Complement activating properties of monoreactive and polyreactive IgM rheumatoid factors

Yukio Sato, Rika Sato, Hiroshi Watanabe, Atsuko Kogure, Kazuo Watanabe, Tomoe Nishimaki, Reiji Kasukawa, Mikio Kuraya, Teizo Fujita

Abstract

Objectives—To estimate the complement activating properties of monoclonal, monoreactive, and polyreactive IgM rheumatoid factors derived from Epstein-Barr virus transformed B cells isolated from peripheral blood and synovial tissue of patients with rheumatoid arthritis (RA).

Methods—An enzyme linked immunosorbent assay (ELISA) was used to measure the activation of the classical pathway of complement by monoclonal IgM rheumatoid factor. Monoclonal IgM rheumatoid factor was bound to IgG Fc adsorbed onto microtitre plates and then reacted with diluted normal human serum as a source of complement. The activation and binding of C4 were measured with F(ab')2 antibody to human C4. The complement activating property of IgM rheumatoid factor bound to IgG Fc was tentatively expressed as the ratio of the amount of bound C4 to the amount of bound IgM rheumatoid factor.

Results—The complement activating property of monoreactive IgM rheumatoid factor was shown to be about three times higher than that of polyreactive IgM rheumatoid factor.

Conclusions—Monoreactive IgM rheumatoid factor with the higher complement activating property would result in a greater degree of complement dependent inflammation and might have a more important pathogenic role in RA than polyreactive IgM rheumatoid factor.

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Patients and methods

PREPARATION OF B CELLS AND B CELLS INFECTED WITH EPSTEIN-BARR VIRUS

One sample of peripheral blood (PB1) was obtained from a 61 year old man with a seven year history of RA. A set of peripheral blood (PB2) and synovial tissue (ST2) samples was obtained from a 37 year old woman with a 14 year history of RA. These two patients were positive for rheumatoid factor (titre>40 IU/ml by routine laser nephelometry) and satisfied the revised criteria for RA established by the American College of Rheumatology (formerly the American Rheumatism Association). Mononuclear cells were isolated from the heparinised peripheral blood through the use of Lymphoprep (Nycomed, Oslo, Norway). Isolation of mononuclear cells from the synovial tissue was performed following the procedure of Robbins and Wistar. Briefly, the synovial tissue was cut in Ca2+ and Mg2+ free Hanks’s balanced salt solution (Grand Island Biological, Grand Island, NY, USA) containing 50 IU/ml penicillin and 50 mg/ml streptomycin, and treated with 0.5 mg/ml collagenase (Sigma Chemical, St Louis, MO, USA) and 0.15 mg/ml DNase (Sigma) at 37°C for 60 minutes followed by filtration through a nylon filter (200 μm). Mononuclear cells were then isolated by density gradient centrifugation using Lymphoprep. Adherent cells were removed from each mononuclear cell
preparation by incubation in a cell culture flask (Nuncalon, Roskilde, Denmark) with RPMI (Biofluids, Rockville, MD, USA) containing 10% fetal calf serum (FCS) at 37°C for two hours. T cells were further removed by rosetting with sheep blood cells and isolating by Lymphoprep. The remaining mononuclear cells contained 30–40% B cells as defined by mouse monoclonal antibody to CD19 (Becton, Mountain View, CA, USA) and were used as enriched B cells.

The enriched B cells were suspended in the supernatant of the Epstein-Barr virus induced cell lines at a concentration of 10^6/ml. After incubation for one hour at 37°C the cells were centrifuged and resuspended in RPMI containing 10% FCS and cultured in tissue culture flasks (Nuncalon) for three to five weeks. When the Epstein-Barr virus infected B cells were expanded, these cells were cloned at 0-3 cells/well in 96 well flat bottomed microculture plates (Nuncalon) which contained 10^5 irradiated (2000 rad) human peripheral blood mononuclear cells as feeders.

ELISA FOR IGM RHEUMATOID FACTOR
Screening for IGM rheumatoid factor was performed by ELISA. A 100 μl volume of 10 μg/ml human IgG Fc (Cappel, West Chester, PA, USA) in carbonate-hydrogen carbonate buffer (pH 9-6) was added to the wells of microtitre plates (Dynatech, Chantilly, VA, USA) and incubated overnight at 4°C. The wells were washed three times with 0.15 M phosphate buffered saline (PBS) (pH 7.3) containing 0.05% Tween 20 (PBS-Tween). The wells were then incubated with PBS supplemented with 1% bovine serum albumin (BSA) for two hours at 37°C. After washing three times with PBS-Tween, the wells were incubated with 100 μl of supernatant taken from the microcultures for two hours at 37°C. After another three washings with PBS-Tween the wells were incubated with 100 μl of alkaline phosphatase conjugated goat F(ab')2 antibody to human IgM (Tago, Burlingame, CA, USA), diluted to 1:1000 in PBS-Tween, for one hour at 37°C. The wells were then developed with p-nitrophenylphosphate substrate (Sigma) and the absorbance was read with a spectrophotometer (Bio-Rad Model 2550 EIA Reader; Bio-Rad, Richmond, CA, USA) at 405 nm. All experiments were performed in duplicate. The ELISA for the IgM rheumatoid factor had an intra-assay variation of 2-4% in the range 0.01–2.0 μg/ml and its sensitivity was 0.005 μg/ml. The L chain isotypes of monoclonal IgM rheumatoid factors were determined using alkaline phosphatase conjugated goat F(ab')2 antibody to human κ or λ chain (The Binding Site, Birmingham, UK).

ELISA TO DETERMINE REACTIVITY OF IGM RHEUMATOID FACTOR WITH SINGLE STRANDED DNA, INSULIN, TETANUS TOXOID, OR TYPE II COLLAGEN
The ELISA for the antibody to single stranded DNA was performed using a commercial ELISA kit. The ELISA for antibodies to other antigens was performed using the same method as for IgM rheumatoid factor described earlier, coating each plate with insulin (Shionogi, Osaka, Japan), tetanus toxoid, or type II collagen (Sigma) at a concentration of 10 μg/ml. The positive threshold of each antibody was determined as values above the mean value plus two standard deviations (SDs) of negative control samples.

MEASUREMENT OF DISSOCIATION CONSTANT (Kᵩ) OF MONOCLONAL IGM RHEUMATOID FACTOR REACTING TO IGG-Fc
The Kᵩ of monoclonal IgM rheumatoid factor reacting to IgG Fc (molecular weight 50 000) was calculated according to the method described by Friguet et al. Briefly, monoclonal IgM rheumatoid factor in solution was incubated with IgG Fc until equilibrium was reached; then the proportion of IgM rheumatoid factor which remained unsaturated at equilibrium was measured by ELISA as previously described. The data used for the determination of Kᵩ were derived from the experiments in which the molarity of IgG Fc exceeded that of monoclonal IgM rheumatoid factor by at least 50 times.

PREPARATION OF BIOTINYLATED RABBIT F(ab')₂ ANTIBODY TO HUMAN C4
Human C4 was purified as described previously. Based on the results from sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli, it was homologous under non-reducing conditions and showed the appropriate molecular weight for its polypeptide chains under reducing conditions.

Antiserum to C4 was prepared by immunising a rabbit with the purified C4. The rabbit antiserum to human C4 was purified using a Sepharose 4B (Pharmacia, Uppsala, Sweden) column coupled with the human C4. The purified rabbit antibody to human C4 was then applied to Sephacryl S-300 (Pharmacia, Uppsala, Sweden) in PBS, and the IgG fractions were collected. The rabbit IgG antibody to human C4 was dialysed against 0.1 M acetic buffer (pH 4.0) and subjected to pepsin digestion. IgG (36 mg) in 10 ml of solution was mixed with 0.72 mg pepsin and incubated for 18 hours at 37°C. The digestion was stopped by adjusting the pH to 7.4 with 1-M Tris-HCl (pH 9.0) and the material was dialysed against PBS. The F(ab')₂ fragment of the IgG antibody to human C4 was separated from Fc and undigested IgG by a Protein A-Sepharose 4B column (Pharmacia, Piscatway, NJ, USA). The purity of the F(ab')₂ fragment of the antibody to human C4 was assessed by SDS-PAGE. No contamination with intact IgG or Fc fragments was observed. Biotinylation of rabbit F(ab')₂ antibody to human C4 was performed by the same method as described previously.

The capability of this biotinylated F(ab')₂ antibody for detecting human C4 was assessed
by immunoblotting. Normal human serum, diluted to 1:100 with PBS, was subjected to SDS-PAGE under non-reducing conditions and then transblotted onto a nitrocellulose membrane. The membrane was blocked with 5% BSA in PBS for one hour at 37°C, and incubated for one hour with 1 µg/ml biotinylated F(ab')2 antibody to human C4, which had been diluted with PBS-Tween containing 1% BSA. After washing the membrane was treated with the Vectastatin ABC-AP kit (Vector Laboratories, Sunnyvale, CA, USA) according to the manufacturer’s instructions. The membrane was then developed using 5-bromo-4-chloro-3-indolylphosphatase toluidine salt/ p-nitroblue tetrazolium chloride as a substrate. For inhibition experiments, the F(ab')2 antibody to human C4 (1 µg/ml) was incubated with the purified human C4 (1 mg/ml) for two hours at room temperature before testing by immunoblotting.

ELISA FOR COMPLEMENT ACTIVATION BY IgM RHEUMATOID FACTOR

The ELISA was performed according to the modified method of Sabharwal et al. A 100 µl volume of the suitably diluted monoclonal IgM rheumatoid factor was added to the wells coated with 1 µg/ml human IgG Fc, as described earlier. After incubation for two hours the wells were washed three times with PBS-Tween. The amounts of IgM rheumatoid factor bound to IgG Fc in 12 monoclonal samples were calculated by taking the absorbance at 405 nm. After each step of the following incubation the wells were washed three times with PBS-Tween. As a source of complement, 100 µl of normal human serum, diluted 1:200 in veronal buffered saline containing 1% gelatine and optimum Ca²⁺ and Mg²⁺ (GVB²⁺), was added to the wells and incubated for one hour at 37°C. Normal human serum heated at 56°C for 30 minutes was also used as an inactivated complement. A 100 µl volume of 1 µg/ml biotinylated rabbit F(ab')2 antibody to human C4 diluted in PBS-Tween was then added and incubated for one hour at 37°C. The wells then received avidin-biotin-alkaline phosphatase complex (ABC) (Vector Laboratories), which had been mixed in PBS-Tween 30 minutes before use. The wells were then incubated for one hour at 37°C. The p-nitrophenylphosphate substrate (Sigma) was then added to the wells and the colour appearing was read under a spectrophotometer with absorbance at 405 nm; the amounts of bound C4 were expressed as absorbance values. The complement activating property of IgM rheumatoid factor bound to IgG Fc was tentatively expressed as a ratio of the amount of bound C4 (absorbance value) to the amount of bound IgM (absorbance value). All experiments were performed in duplicate.

To determine the optimum concentration of each monoclonal IgM rheumatoid factor solution for the ELISA, preliminary experiments were performed using various concentrations (data not shown). The different solutions showed similar levels of IgM rheumatoid factor binding; the results all fell within the C4 binding curve representing values obtained from serial dilution of each IgM rheumatoid factor. The IgM concentration in each IgM rheumatoid factor solution was determined by ELISA using the same method for IgM rheumatoid factor as described earlier, except for coating of each plate with unconjugated goat F(ab')2 antibody to human IgM (Tago), diluted to 1:1000 with carbonate-hydrogen-carbonate buffer, pH 9.6. The dose dependent binding curve for IgM at a known concentration was used as a standard curve. As a result of this procedure, we found that the optimum concentrations of monoreactive IgM rheumatoid factors ranged from 0.3 to 1 µg/ml, and those polyreactive IgM rheumatoid factors ranged from 1 to 2 µg/ml. At these optimum concentrations, the amounts of bound IgM rheumatoid factors were such that non-specific binding of IgM from normal human serum was negligible.

STATISTICAL ANALYSIS

A non-parametric method (Wilcoxon rank sum test) was used to calculate the statistical difference in complement activating properties between monoreactive and polyreactive IgM rheumatoid factors.

Results

SPECIFICITY OF BIOTINYLATED RABBIT F(ab')2 ANTIBODY TO HUMAN C4

As shown in fig 1, the rabbit F(ab')2 antibody reacted to a single protein moiety in normal human serum, showing a single band at the approximate molecular weight of human C4 (lane 2). When the rabbit F(ab')2 antibody was preincubated with the purified human C4, its reactivity to the single band was inhibited (lane 3). These results show the monospecificity of the rabbit F(ab')2 antibody to human C4.

ESTABLISHMENT OF MONOCLONAL IgM RHEUMATOID FACTOR PRODUCING CELL LINES FROM EPSTEIN-BARR VIRUS TRANSFORMED B CELLS AND CHARACTERISATION OF THE MONOCLONAL IgM RHEUMATOID FACTOR

Twelve Epstein-Barr virus transformed B cell lines producing IgM rheumatoid factor were obtained after two sequential cloning steps from two samples of peripheral blood from the two patients with RA (PB1 and PB2 respectively) and one sample of synovial tissue from one of the patients with RA (ST2). Antibodies produced from some of these B cell lines reacted to not only IgG Fc but also to single stranded DNA, type II collagen, tetanus toxoid, and insulin. Therefore these 12 B cell lines were divided into two groups: one reacting only to IgG Fc (monoreactive) and the other reacting to various antigens in addition to IgG Fc (polyreactive). Monoclonal monoreactive IgM rheumatoid factor was observed in two B cell lines from PB2 and in four B cell lines from ST2. Monoclonal polyreactive IgM rheumatoid factor was observed in two cell
Figure 1  Immunoblots of normal human serum probed with biotinylated F(ab')$_2$, antibody to human C4. Normal human serum was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis under non-reducing conditions, and transferred onto nitrocellulose strips. All three strips were run and processed simultaneously (see under patients and methods for details). Lane 1, total protein stained with Coomassie brilliant blue PR-250; lane 2, binding of biotinylated F(ab')$_2$, antibody to human C4; lane 3, inhibition of the biotinylated F(ab')$_2$, antibody binding to human C4 by preincubation with purified human C4. Molecular weight markers are shown on the left.

Table 1  Antibody binding activity of monoclonal IgM rheumatoid factors (RFs) produced by Epstein-Barr virus transformed B cell lines from patients with rheumatoid arthritis

<table>
<thead>
<tr>
<th>Monoclonal IgM RF</th>
<th>L Chain</th>
<th>Antigen binding activity*</th>
<th>IgG Fc</th>
<th>ss DNA</th>
<th>Ins</th>
<th>TT</th>
<th>C II</th>
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<tr>
<td>Monoreactive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PB2-A11B</td>
<td>$\lambda$</td>
<td>1-405</td>
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<td>0-079</td>
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<td>1-366</td>
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<td>PB2-D10D</td>
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<td>0-834</td>
<td>0-304</td>
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<td>0-466</td>
<td>0-475</td>
<td>0-588</td>
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</table>

*Binding activity of monoclonal IgM RFs to IgG Fc, single stranded (ss) DNA, insulin (Ins), tetanus toxoid (TT), and type II collagen (C II) is expressed as absorbance at 405 nm. Threshold of activity is 0-1. Positive values are in italic.

Table 2  Dissociation constant ($K_d$, mol/l) of monoclonal IgM rheumatoid factors (RFs) to IgG Fc

<table>
<thead>
<tr>
<th>Monoclonal IgM RF</th>
<th>$K_d$ to IgG Fc* (mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoreactive</td>
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</tr>
<tr>
<td>PB2-A11B</td>
<td>1.8 $\times 10^{-5}$</td>
</tr>
<tr>
<td>PB2-B3C</td>
<td>4.6 $\times 10^{-5}$</td>
</tr>
<tr>
<td>ST2-A3B</td>
<td>1.3 $\times 10^{-4}$</td>
</tr>
<tr>
<td>ST2-A10E</td>
<td>7.8 $\times 10^{-4}$</td>
</tr>
<tr>
<td>ST2-E11F</td>
<td>5.3 $\times 10^{-4}$</td>
</tr>
<tr>
<td>ST2-G4E</td>
<td>3.2 $\times 10^{-4}$</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>4.0 (2.4) $\times 10^{-4}$</td>
</tr>
<tr>
<td>Polyreactive</td>
<td></td>
</tr>
<tr>
<td>PB1-D10C</td>
<td>2.7 $\times 10^{-5}$</td>
</tr>
<tr>
<td>PB1-A11D</td>
<td>1.8 $\times 10^{-4}$</td>
</tr>
<tr>
<td>PB2-D10D</td>
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<tr>
<td>PB2-E10A</td>
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<tr>
<td>PB2-E11F</td>
<td>5.4 $\times 10^{-4}$</td>
</tr>
<tr>
<td>PB2-F5G</td>
<td>3.1 $\times 10^{-4}$</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>3.1 (1.4) $\times 10^{-4}$</td>
</tr>
</tbody>
</table>

*$K_d$ to IgG Fc* fragment used for the calculation of $K_d$ is 50 000.

ELISA FOR COMPLEMENT ACTIVATION BY MONOCLONAL IgM RHEUMATOID FACTOR

Figure 2 gives a representative result of C4 binding by monoreactive monoclonal IgM rheumatoid factor (PB2-A11B) in a reaction with IgG Fc. The IgM rheumatoid factor solution (2 µg/ml) was diluted serially from 1:2 to 1:2000 with PBS-Tween, and each dilution was added to a well precoated with IgG Fc. The amount of bound C4 (absorbance value) decreased with the amount of monoclonal IgM rheumatoid factor added in the dilution range 1:2 to 1:2000. No binding of C4 was observed when heated normal human serum was used as a complement source. Therefore the amount of bound C4 represented complement activation by the IgM rheumatoid factor in a reaction with IgG Fc.
Complement activation by monoclonal rheumatoid factors

Discussion

By introducing the Epstein-Barr virus to B lymphocytes taken from two peripheral blood samples and one synovial tissue sample originating from two patients with RA positive for rheumatoid factor, we obtained 12 monoclonal IgM rheumatoid factor producing cell clones. Examination of their reactivity with a panel of self and various non-self antigens showed that six IgM rheumatoid factors were monoreactive, binding restrictively to IgG Fc, whereas the other six IgM rheumatoid factors were polyreactive, binding not only to IgG Fc but also to other antigens, such as single stranded DNA, insulin, tetanus toxoid, and type II collagen. The study conducted by Burastero et al showed that monoreactive and polyreactive rheumatoid factors were obtained from B lymphocytes of patients with RA, but no monoreactive rheumatoid factors could be obtained from B lymphocytes of healthy subjects.  

In this study, among eight monoclonal IgM rheumatoid factor producing cell clones obtained from the peripheral blood of two patients with RA, only two clones were found to produce monoreactive IgM rheumatoid factor. In contrast, four monoclonal IgM rheumatoid factor producing cell clones derived from one synovial tissue sample all produced monoreactive IgM rheumatoid factor. Although the number of clones obtained was too small to reach a definite conclusion, our results suggest that the proportion of monoreactive rheumatoid factor producing B cell precursors is higher in synovial tissue than in peripheral blood in RA. Our finding is in agreement with the studies reported by Randen et al, which showed that 14 monoclonal rheumatoid factors were monoreactive among 20 monoclonal rheumatoid factor producing clones isolated from the synovial fluid of two patients with RA and one patient with juvenile RA.  

The observed higher affinity of monoreactive IgM rheumatoid factor to IgG Fc, shown by $K_a$ values two orders of magnitude larger than those of polyreactive IgM rheumatoid factors, coincides with the findings published by other workers.  

![Figure 3](image_url)  

Figure 3 shows that the mean (SD) value, 2.66 (0.78), of bound C4 to monoreactive IgM rheumatoid factors was approximately three times larger than that (0.96 (0.22)) of bound C4 to polyreactive IgM rheumatoid factors. This difference was significant (p<0.01).

The precise mechanism by which monoreactive IgM rheumatoid factor shows higher complement activating properties is unclear. Feinstein and coworkers showed that the IgM molecule, which existed in a planar 'star' conformation with its $F(ab')_2$ regions in the same plane as the central (Fc)2 disc, did not bind complement, whereas the IgM molecule, which existed in a 'staple' conformation with its $F(ab')_2$ regions folded down to enable attachment to an antigen, bound complement. Lucisano Valim and Lachmann investigated the complement fixing activity of immune complexes formed in the fluid phase and showed that IgM activated the classical pathway but showed a prozone phenomenon where an immune complex formed in excess IgM antibody inhibited complement activation; this is presumed to reflect the relative amounts of IgM that were in the 'star' and 'staple' conformations.  

In our study, the concentrations of polyreactive IgM rheumatoid factors were higher than those of monoreactive IgM rheumatoid factors, hence all IgM rheumatoid factors bound to IgG Fc precoated wells to a similar
extent. Although Lucisano Valim and Lachmann investigated complement activation by IgM bound to antigen in the fluid phase, whereas we examined complement activation to IgM rheumatoid factor bound to IgG Fc on the solid phase, it may be possible for polyreactive IgM rheumatoid factors to appear less effective in complement activation because of the prozone phenomenon. This seems unlikely, however, because the amounts of C4 bound to polyreactive IgM rheumatoid factors decreased with the dilution of polyreactive IgM rheumatoid factors in the same manner as monoreactive IgM rheumatoid factors (data not shown); the optimum concentration of each polyreactive IgM rheumatoid factor fell to within the steep portion of the C4 binding curve produced by serial dilution of each IgM rheumatoid factor. It is therefore likely that a higher avidity between monoreactive IgM rheumatoid factor and IgG Fc increases the proportion of IgM rheumatoid factor molecules in the 'staple' conformation, thereby resulting in efficient binding of C1q and C4b.

Immune complexes containing IgG, IgM, and complement components have been shown to be present on the surface of articular cartilage in patients with RA. In addition, Ishikawa et al showed, using immunoelectron microscopy, that the clusters of immune deposits on cartilage contained rheumatoid factor. These findings suggest that rheumatoid factor may be an important constituent of the immune complex bound to cartilage. Along these lines, monoreactive rheumatoid factor may be more important in the synovitis of RA, as monoreactive IgM rheumatoid factor bound to IgG Fc on the solid phase is more reactive than polyreactive IgM rheumatoid factor in complement activation, and the binding of monoreactive IgM rheumatoid factor to IgG in immune complexes on articular cartilage would produce more severe complement mediated tissue injury.

The commercial ELISA kit for single stranded DNA was a kind gift from Med. Biol. Lab., Nagoya, Japan. Tetanus toxoid was a kind gift from Green Cross Co., Osaka, Japan.

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