Inhibition of Fc-rosette formation by synovial fluids from patients with rheumatoid arthritis

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SUMMARY Human red cells sensitized with one of the Rh antisera (Ripley) form rosettes (Fc-rosettes) with human B lymphocytes and the rosettes are well inhibited by aggregated human IgG. As synovial fluids from patients with rheumatoid arthritis are usually hypocomplementaemic and frequently contain immune complexes, they were used for the inhibition of Fc-rosette formations in this study.

The Fc-rosette inhibitory rates were inversely correlated with the levels of CH₅₀ and C4 in the synovial fluids. Fractionation studies of the synovial fluids by ultracentrifugation and Sephadex G200 gel filtration indicated that main inhibitory activities were present in macromolecular fractions. Reduction and alkylation of the synovial fluids resulted in marked reductions of inhibitory activities. The tested synovial fluids did not contain anti-HLA activity as assessed by the microcytotoxicity test. The Fc-rosette inhibitory rates were not correlated with the levels of C3 and immunoglobulins, nor with the activity of rheumatoid factors in the synovial fluids.

These results indicate that the Fc-rosette formations are inhibited by the immune complexes contained in the synovial fluids and it was confirmed that the Fc-rosette inhibition test is a simple and sensitive method for the detection of circulating immune complexes.

Our previous study showed that human red cells sensitized with one of the Rh antisera (Ripley) formed rosettes (Fc-rosette) with some human lymphocytes and these rosettes were well inhibited by hypocomplementaemic systemic lupus erythematosus sera (Morito *et al.*, 1976). The inhibitions were ascribed to the presence of immune complexes in the sera. However, it is well known that complement levels of synovial fluids from patients with rheumatoid arthritis are usually depressed in spite of their relatively high content of total protein (Pekin and Zvaifler, 1964; Bunch *et al.*, 1974), and that immune complexes are frequently detectable in these synovial fluids (Hannestad, 1967; Winchester *et al.*, 1971; Luthra *et al.*, 1975).

In the present study synovial fluids from patients with rheumatoid arthritis were used for the inhibition of Fc-rosette formation, and the inhibitory rates were compared with several parameters including complement levels, immunoglobulin levels, and rheumatoid factor activities. The nature of inhibitory factors in the synovial fluids was analysed using ultracentrifugation and gel filtration.

Materials and methods

SYNOVIAL FLUIDS

Thirty-two synovial fluid samples were obtained from 20 patients with rheumatoid arthritis. All the patients were classified as definite or classical rheumatoid arthritis according to the criteria of the American Rheumatism Association (Ropes *et al.*, 1958). In addition, four synovial fluid samples from patients with degenerative joint disease were used in this study. 20 units/ml hyaluronidase (Spray, Mochida Pharmaceutical, Tokyo) was added to each synovial fluid and mucin contained in the synovial fluid was dissolved. All the synovial fluid samples were heat inactivated at 56°C for 30 minutes.

INHIBITION OF FC-ROSETTE FORMATION

Human blood type O (C⁺D⁺) red cells were washed three times with physiological saline and twice with 0.15 mol/l, pH 7.4 gelatin veronal buffer (GVB⁺) as previously described (Mayer, 1961), and suspended at 2 × 10⁸/ml. An equal volume of Ripley's serum 20 times diluted (Waller and Vaughan, 1956) was added to the red cell suspensions and incubated at 37°C for 60 minutes, and these were used as sensitized red cells. Lymphocytes were separated

from normal human peripheral blood by the differential centrifugation method using Ficoll-Conray's solution as described previously (Morito *et al.*, 1976).

For the Fc-rosette inhibition, 0.2 ml of the synovial fluid was added to 0.1 ml of 7×10^6 /ml of lymphocyte suspensions and incubated at 37°C for 30 minutes. After incubation the lymphocytes were washed five times with gelatin veronal buffer containing 0.01 mol/l ethylene diamine tetra-acetate (EDTA). Equal volumes of 7×10^6 /ml of the lymphocytes and 1×10^8 /ml of the sensitized red cells were incubated at 37°C for 15 minutes. After centrifugation at 500 rpm for 5 minutes and gentle resuspension, one drop of the suspensions was placed on the microscope slide and lymphocytes surrounded by more than four red cells were considered to be positive rosette forming cells. Fc-rosette formation rates were calculated from a total of 200 lymphocytes. Fc-rosette inhibitory rates of the synovial fluids were estimated in comparison with the rosette forming rate with normal human serum used as control.

FRACTIONATION OF SYNOVIAL FLUIDS BY ULTRACENTRIFUGATION

Each 3 ml of synovial fluid was centrifuged at 140 000 *g* for 4 hours using a Hitachi 65p centrifuge. The synovial fluid was separated into three fractions of 1 ml each, i.e. top, middle, and bottom fractions.

FRACTIONATION OF SYNOVIAL FLUIDS BY SEPHADEX G200 GEL FILTRATION

Three ml of synovial fluid was applied on Sephadex G200 column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.15 mol/l, pH 7.4, phosphate buffered saline (PBS), and separated into five fractions consisting of 1st peak (void volume, 19S fraction), 2nd peak (7S fraction), 3rd peak (albumin fraction), and fractions between them. Each fraction was concentrated by evaporation and used for the Fc-rosette inhibition.

REDUCTION AND ALKYLATION OF SYNOVIAL FLUIDS

Synovial fluids were reduced by 0.01 mol/l dithiothreitol at room temperature for 30 minutes and alkylated by 0.015 mol/l iodoacetamide at 0°C for 2 hours as described previously (Tanimoto *et al.*, 1975). They were dialysed against 0.15 mol/l, pH 7.4, PBS for 48 hours and used for the experiments.

MEASUREMENTS OF COMPLEMENT AND IMMUNOGLOBULIN LEVELS

The CH_{50} of synovial fluid was measured by Mayer's 50% haemolytic method (Mayer, 1961).

Levels of C3, C4, IgG, IgA, and IgM were determined by single radial immunodiffusion using Partigen's Immunoplate (Behring Institute, W. Germany) (Mancini *et al.*, 1975).

DETECTION OF RHEUMATOID FACTOR ACTIVITY

Rheumatoid factor activities in synovial fluids were detected by the latex fixation test using the RA-test (Hyland Lab., Calif.) (Singer and Plotz, 1956) and by the sensitized sheep cell agglutination test described by Heller *et al.* (1954).

DETECTION OF ANTI-HLA ACTIVITY

All the synovial fluids were heat inactivated at 56°C for 30 minutes and their anti-HLA activities were detected by Terasaki's microcytotoxicity test using 12 different panels of well typed lymphocytes (Terasaki and McClelland, 1964).

Results

When the Fc-rosette inhibitory rates of synovial fluids from patients with rheumatoid arthritis were compared with those from patients with degenerative joint disease many of the former showed considerable inhibition, while all of the latter failed to inhibit rosette formation significantly (Fig. 1).

Fig. 1 *Fc-rosette inhibition by synovial fluids of patients with rheumatoid arthritis and degenerative joint disease. The CH₅₀ of each synovial fluid was divided by corresponding total protein level (g/100 ml) and the ratio plotted against Fc-rosette inhibitory rates. Synovial fluid from patients with rheumatoid arthritis was divided into two groups. In one group CH₅₀/total protein was >2.0, and in the other <2.0. The hypocomplementaemic group showed slightly higher inhibition rates than the normocomplementaemic group and patients with degenerative joint disease.*

Each CH_{50} value of synovial fluid was divided by the corresponding total protein (TP) level. On the basis of the ratio of CH_{50} to TP, the synovial fluids from patients with rheumatoid arthritis were divided into two groups. In the hypocomplementaemic group CH_{50}/TP values were <2.0 , and in the normocomplementaemic group the values were >2.0 . The hypocomplementaemic group showed higher inhibitory rates than the normocomplementaemic group (Fig. 1). The correlation coefficient

Fig. 2 Relation between Fc-rosette inhibition and $CH_{50}/total\ protein$ of rheumatoid synovial fluids. The ratio of CH_{50} to total protein was plotted against Fc-rosette inhibitory rates. Negative correlation was found between them ($r = -0.49$, $P < 0.01$).

Fig. 3 Relation between Fc-rosette inhibitory rates and C3/total protein and C4/total protein of rheumatoid synovial fluid. The values of C3 and C4 (mg/100 ml) in the synovial fluids were divided by total protein levels (g/100 ml). The rates of C3 and C4 to total protein were plotted against Fc-rosette inhibitory rates. The correlation between the inhibitory rates and C3/total protein was not significant, but a negative correlation was found between Fc-rosette inhibitory rates and C4/total protein ($r = -0.56$, $P < 0.05$).

between the Fc-rosette inhibitory rates and CH_{50}/TP values was $r = -0.49$ ($P < 0.01$) (Fig. 2), indicating that the lower the complement levels in the synovial fluids the higher the Fc-rosette inhibitory rate.

C3 and C4 levels in synovial fluid were also compared with Fc-rosette inhibitory rates. Although the correlation between C3 levels and the inhibitory rates did not reach significance, the correlation coefficient of C4 levels with the inhibitory rates was significant ($r = -0.56$, $P < 0.05$) (Fig. 3).

Fig. 4 Relation between Fc-rosette inhibitory rate and RA-test positivity of rheumatoid synovial fluids. The synovial fluids were divided into RA-test positive and negative groups. When the Fc-rosette inhibitory rates of these two groups were compared there were no significant differences.

Fig. 5 Relation between Fc-rosette inhibitory rates and sensitized sheep cell agglutination (SSCA) titres of rheumatoid synovial fluids. SSCA titres in the synovial fluids were determined as described by Heller et al. (1954). The titres were plotted against Fc-rosette inhibitory rates but no significant correlation was observed.

Fig. 6 Relation between Fc-rosette inhibitory rates and immunoglobulin levels in rheumatoid synovial fluids. IgG, IgM, and IgA levels in the synovial fluids were measured by single radial immunodiffusion. The results compared with the Fc-rosette inhibitory rates showed no significant correlations.

Fc-rosette inhibitory rates were studied in comparison with rheumatoid factor activities and immunoglobulin levels. Neither the results of positive latex fixation tests nor of SSCA titres gave significant correlations with Fc-rosette inhibitory rates (Figs. 4, 5). Levels of IgG, IgA, and IgM in the synovial fluids plotted against the corresponding Fc-rosette inhibitory rates (Fig. 6) showed no significant correlations with the inhibitory rates.

Three highly inhibitory synovial fluid samples were centrifuged at 140 000 *g* for 4 hours, and separated into top, middle, and bottom fractions. When each fraction was tested by the Fc-rosette inhibition test, the bottom fractions were found to be the most inhibitory (Fig. 7).

One synovial fluid sample was applied on Sephadex G200 column and separated into five fractions: 1st peak (void volume 19S fraction), 2nd peak (7S fraction), 3rd peak (albumin fraction), and fractions between them (Fig. 8). These fractions were also used in Fc-rosette inhibition. The strongest inhibition

was observed in the 1st peak, some inhibition was noted in the 2nd peak and in the fraction between the 1st and 2nd peaks, while the other fractions showed no inhibition.

Synovial fluids conspicuously lost their inhibitory activities after reduction and alkylation (Fig. 9). Anti-HLA activities were not found in the synovial fluids used in this study by the microcytotoxicity test against 12 different panels of lymphocytes.

Fig. 8 Inhibition of Fc-rosette by rheumatoid synovial fluids fractionated by Sephadex G200 gel filtration. One synovial fluid was applied on Sephadex G200 column and separated into five fractions. Fc-rosette was most strongly inhibited by Fraction I (Fr-I, 1st peak, void volume); Fr-II and Fr-III (7S fraction) showed moderate inhibitions; neither Fr-IV nor Fr-V inhibited rosette formation.

Fig. 7 Inhibition of Fc-rosette by rheumatoid synovial fluids fractionated by ultracentrifugation. Three synovial fluids were separated into top, middle, and bottom fractions by ultracentrifugation at 140 000 *g* for 4 hours. Each fraction was tested in Fc-rosette inhibition test and bottom fractions were found to be the most inhibitory.

Fig. 9 Effect of reduction and alkylation of rheumatoid synovial fluids on Fc-rosette inhibition. Three synovial fluids were reduced with 0.01 mol/l dithiothreitol and alkylated with 0.015 mol/l iodoacetamide. Their Fc-rosette inhibitory activities were compared before and after reduction and alkylation. The inhibitory activities were moderately to markedly reduced after reduction and alkylation.

Discussion

Reports have indicated that immune complexes are frequently present in synovial fluids from patients with rheumatoid arthritis. The methods used were precipitation reactions with rheumatoid factor or C1q, but these methods are generally insensitive. More sensitive methods have also been used, including radioimmunoassay using rheumatoid factor (Cowdery *et al.*, 1975) and the precipitation reaction with monoclonal rheumatoid factor (Winchester *et al.*, 1971). However, radioimmunoassay is not a simple procedure, and it is difficult to determine monoclonal rheumatoid factor.

As noted above, we consider that the Fc-rosette inhibition test used in this study is a simple and sensitive method. With regard to the specificity of this test, Fc-rosette formation may be inhibited not only by immune complexes or aggregated IgG, but also by other factors such as antilymphocyte antibody (Morito *et al.*, 1976). Nevertheless, for the following reasons we think that Fc-rosette inhibition is caused mainly by the presence of immune complexes in synovial fluids.

(1) The hypocomplementaemic group of synovial fluids showed higher Fc-rosette inhibitory rates than the normocomplementaemic group, and the levels of CH₅₀ and C4 were inversely correlated with the Fc-rosette inhibitory rates. (2) Ultracentrifugation and gel filtration studies indicated that the main inhibitory activities are present in macromolecular fractions. (3) Reduction and alkylation of synovial fluids resulted in marked reduction of the inhibitory activities. (4) None of the tested synovial fluid samples contained anti-HLA activities.

It has been reported that the reactivity of immune complexes with Fc-receptors on lymphocytes disappears after reduction and alkylation (Dickler, 1974), whereas the inhibitory activities of antilymphocyte antibodies are not affected by reduction and alkylation. It may be that rheumatoid factor contained in synovial fluids interferes with rosette formation of the lymphocytes with sensitized red cells. In our study the lymphocyte preparations after reaction with synovial fluids were thoroughly washed and rheumatoid factor activity was no longer detected in the supernates. In addition, there were no significant differences of Fc-rosette inhibitory rates between the rheumatoid factor positive group and the negative group in the synovial fluids. It is therefore unlikely that rheumatoid factor contained in the synovial fluid would inhibit Fc-rosette formation.

Antilymphocyte antibodies such as anti-HLA antibodies may inhibit Fc-rosette formation, but the microcytotoxicity test showed none of these

antibodies in the synovial fluids. In addition, such antibodies do not usually lose their inhibitory activity after reduction and alkylation. In our experiments, the 7S fraction from synovial fluids showed slight inhibition. Since the fraction might contain peculiar antibodies which are not detected by the microcytotoxicity test, it is possible that these antibodies inhibit Fc-rosette formation.

In synovial fluids from patients with rheumatoid arthritis complement levels are usually low in spite of a high protein content (Pekin and Zvaifler, 1964; Bunch *et al.*, 1974). It is generally believed that immune complexes formed in synovial fluids activate the classical complement sequences and result in the reduction of complement levels. In leucocytes from the synovial fluids immune complexes and complement components have been observed as inclusion bodies (Hollander *et al.*, 1965; Vaughan *et al.*, 1968), lysosomal enzymes being released from the leucocytes after phagocytosis. Lysosomal enzymes are thought to play an important role in the inflammatory process in rheumatoid arthritis. Thus, immune complexes will be one of the most important factors in the pathogenesis of rheumatoid arthritis, and the detection of immune complexes has significant value in rheumatoid arthritis. We stress that the Fc-rosette inhibition test is one of the simplest and most sensitive methods for the detection of circulating immune complexes in clinical material.

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References

- Bunch, T. W., Hunder, G. G., McDuffie, F. C., O'Brien, P. C., and Markowitz, H. (1974). Synovial fluid complement determination as a diagnostic aid in inflammatory joint disease. *Mayo Clinic Proceedings*, **49**, 715-720.
- Cowdery, J. S., Jr., Treadwell, P. E., and Fritz, R. B. (1975). A radioimmunoassay for human antigen-antibody complexes in clinical material. *Journal of Immunology*, **114**, 5-9.
- Dickler, H. B. (1974). Studies of the human lymphocyte receptor for heat-aggregated or antigen-complexed immunoglobulin. *Journal of Experimental Medicine*, **140**, 508-522.
- Hannestad, K. (1967). Presence of aggregated γ G-globulin in certain rheumatoid synovial effusions. *Clinical and Experimental Immunology*, **2**, 511-529.
- Heller, G., Jacobson, A. S., Kolodny, M. H., and Kammerer, W. H. (1954). The hemagglutination test for rheumatoid arthritis. II. The influence of human plasma fraction (gamma globulin) on the reaction. *Journal of Immunology*, **72**, 66-78.
- Hollander, J. L., McCarty, D. J., Astorga, G., and Castro-Murillo, E. (1965). Studies on the pathogenesis of rheumatoid joint inflammation. *Annals of Internal Medicine*, **67**, 271-280.

- Luthra, H. S., McDuffie, F. C., Hunder, G. G., and Samayoa, E. A. (1975). Immune complexes in sera and synovial fluids of patients with rheumatoid arthritis. *Journal of Clinical Investigation*, **56**, 458-466.
- Mancini, G., Carbonara, A. O., and Heremans, J. F. (1965). Immunochemical quantitation of antigens by single immunodiffusion. *Immunochemistry*, **2**, 235-254.
- Mayer, M. M. (1961). *Experimental Immunochemistry*, 2nd ed., p. 133. Thomas, Springfield, Illinois.
- Morito, T., Tanimoto, K., Hashimoto, Y., Horiuchi, Y., and Juji, T. (1976). Fc-rosette inhibition by hypocomplementaemic systemic lupus erythematosus sera. *Annals of the Rheumatic Diseases*, **35**, 415-421.
- Pekin, T. J., Jr., and Zvaifler, N. J. (1964). Hemolytic complement in synovial fluid. *Journal of Clinical Investigation*, **43**, 1372-1382.
- Ropes, M. W., Bennett, G. A., Cobb, S., Jacox, R. F., and Jesser, R. A. (1958). Revision of diagnostic criteria for rheumatoid arthritis. *Bulletin on Rheumatic Diseases*, **9**, 175-176.
- Singer, J. M., and Plotz, C. M. (1956). The latex fixation test. I. Application to the serologic diagnosis of rheumatoid arthritis. *American Journal of Medicine*, **21**, 888-892.
- Tanimoto, K., Cooper, N. R., Johnson, J. S., and Vaughan, J. H. (1975). Complement fixation by rheumatoid factor. *Journal of Clinical Investigation*, **55**, 437-445.
- Terasaki, P. I., and McClelland, J. D. (1964). Microdroplet assay of human serum cytotoxins. *Nature*, **204**, 998-1000.
- Vaughan, J. H., Barnett, E. V., Sobel, M. V., and Jacox, R. F. (1968). Intracytoplasmic inclusions of immunoglobulins in rheumatoid arthritis and other diseases. *Arthritis and Rheumatism*, **11**, 125-134.
- Waller, M. V., and Vaughan, J. H. (1956). Use of anti-Rh sera for demonstrating agglutination activating factor in rheumatoid arthritis. *Proceedings of the Society for Experimental Biology and Medicine*, **92**, 198-200.
- Winchester, R. J., Kunkel, H. G., and Agnello, V. (1971). Occurrence of γ -globulin complexes in serum and joint fluid of rheumatoid arthritis patients: use of monoclonal rheumatoid factors as reagents for their demonstration. *Journal of Experimental Medicine*, **134**, 286-295S.