Fc-rosette inhibition by hypocomplementaemic systemic lupus erythematosus sera

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Morito, T., Tanimoto, K., Hashimoto, Y., Horiuchi, Y., and Juji, T. (1976). Annals of the Rheumatic Diseases, 35, 415–421. Fc-rosette inhibition by hypocomplementaemic systemic lupus erythematosus sera. Human red cells sensitized with one of the Rh antisera (Ripley) form rosettes (Fc-rosette) with human B lymphocytes and the rosettes are well inhibited by aggregated human IgG. Since sera of hypocomplementaemic patients with systemic lupus erythematosus (SLE) have frequently been reported to contain immune complexes, they were used for the inhibition of Fc-rosette formation in this study.

The results of Fc-rosette inhibition rates of the sera were inversely correlated with the serum CH₅₀ levels. When the sera were separated into top, middle, and bottom fractions by ultracentrifugation, the bottom fractions showed more effective inhibitions than the others. Similarly, the strongest inhibition was found in the void volume of the serum separated by Sephadex G200 gel filtration. Reduction and alkylation of IgG resulted in the loss of reactivity with Fc receptor of B lymphocytes, and the rosette inhibiting activities of the SLE sera were markedly reduced after reduction and alkylation. Some of anti-HLA sera were inhibitory for the Fc-rosette formation, while the tested sera did not contain anti-HLA activity assessed by the microcytotoxicity test. These results indicated that circulating immune complexes contained in the sera inhibit the rosette formation, and that the Fc-rosette inhibition test is a simple and relatively sensitive method for the detection of circulating immune complexes.

Antinuclear antibody activities of the sera were tested by the indirect immunofluorescent method; however, clear correlations were not obtained between Fc-rosette inhibition rates and staining patterns of antinuclear antibodies. On the other hand, the positive groups of LE-test exhibited slightly greater inhibition rates of the rosette than the negative groups.

A previous report (Morito and others, 1976) showed that Fc-receptors mainly on human B lymphocytes are detected by the rosette formation (Fc-rosette) using the human red cells sensitized with one of the Rh-antisera (Ripley), and that the Fc-rosette is strongly inhibited by aggregated human IgG. This suggested that circulating immune complexes in systemic lupus erythematosus (SLE) sera will inhibit the Fc-rosette formation when the complexes contain aggregated IgG. In the present study it was found that all the tested hypocomplementaemic SLE sera inhibited the Fc-rosette formation. Analysis of the sera suggested that the inhibition was caused mainly by the immune complexes present in the sera.

Materials and methods

SLE SERA

Eleven sera from patients with SLE who fit the criteria of the American Rheumatism Association (Cohen and others, 1971) were used after heat inactivation at 56°C for 30 minutes for the inhibition of Fc-rosette formation. Serum CH₅₀ levels of these sera were < 20 units. 7 normal human sera and 8 normocomplementaemic SLE sera were used as controls.

PREPARATION OF AGGREGATED HUMAN IgG

Human Cohn Fraction II (purchased from Midori Juji, Tokyo) was used as human IgG, and 10 mg/ml diluted in 0-15 mol/l phosphate-buffered saline, pH 7-4, were heat aggregated at 63°C for 20 minutes. Under this condition, IgG showed moderate turbidity but no

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precipitation. Aggregated IgG were isolated in void volume from nonaggregated IgG on Sephadex G200 (Pharmacia, Uppsala, Sweden) gel filtration. They were added to heat inactivated normal human serum and used for the inhibition of Fc-rosette formation.

**Inhibition of Fc-Rosette Formation**

Detailed procedures for Fc-rosette formation have been described (Morito and others, 1976) and are briefly mentioned in the present study. Human blood type O (C+D+) red cells were washed three times with 0.15 mol/l NaCl and twice with 0.15 mol/l gelatin veronal buffer (GVB+), pH 7.4, and suspended to 2 x 10^9/ml in GVB+. An equal amount of Ripley's serum diluted 20 times (Waller and Vaughan, 1956; Waller and Lawler, 1962) was added to the cell suspensions and incubated at 37°C for 60 minutes. (Ripley's serum was kindly given by Dr. M. Waller, Richmond, Dr. J. S. Johnson, La Jolla, Calif., and Dr. K. Kano, Buffalo, N.Y.) Lymphocytes were separated from normal human peripheral blood by the differential centrifugation method using Ficoll-Conray's solution (Ficoll, Pharmacia, Uppsala, Sweden; Conray, meglumine tatalamate, Daiichi Pharmaceutical, Tokyo, specific gravity 1.076).

For the rosette formation equal volumes of the sensitized red cells (1 x 10^5/ml) and lymphocytes (5 x 10^9/ml) were incubated at 37°C for 15 minutes, and centrifuged at 500 rpm for 5 minutes. Pellets were gently resuspended and lymphocytes surrounded by more than four red cells were counted as positive rosette-forming cells. The percentage of positive rosette formation was calculated from a total of 200 lymphocytes. The normal mean ± SD of the rate of Fc-rosette formation from peripheral blood lymphocytes was 27.1 ± 6.9%.

For the rosette inhibition test, 0.2 ml of tested serum was added to the lymphocyte suspensions and incubated at 37°C for 30 minutes. Lymphocyte suspensions were washed three times with Hank's balanced salt solution, and the rosette formations were observed with the sensitized red cells. Fc-rosette inhibition rates were evaluated in comparison with the rosette forming rate when normal human serum was used instead of SLE sera.

**Fractionation of SLE Serum by Ultracentrifugation**

SLE sera were ultracentrifuged at 140 000 g for 4 hours using Hitachi 65p automatic preparative ultracentrifuge and separated into equal volumes of top, middle, and bottom fractions. Each fraction was used for the rosette inhibition test. Anti-HLA-2 serum (no. 97), obtained from a multiparous woman, which was strongly inhibitory for Fc-rosette formation was separated in the same manner by the ultracentrifugation and used as a control.

**Fractionation of SLE Serum by Sephadex G200 Gel Filtration**

One of the most inhibitory SLE sera was applied on Sephadex G200 column and separated into five fractions. They consisted of 19S fraction (void volume), 7S fraction, albumin fraction, and troughs between them. Each fraction was concentrated by evaporation and used for the experiment. Normal human serum was separated into five fractions in the same manner and used as a control.

**Measurement of Serum CHs0 Levels**

Serum CHs0 levels were measured by Mayer's method (1961). Normal ranges were 29–3–43·9 unit/ml.

**Assessment of Antinuclear Antibody Activities**

Smears of normal human peripheral blood fixed on the microscope slides were incubated with SLE sera at 37°C for 30 minutes. The slides were washed several times with 0.15 mol/l phosphate-buffered saline, pH 7.4, and stained with fluorescein isothiocyanate labelled rabbit antihuman IgG (Behring, Germany). Nuclear staining patterns were classified as peripheral, homogeneous, and speckled as described by Tan (1967).

Final dilutions of the sera which gave the positive strainings of each pattern were determined as titres of antinuclear antibodies. LE-tests (Hyland, Calif.) were performed simultaneously in undiluted sera using the latex slide test. The observed macroscopical agglutination was determined as positive.

**Detection of Anti-HLA Activity**

Heat inactivated SLE sera were added to 12 different HLA-A types of lymphocytes and incubated with fresh rabbit serum as a complement source. The microcytotoxicity test was performed by Terasaki's method (Terasaki and others, 1974) to detect anti-HLA activities in the SLE sera.

**Reduction and Alkylation of the Sera**

Three SLE sera which strongly inhibited Fc-rosette formation were reduced with 0·01 mol/l dithiothreitol at room temperature for 30 minutes and alkylated with 0·015 mol/l iodoacetamide at 0°C for 2 hours (Tanimoto and others, 1975). After dialysis against 0·15 mol/l phosphate-buffered saline, pH 7·4, the reduced and alkylated sera were tested for Fc-rosette inhibition in the same manner as the nontreated sera. Anti-HLA-2 (no. 97) and anti-HLA-5 (no. 191) sera, which are also inhibitory for the Fc-rosette formation, were reduced and alkylated in the same manner as described above. In this inhibition study, HLA-2 and 5 types of lymphocytes were used.

**Results**

When aggregated human IgG were added to normal human serum in the Fc-rosette inhibition test, dose response was clearly observed as shown in Fig. 1. The experiments were repeated five times. Mean values and standard deviations of the inhibition rates are given in Fig. 1. Considerable inhibition, i.e. about 50%, was observed when 0·1 mg aggregated IgG was added to the serum, while normal human serum used as control which did not contain aggregated IgG did not inhibit Fc-rosette formation.

Under the same condition 11 hypocomplement-aemic SLE sera inhibited Fc-rosette formation. The inhibitory rates were inversely correlated with serum CHs0 levels as shown in Fig. 2 (r = −0·80,
P < 0.01), indicating that the lower the serum CH₅₀ levels the higher the inhibitory rates of Fc-rosette. Mean inhibitory rates of the hypocomplementaemic SLE sera were 44·4 ± 12·8 SD, whereas 7 normal human sera and 8 normocomplementaemic SLE sera used as controls gave 6·4 ± 4·6% and 2·7 ± 3·9% of the Fc-rosette inhibitions. Mean CH₅₀ levels of the hypocomplementaemic SLE sera were 16·4 ± 4·1 unit/ml, and those of the normal human sera and normocomplementaemic sera were 41·9 ± 7·5 unit/ml. In order to characterize the inhibitory factor in the hypocomplementaemic SLE sera, three strongly inhibitory SLE sera were separated into top, middle, and bottom fractions by ultracentrifugation. When these fractions were tested in the Fc-rosette inhibition, bottom fractions were found to be the most inhibitory, while middle or top fractions were weakly inhibitory (Fig. 3). On the contrary, the top fraction of anti-HLA-2 serum used as a control was still highly inhibitory.

Fractionation of one of the SLE sera by Sephadex G200 gel filtration showed that the most inhibitory

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**FIG 1** Fc-rosette inhibition by aggregated human IgG in heat inactivated normal human serum. Lymphocytes from a normal healthy adult were incubated with aggregated human IgG in normal human serum and Fc-rosettes were formed with the sensitized human red cells. Fc-rosette inhibition rates were compared with controls in the absence of aggregated IgG. Clear dose response was observed by the included aggregated IgG.

**FIG 2** Inhibition of Fc-rosette formation by hypocomplementaemic SLE sera. Normal lymphocytes were incubated with the SLE sera and Fc-rosettes were formed. The rosette inhibitory rates were compared with serum CH₅₀ levels of these sera, showing an inverse correlation between them (r = -0.80, P < 0.01)

**FIG 3** Inhibition of Fc-rosette formation by SLE sera fractionated by ultracentrifugation. Three hypocomplementaemic SLE sera were ultracentrifuged at 140,000 g for 4 hours and separated into top, middle, and bottom fractions. Fc-rosette inhibition test was performed with each fraction.

Anti-HLA-2 serum (no. 97), which was strongly inhibitory of Fc-rosette formation, was separated in the same manner and Fc-rosette inhibition by each fraction was observed. The bottom fractions of SLE sera were more inhibitory than other two fractions, i.e. the middle and the top fractions, while the top fraction of anti-HLA serum was still highly inhibitory.
fraction was present in void volume (19S fraction, Fr-I) and relatively weak inhibition was observed in 7S fraction (Fr-III) and between them (Fr-II), but none was seen in the albumin fraction (Fr-V) (Fig. 4). None of the fractions from normal human serum used as controls showed significant inhibitions of the rosette formation (Fig. 5).

Reduction and alkylation of three SLE sera resulted in marked reduction of Fc-rosette inhibition, while anti-HLA sera (nos. 97, 191) still retained the inhibitory activity after reduction and alkylation (Fig. 6).

None of the tested SLE sera contained anti-HLA activities as assessed by Terasaki's microcytotoxicity test (Terasaki and others, 1974) using 12 different HL-A types of lymphocytes.

Final dilutions of these 11 sera which gave positive stainings classified as peripheral, homogeneous, and speckled types were determined as titres of respective antinuclear antibodies by immunofluorescent method, and the results were compared with Fc-rosette inhibition rates. As shown in Fig. 7, no clear correlation except slightly higher Fc-rosette inhibition rates in positive groups of peripheral patterns were observed between Fc-rosette inhibition rates and titres of each pattern.

The activities of antinuclear antibodies were also assessed by the LE test. When Fc-rosette inhibitory rates were compared between LE test positive and negative groups, the former group showed slightly higher inhibitory rates than the latter (Fig. 8).

**Discussion**

Several reports (Tan and others, 1966; Agnello and others, 1971; Bombardieri, Lightfoot, and Christian, 1973) have indicated that circulating immune complexes are frequently detectable in hypocomplementaemic SLE sera. The methods used for the detection of these immune complexes include precipitation.
reaction by rheumatoid factor or Clq (Hannestad, 1967; Agnello and others, 1971). Since these methods are generally not sensitive, improved methods such as radioimmunoassay using rheumatoid factor (Cowdery, Treadwell, and Fritz, 1975) and precipitation reaction with a high titre of monoclonal rheumatoid factor (Winchester, Kunkel, and Agnello, 1971) have been reported. However, it is difficult to find monoclonal rheumatoid factor reactive only with aggregated IgG, and the radioimmunoassay is not a simple method. The Fc-rosette inhibition test described in this study is a simple and relatively sensitive method, i.e. only 0.1 mg aggregated human IgG was needed to inhibit about 50% of Fc-rosette formation.

The Fc-rosette inhibition test is not a direct method such as the rheumatoid factor or Clq precipitation test. In this regard Fc-rosette may be inhibited not only by aggregated IgG or immune complexes but also by other factors in the sera such as antilymphocyte antibody. In mice it was reported that anti-Ia (I-region associated antigen) and heterologous anti-immunoglobulin were also inhibitory for Fc-rosette formation (Schirrmacher, Halloran, and David, 1975), and another report suggested that Ia antigens may be identical or closely related to Fc receptors on the lymphocytes (Dickler and Sachs, 1974). In humans it is not known whether Fc receptors have some relation with Ia antigens or not, nor is the presence of Ia antigen clear. In our experience, some of anti-HLA sera also inhibited Fc-rosette formation, and this inhibiting activity has virtually no relation with type-specific anti-HLA activities (Morito and others, 1976). If HL-A sera in humans contain anti-Ia like activities as observed in alloantisera of mice (David and Shreffler, 1974; Dickler and Sachs, 1974; Sachs and Cone, 1975), and if Fc receptors in humans are identical or very closely related to Ia antigens, these anti-Ia like activities in the HL-A sera will inhibit Fc-rosette formation.

Published reports indicate that SLE sera frequently contain antilymphocyte antibodies (Terasaki, Mottironi, and Barnett, 1970; Mittal and others, 1970; Lies, Messner, and Williams, 1973; Messner, Kennedy, and Jelinek, 1975; Winfield and others, 1975). As far as detected in the cytotoxicity test using 12 different panels of lymphocytes, however, the tested SLE sera did not contain either anti-HLA or anti-Ia like activities. If the sera contain antilymphocyte activities not detectable by the cytotoxicity test, such as cold reactive antilymphocyte antibodies (Winfield and others, 1975) or non-complement-fixing antibodies, it is still possible that these peculiar antibodies may inhibit the Fc-rosette formation. In addition, 11 cases of hypocomplementaemic SLE sera were tested in our study. When additional cases are used, some of them may show cytotoxicity against lymphocytes, and their antilymphocyte antibodies may participate in the Fc-rosette inhibition.

Fractionation studies of the sera by ultracentri-
fugation or by Sephadex G200 gel filtration indicated that the main inhibitory activities were present in macromolecular fractions. In addition, reduction and alkylation of the SLE sera resulted in marked reduction of inhibitory activities. As already reported, aggregated IgG or immune complexes lose their reactivity with Fc receptors on B lymphocytes after reduction and alkylation (Dickler, 1974; Morito and others, 1976). These findings strongly suggested that inhibitory factors within the sera represent immune complexes. In the present study, however, relatively weak inhibitory activity was also found in 7S fractions, even after reduction and alkylation of the sera (Figs. 4 & 6). These results may indicate that some unidentified IgG antibody to lymphocyte as described above may participate in Fc-rosette inhibition to some extent.

It is also conceivable that native IgG in the sera may have some relation with Fc-rosette inhibition since a large amount of IgG are inhibitory for the rosette formation. Although the precise mechanism of Fc-rosette inhibition with native IgG is not clear, it is possible that native IgG is also reactive with the Fc-receptor on the lymphocyte, but their affinity to the receptor will be much less than aggregated forms. Therefore small amounts of native IgG are not inhibitory, while large amounts of them are inhibitory.

Although circulating immune complexes in the sera are thought to play a main role in Fc-rosette inhibition, it is advisable to use this test as one of the screening tests for the detection of immune complexes, and confirmation should be made after exclusion of antilymphocyte activity.

It is generally accepted that DNA-anti-DNA complexes are pathogenetically most important among many immune complexes in SLE sera (Kohler, 1973). In general, peripheral patterns of immunofluorescent stainings correspond well to anti-DNA activities. In our study the correlation between Fc-rosette inhibition rates and titres of peripheral patterns is not as remarkable as expected. Although LE-test positive groups showed more inhibition than negative groups, further analysis will be necessary to include the relation of Fc-rosette inhibition with multiple antinuclear antibody specifications.

The in vivo role of Fc-rosette inhibition of immune complexes is unknown, but several points can be made. First, lymphocytes bearing Fc receptors on their surface are known to show cytotoxicity against cells sensitized with IgG antibody (antibody-dependent lymphocyte-mediated cytotoxicity, ADLC) (Perlmann, Perlmann, and Wigzell, 1972). This cytotoxicity is inhibited by immune complexes. For the same reason, phagocytosis of cells sensitized with IgG antibody by neutrophils or macrophages is inhibited by immune complexes. Therefore, it is possible that in some cases circulating immune complexes may be protective since they are capable of inhibiting ADLC or phagocytosis.

Second, as it was suggested in mice that Fc receptor on B lymphocytes may be identical to Ia antigen (Dickler and Sachs, 1974), immune complexes will react with Ia antigens, and may inhibit their activities. They may suppress T-cell dependent antibody production because helper substances from T-cells will react with Ia antigens on B-cells (Katz, 1974). Thus it is likely that once circulating immune complexes are formed in SLE patients, antibody production would be suppressed by a feedback-like mechanism. Another report (Lozner and others, 1974) suggested that Ia antigen may be identical to the stimulator locus of B lymphocyte in mixed leucocyte culture. This finding also suggested that immune complexes might be inhibitory for mixed lymphocyte reaction. All these possibilities are not yet proved in vivo, but we suggest that at least some of them are working in disease processes.

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