rheumatoid factors. But interference by anti-F(ab')2 antibodies (Abs), as illustrated in Fig. 1-b, has

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been reported (Olds and Miller 1984; Fukuda et al. 1985), indicating that anti-F(ab')\textsubscript{2} Abs were erroneously measured as C3 bearing CIC by the SP anti-C3 assay. These anti-F(ab')\textsubscript{2} Abs in human sera have partial cross reactivity with Fab or F(ab')\textsubscript{2} fragments of another species such as rabbit, bovine, goat, guinea pig and so on (Ling and Drysdale 1981; Olds and Miller 1984). Moreover, anti-F(ab')\textsubscript{2} Abs react Fab or F(ab')\textsubscript{2} fragments obtained by any enzymatical methods (Waller et al. 1968, 1969). Therefore the interference by anti-F(ab')\textsubscript{2} Abs is inevitable in the SP anti-C3 assay when no procedure was taken to eliminate them prior to the assay. Besides, interference by free C3 with the assay must be considered, because free C3 far exceeds the CIC-bound C3 in quantity.

In order to separate CIC from free C3 and IgG anti-F(ab')\textsubscript{2} Abs, we precipitated CIC by 5% polyethylene glycol (PEG) and the precipitates were tested by SP anti-C3 assay (modified SP anti-C3 assay).

**Materials and Methods**

**Sera of patients**

Blood samples were collected from 35 patients with lupus nephritis, who were receiving steroid therapy. Blood was kept at room temperature for 1 hr and centrifuged at 3,000 \( \times \) g for 15 min. Serum samples were stored at \(-80^\circ\)C and examined within 4 weeks. The sera, once thawed, were not stored again.

To test the reliability of Clq solid phase assay, conglutinin binding assay and modified SP anti-C3 assay, the sera from 16 patients in an active state of lupus erythematosus with hypocomplementemia (CH50 lower than 15 U/ml) were studied, and these 16 patients were again checked later when they were improved by treatment and their complement levels were restored to normal. The average interval of these two samplings was 2 months. As a control group, 6 patients with lupus nephritis in stable state with normal serum complement activity and 6 patients with membranoproliferative glomerulonephritis (MPGN) with lowered CH50 were studied. Twice samplings were done from these 12 control patients at
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a mean interval of 6 months and the sera were frozen at –80°C and tested simultaneously.

\( F(\text{ab}'_2) \) anti-C3

Hyperimmune anti-C3 serum was prepared in rabbit injected repeatedly with purified C3b (Mardiney and Mueller-Eberhart 1965).

The rabbit anti-C3 antiserum was dialysed against 0.0175 M phosphate buffer, pH 6.4, then applied to a DEAE-Sepharose column in the same buffer. The IgG fraction was digested by pepsin, and the digest was applied to a Sephadex G-100 column. The first peak was collected and was further incubated with protein A-Sepharose to remove intact IgG. The antibody activity of \( F(\text{ab}'_2) \) anti-C3 was confirmed by the agglutinating activity to EAC3b and by the reproducibility of standard aggregated human globulin (AHG) curve.

Rabbit \( F(\text{ab}'_2) \) fragments without specific antibody-activity was prepared from normal rabbit serum by the same method as described above.

The purity of \( F(\text{ab}'_2) \) was proved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It had no reactivity to protein A.

Anti-C3 assay

One hundred \( \mu l \) of \( F(\text{ab}'_2) \) anti-C3 as 0.1% solution in 0.01 M carbonate buffer (pH 9.5) was added to each well of a polyvinyl chloride microtiter plate (Cooke Co., Virginia, USA) and incubated at 37°C for 3 hr, then kept at 4°C overnight. Unbound \( F(\text{ab}'_2) \) anti-C3 was removed and stored at 4°C until next use. After washing the wells with phosphate buffer saline (PBS)-0.05% Tween 20, 200 \( \mu l \) of 1% bovine serum albumin (BSA) in PBS was added and incubated at 37°C for 1 hr to prevent non-specific adsorption of proteins. The wells were washed with PBS-0.05% Tween 20 again. 100 \( \mu l \) of serum samples or PEG precipitates of the sera finally diluted 1:12 with dilution buffer (PBS containing 0.05% Tween 20, 0.5% BSA and 10 mM EDTA) was added and incubated at 37°C for 1 hr. After washing with PBS-Tween 20, anti-IgG labeled with horseradish peroxidase (HRPO : Sigma Co., St. Louis, USA) was added instead of \( ^{125}\text{I}-\text{anti-IgG} \) in the original method (Pereira et al. 1980), then incubated at 37°C for 1 hr. The bound HRPO activity was determined as CIC values by colorimetry with o-phenylenediamine at OD492.

To separate CIC from free IgG and free C3, 20 volumes of 5% PEG in PBS containing 10 mM EDTA were added to one volume of the serum. The mixture was incubated at 0°C for 1 hr, then centrifuged at 2,000 g for 30 min. After washing the precipitates three times, the pellets were dissolved in the dilution buffer at the final concentration of 1:12 and tested by SP anti-C3.

Measurement of anti-rabbit \( F(\text{ab}'_2) \) activity

One hundred \( \mu l \) of rabbit \( F(\text{ab}'_2) \) fragments as 0.1% solution in 0.01 M carbonate buffer (pH 9.5) was added to each well. The method outlined in anti-C3 assay was then followed.

Purification of anti-\( F(\text{ab}'_2) \) Abs by affinity chromatography

Each 1 ml of serum from 50 patients with glomerulonephritis was mixed, and was made in 10 mM EDTA and the mixture was applied to a human \( F(\text{ab}'_2) \)-Sepharose column. The column was washed with 500 ml of PBS and anti-\( F(\text{ab}'_2) \) Abs were eluted with 0.1 M glycine buffer, pH 2.5. The anti-\( F(\text{ab}'_2) \) activity of the eluted material was measured by enzyme linked immunoassay (EIA).

Gel filtration analysis of CIC-like activity

0.5 ml of serum samples were applied to a 1.5 x 100 cm column of Sephadex G-200 equilibrated with PBS containing 2 mM EDTA, and 3 ml fractions were collected at a flow rate of 0.2 ml/min. Serum samples were incubated with or without rabbit \( F(\text{ab}'_2) \)-Sepharose prior to gel filtration. Each fraction was analysed subsequently by SP anti-C3 assay.
Complement activity

Total hemolytic activity (CH50) was measured according to Mayer's method. Alternative complement pathway activity (AH50) was measured using rabbit erythrocytes (Platts-Mills and Ishizaka 1974).

RESULTS

CIC values determined by SP anti-C3 assay and anti-rabbit F(ab')2 activity

Fig. 2 shows the relationship between CIC-like activity by the conventional SP anti-C3 assay and anti-rabbit F(ab')2 activity. The two results are almost identical.

Gel filtration analysis of CIC-like activity by SP anti-C3 assay

Three sera with high CIC-like activity were further analyzed by gel filtration on Sephadex G-200. As shown in Fig. 3, all samples exhibited reactive peaks of CIC-like activity in IgG fractions. The peak of anti-F(ab')2 activity was also found in IgG fractions in each case when unprocessed sera were applied. On the other hand, after the anti-rabbit F(ab')2 Abs were absorbed by rabbit F(ab')2-Sepharose, the peak in IgG fractions disappeared and the reactivity remained in a heavier fraction alone.

Analysis of purified anti-F(ab')2 Abs

Purified anti-F(ab')2 Abs contained IgM, IgG and IgA but no complement component C3, C4, factor B(Fig. 4). The IgG content was 1 mg and that of IgA...
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Fig. 3. Gel filtration analysis by Sephadex G-200 column on three sera in which CIC-like activity was high by conventional SP anti-C3 assay. ○: CIC-like activity by SP anti-C3. ▲: Anti-F(ab')₂ activity in the fractions when the neat sera were applied to gel filtration. ●: CIC values by SP anti-C3 assay in the fractions when the sera preincubated with rabbit F(ab')₂-Sepharose were applied.

Fig. 4. Double immunodiffusion analysis of affinity purified anti-F(ab')₂ antibodies. F: purified anti-F(ab')₂ Abs. M: anti-IgM. G: anti-IgG. A: anti-IgA. B: anti-factor B. 3: anti-C3. 4: anti-C4.
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was 0.15 mg. The obtained IgG anti-F(ab')2 Abs reacted well with solid phase human F(ab')2 fragments and the reaction was not inhibited by 100 μg/ml of human F(ab')2 fragments but was inhibited by 100 μg/ml of heat aggregated human F(ab')2 fragments. The IgG anti-F(ab')2 Abs also reacted with solid phase rabbit F(ab')2 fragments weakly but did not react with solid phase rabbit IgG (Fig. 5). C3-IgG complexes were not detected in the affinity purified anti-F(ab')2 Abs by SP anti-C3 using rabbit IgG anti-C3. Thus, anti-F(ab')2 Abs that interfere with the SP anti-C3 assay were proved not to contain C3 bearing CIC.

**Anti-C3 assay on 5% PEG precipitates of sera and the sera preincubated with rabbit F(ab')2-Sepharose**

Fig. 6 shows results of the two assays. When the sera were preincubated with rabbit F(ab')2-Sepharose and tested by SP anti-C3 assay, free C3 remained in the sera and interfered with the assay, and false negative results were obtained in sera with high CIC titers. On the other hand, when 5% PEG precipitates of the sera were tested by SP anti-C3 assay, CIC were detected undisturbed by C3 because free C3 had not been eliminated by 5% PEG precipitation.

As anti-F(ab')2 Abs are thought to be autoantibodies to immune complexes, anti-F(ab')2 Abs may bind to CIC and may be precipitated by 5% PEG if anti-F(ab')2 Abs coexist with CIC. Indeed, anti-human F(ab')2 Abs were sometimes found in 5% PEG precipitates (4/40). But anti-rabbit F(ab')2 Abs were
rarely precipitated by 5% PEG even if CIC were precipitated (Fig. 7).

*CIC-values determined by SP anti-C3 assay on the 5% PEG precipitates of the sera and complement activity of the sera*

CIC titers in the 5% PEG precipitates of the sera were found to correlate well
Fig. 8. Correlation between CIC in 5% PEG precipitates detected by SP anti-C3 assay and complement hemolytic activity. AH50: hemolytic activity of the alternative pathway.

Fig. 9. Comparison of CIC values measured by three different methods. a, d: conglutinin binding assay. b, e: Clq solid phase assay. c, f: modified anti-C3 assay on the 5% PEG precipitates of the sera. a, b, c: control group in clinically stable state. Solid line: SLE with normal CH50. Dotted line: MPGN with continuous low CH50. d, e, f: SLE in active stage and in inactive stage.

with the values of complement activity (CH50 and AH50) of the same sera. AH50 had better correlation with CIC titers than CH50 did (Fig. 8).
Clinical usefulness of Clq solid phase assay, conglutinin binding assay and modified anti-C3 assay

Fig. 9 shows the results of these three assays. Modified anti-C3 assay is thought to be the best in the three. CIC measured by modified anti-C3 assay were positive in 14/16 at active stage of SLE and positive only in 2/16 at inactive stage (Fig. 9-f). CIC measured by conglutinin binding assay or Clq solid phase assay did not correlate with the activity of the disease.

DISCUSSION

Anti-F(ab')₂ Abs were initially called pepsin agglutinators because of their ability to agglutinate blood cells coated with F(ab')₂ which was prepared from pepsin-treated IgG (Osterland et al. 1963). They are usually seen in human sera as IgG antibodies to the so-called pepsin sites of pepsin treated IgG (Mellbye and Natvig 1970; Davey and Korngold 1982; Birdsall and Rossen 1983). However, anti-F(ab')₂ Abs also react with F(ab')₂ or Fab fragments obtained by other enzymes such as trypsin or papain (Waller et al. 1968, 1969). They react not only with human F(ab')₂ fragments but also though partially, with F(ab')₂ fragments of another species such as rabbit, bovine and goat (Ling and Drysdale 1981).

We have demonstrated that levels as measured by the SP anti-C3 assay are unreliable in the presence of anti-F(ab')₂ Abs. The CIC-like activity detected by conventional SP anti-C3 assay is usually found in the 7S fractions. This has previously been explained by the concept of C3d bearing IgG (Pereira et al. 1981) and of low molecular weight C3 bearing immunoglobulin (Jacobs and Richilin 1983). But we revealed that low molecular weight CIC-like activity is anti-F(ab')₂ activity.

It is necessary for the detection of C3 bearing immunoglobulins that anti-F(ab')₂ Abs are removed before the assay. We used two methods for this purpose. First, sera were preincubated with rabbit F(ab')₂-Sepharose and secondly CIC were precipitated by 5% PEG which excludes free C3 and free IgG. When the sera preincubated with rabbit F(ab')₂-Sepharose were tested by SP anti-C3 assay, free C3 interfered with the assay and false negative results were obtained not infrequently. As C3 bearing IgG occupies heavier molecular weight fraction in gel filtration and free IgG and free C3 are not precipitated by 5% PEG, anti-C3 assay of the 5% PEG precipitates of the sera is thought to be beneficial. CIC values in 5% PEG precipitates correlated well with complement activity in SLE sera. AH50 correlated to CIC values better than CH50. This may be explained by the fact that alternative pathway of the complement is essential for solubilization of immune complexes (Takahashi et al. 1980). CIC are thought to consume essentially the components of alternative pathway.

CIC detected by SP anti-C3 on 5% PEG precipitates of the sera reflected clinical activity in SLE patients. The high CIC values are not due to IgG
aggregation, because −80°C storage of sera for more than 6 months did not affect CIC values.

In the case of MPGN where C3 nephritic factor (C3NeF) is present in serum, IgG-C3-factor B complex must be detected by the modified SP anti-C3 assay. CIC were negative when C3NeF positive sera were tested by modified SP anti-C3 assay. But when C3NeF positive sera were preincubated with normal human sera at 37°C for 15 min in glycolethylenediamine tetraacetic acid (EGTA)-Mg gelatin veronal buffer and were tested by modified SP anti-C3 assay, CIC were highly positive.

If CIC coexist with anti-F(ab’)2 Abs, anti-F(ab’)2 Abs may bind to CIC and may be precipitated by PEG. But anti-rabbit F(ab’)2 Abs were rarely found (1/80) in 5% PEG precipitates, while anti-human F(ab’)2 Abs were found more frequently (4/40). This may be due to differences in quantity and affinity of antibodies. Anti-human F(ab’)2 Abs exceed anti-rabbit F(ab’)2 Abs in quantity (Ling et al 1981) and probably in affinity. C3 or C3-IgG complexes are not detected in the affinity purified anti-F(ab’)2 Abs. This suggests that anti-F(ab’)2 Abs do not react with C3-bound CIC.

Except the rare cases where anti-rabbit F(ab’)2 Abs are precipitated by 5% PEG, it is possible to avoid the interference by anti-F(ab’)2 Abs with the SP anti-C3 assay by means of 5% PEG precipitation.

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References


