Circulating and intra-articular immune complexes in rheumatoid arthritis: a comparative study of the C1q binding and monoclonal rheumatoid factor assays

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SUMMARY The C1q binding assay and the nephelometric monoclonal rheumatoid factor assay were able to discriminate 79% and 57% respectively of rheumatoid arthritis (RA) patients from healthy blood donors. In addition these assays could distinguish those patients with active arthritis from those with inactive disease, and the C1q binding assay correlated significantly with other laboratory indices of the rheumatoid process, including the erythrocyte sedimentation rate, low molecular weight or 7S IgM, and the rheumatoid factor titre. High levels of C1q binding were also seen in rheumatoid vasculitis. Both assays gave higher mean values in synovial fluid compared with the corresponding serum, but it appeared from ultracentrifugal analysis and from a lack of a consistent correlation between these assays that each assay was measuring different forms of immune complex-like material which may be involved in the immunopathogenesis of this disease. The C1q binding assay is of some value in the laboratory assessment of rheumatoid arthritis and appears to offer greater advantages than the monoclonal rheumatoid factor assay, although the usefulness of this latter assay may be very dependent on the monoclonal rheumatoid factor used.

Activation and inhibition of various immunological effector mechanisms by circulating and intra-articular immune complexes is thought to explain many of the pathological manifestations of rheumatoid arthritis (RA).¹ Consequently it is hoped that sensitive and simple techniques for measuring these complexes might be of value in the diagnosis of this disease and in assessing disease activity and monitoring treatment response. A recent study involving 18 different assays for immune complexes showed that the 2 assays giving the best discrimination between RA patients and controls were the C1q binding assay (C1q BA) and the monoclonal rheumatoid factor (MCRF) assay.² Furthermore, Zubler *et al.*³ and Luthra *et al.*⁴ using these assays have shown high levels of immune-complex-like material in rheumatoid sera and synovial fluid and have correlated serum levels with some indices of disease activity.

In this communication we describe our experience with these 2 assays in a comparative study of a group of 42 patients with RA. Our aim has been (1) to find if these assays correlate with the disease activity and with other variables of the rheumatoid process, and (2) to compare these assays to find if they are measuring similar immune-complex-like material in sera and synovial fluid.

Materials and methods

PATIENTS AND CONTROLS

Forty-two patients with definite or probable RA (American Rheumatism Association criteria) were selected from the rheumatological ward (19 patients) and the outpatient clinic (23 patients). Twenty-five of the patients were female and 17 male, the mean age was 52 years (range 14-78 years), and the mean duration of the illness was 13 years (range 2 months to 27 years). Twenty-nine of the patients were seropositive, defined as a Rose-Waaler titre of >1:32. The patients were divided into 3 groups: (1) those with active disease, defined by the presence of joint

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swelling, tenderness, and pain in one or more joints (15 patients); (2) those with active disease complicated by rheumatoid vasculitis defined by the presence (within 1 month of testing) of mononeuritis multiplex, a necrotising vasculitis, or the presence of skin ulceration not attributable to other causes (6 patients); and (3) those with inactive or suppressed disease defined by the absence of active joint inflammation (11 patients). Thirty-five of the patients were taking nonsteroidal anti-inflammatory drugs, 14 were taking low-dose prednisolone, 8 were taking penicillamine, 6 were on gold, 6 were taking antimalarials, and 1 was taking azathioprine. Three patients were taking no drugs. Control sera were obtained from healthy blood donors.

Venous blood was allowed to clot at room temperature, and serum, obtained within 4 hours, was divided into aliquots and stored at -80°C . Joint fluid was aspirated from the knees in 10 patients, divided into aliquots, and stored at -80°C .

C1Q BINDING ASSAY

The method used was that described by Zubler *et al.*³ The results were expressed as μg of a preparation of heat-aggregated human IgG (HAGG) per ml of serum. HAGG was prepared by heating Cohn fraction II γ -globulin (20 mg/ml) at 63°C for 30 min and diluting in normal human serum (NHS).

MONOCLONAL RHEUMATOID FACTOR (MCRF) ASSAY

IgG-containing complexes were measured by a laser nephelometric technique with an IgM monoclonal rheumatoid factor isolated from a patient with a lymphoproliferative disorder.⁵ $5\ \mu\text{l}$ of this reagent (14 mg/ml) were added to serum diluted 1:25 with phosphate buffered saline (PBS, pH 7.3) or gradient fraction, to give a final volume of $500\ \mu\text{l}$, incubated at room temperature for 1 h, and the resultant precipitate formation quantitated by laser nephelometry. All estimates were performed in duplicate and blanks (without MCRF) were included in every assay. Precipitate formation was compared against a standard curve utilising known amounts of HAGG (diluted in NHS) which was detectable to a concentration of $6\ \mu\text{g}/\text{ml}$, and all the results were expressed as $\mu\text{g}/\text{ml}$ of HAGG equivalents. Polyclonal rheumatoid factor did not interfere with the reactivity of the MCRF. Monomeric IgG would precipitate with MCRF but only at a final concentration greater than $200\ \text{mg}/100\ \text{ml}$.

SUCROSE GRADIENT ULTRACENTRIFUGATION

Sucrose gradient ultracentrifugation was performed at 4°C in a Beckman L5-50 ultracentrifuge using a

10–40% (w/v) continuous sucrose gradient in PBS. Serum was applied to the top of the gradient at a final dilution of 1:4 in PBS. Serum was centrifuged for 18 h at $285\ 000\ g$, and 5-drop aliquot fractions were collected in a dropwise fashion from the bottom of the gradient. $1\ \text{ml}$ of PBS was added to each fraction, which was then assayed at room temperature for optical density at $280\ \text{nm}$, specific immunoglobulins, and immune complexes.

ESTIMATION OF 7S IGM

1 to $2\ \text{ml}$ of serum was applied to an $85 \times 2.5\ \text{cm}$ Sepharose 6B (Pharmacia) column. Gel filtration was performed in PBS at room temperature with an upward flow at the rate of $20\ \text{ml}/\text{h}$, and 5-ml fractions were collected. IgM concentrations were determined in alternate fractions by a laser nephelometer (Behring) adapted with a single flow cell and a graph recorder (Camag Wang W 1100) to record mV of light scatter. $20\ \mu\text{l}$ of anti-IgM (Dako Immunglobulins) were added to $480\ \mu\text{l}$ of eluant taken from each alternate column fraction, allowed to stand at room temperature for 1 h, and the resultant precipitation measured and compared with a standard curve constructed from normal human serum of known IgM concentration. Blanks (i.e., $500\ \mu\text{l}$ of eluant from column fractions without IgM antisera) for subtraction were included in every assay. The minimum amount of IgM detected by this method is $0.1\ \text{mg}$ per $100\ \text{ml}$. To determine IgM concentrations within the standard curve some column fractions from sera with high IgM levels had to be diluted several-fold. An IgM profile was then obtained, and the percentage 7S IgM was calculated by planimetry where the relative amounts of 19S and 7S fractions were derived. The absolute quantity of 7S IgM was then calculated by multiplying the % 7S IgM with the total serum IgM level. Duplicate determinations of the % 7S IgM for 3 pathological sera did not vary by more than 3.1% (mean difference).

OTHER HAEMATOLOGICAL AND IMMUNOLOGICAL MEASUREMENTS

The erythrocyte sedimentation rate (ESR, Westergren) and Rose-Waaler titre were measured by standard laboratory techniques. Serum immunoglobulins C3 and C4 were measured by an automated immune precipitation nephelometer (Technicon). The anti-IgG (rheumatoid factor) titre was measured by observing the degree of precipitation in the laser nephelometer between $100\ \mu\text{g}$ of HAGG and test serum diluted 25-fold in PBS to give a final volume of $500\ \mu\text{l}$. There is an excellent correlation between this method and the Behring latex titre ($r=0.95$, $P<0.001$).

STATISTICAL ANALYSIS

Comparison between variables was calculated by linear regression, while comparison between groups was by the Wilcoxon sum of ranks method.

Results

C1Q BINDING ASSAY

Serum (Fig. 1). An upper normal limit of 40 µg HAGG equivalents/ml serum (mean + 2 SD) was derived from the C1q binding of 200 blood donors. Thirty-three of the 42 RA patients (79%) gave a binding greater than this value. Those patients with active disease had significantly greater C1q binding ($P < 0.01$) than those with inactive disease, while those with rheumatoid vasculitis all gave high levels (Fig. 1). Significant correlations of serum C1q binding were obtained with the ESR, anti-IgG level, IgA, IgM, and 7S IgM, and inversely with the C4 level (Table 1).

Synovial fluid (Fig. 2). The mean C1q binding for the 10 synovial fluids (311 µg HAGG equivalents/ml) was greater than that of the corresponding sera

(243 µg HAGG equivalents/ml), but this difference was not significant. Significant correlations occurred between C1q binding in synovial fluid and C1q binding in serum ($P < 0.05$) and the synovial fluid anti-IgG, IgA, and MCRF binding (Table 2).

Ultracentrifugation studies (Fig. 3). Ultracentrifugal analysis of 28 RA sera and 7 paired synovial

Fig. 1 C1q binding in inactive RA, active RA, and RA complicated by vasculitis. The upper limit of normal for the assay (40 µg HAGG eq/ml) is indicated by the dashed line.

Fig. 2 C1q binding in paired sera and synovial fluid from 10 patients. The dashed line indicates the normal upper limit for serum.

Table 1 Correlation of serum C1q binding with other clinical and laboratory variables

	Age	Duration	ESR	Anti-IgG	IgG	IgA	IgM	C3	C4	7S IgM	MCRF
No.*	42	40	41	42	42	41§	42	41	41	28	42
r†	0.23	0.12	0.42	0.79	0.09	0.33	0.43	-0.19	-0.39	0.69	0.03
p‡	NS	NS	<0.01	<0.01	NS	<0.05	<0.01	NS	<0.05	<0.01	NS

*Number of patients studied. †Regression coefficient. ‡Probability: NS=not significant, $P > 0.05$. §One patient with IgA deficiency has been excluded.

Table 2 Correlation of synovial fluid C1q binding with other synovial fluid laboratory variables

	C1q (s)*	IgG	IgA	IgM	C3	C4	Anti-IgG	7S IgM	MCRF
No.	10	10	10	10	10	10	10	10	10
r	0.64	0.31	0.73	-0.18	0.0	-0.17	0.57	0.42	0.66
p	<0.05	NS	<0.05	NS	NS	NS	0.05	NS	<0.05

*C1q (s) C1q binding in corresponding serum.

Fig. 3 Sucrose gradient ultracentrifugal analysis of serum and synovial fluid from an illustrative RA patient. The optical density and C1q binding profiles are shown together with the sedimenting position of IgG and IgM.

fluids was performed, and a typical optical density and C1q binding profile is shown in Fig. 3. In general, 3 C1q binding peaks were observed, a high molecular weight peak, a peak sedimenting in the region a little heavier than IgG, and a lighter peak. This latter peak tended to be variable and in some

Fig. 4 MCRF binding in inactive RA, active RA and in rheumatoid vasculitis. The upper limit of normal for the assay (10 μ g/ml HAGG) is indicated by the dashed line.

Table 3 Correlation of serum MCRF assay with other clinical and laboratory variables

	Age	Duration	ESR	Anti-IgG	IgG	IgA	IgM	C3	C4	7S IgM	C1q
No.	42	40	41	42	42	41	42	41	41	28	42
r	0.02	-0.22	-0.01	-0.06	0.37	0.2	-0.25	0.22	0.06	-0.03	0.03
p	NS	NS	NS	NS	<0.05	NS	NS	NS	NS	NS	NS

Table 4 Correlation of synovial fluid MCRF assay with other synovial fluid laboratory variables

	MCRF (s)*	IgG	IgA	IgM	C3	C4	Anti-IgG	7S IgM	C1q
No.	10	10	10	10	10	10	10	10	10
r	0.65	0.63	0.82	-0.38	-0.2	-0.09	0.28	0.21	0.66
p	0.05	0.05	NS	NS	NS	NS	NS	NS	<0.05

*MCRF(s) MCRF binding in corresponding serum.

sera was only small or absent. Surprisingly, there was no agreement between the total areas beneath the peaks and the total C1q binding level in sera or synovial fluid. In addition, serial ultracentrifugal analysis of serum C1q binding was performed in

2 patients over a 6-month period. Despite rising levels of the serum C1q binding and the development of a florid vasculitis in 1 patient, there was no apparent alteration in peak size or sedimentation position.

Fig. 5 MCRF binding in paired sera and synovial fluid from 10 patients. The dashed line indicates the normal upper limit for serum.

MCRF ASSAY

Serum (Fig. 4). Only 2 of 232 blood donors had a binding greater than 10 µg/ml of HAGG equivalents, and this value was therefore taken as an upper normal limit. Twenty-four of the 42 RA patients (57%) had an MCRF binding greater than this upper limit, and those patients with active disease had significantly more MCRF binding ($P < 0.05$) than those with inactive disease. Five of the 6 patients with rheumatoid vasculitis had raised levels. A significant correlation was found between MCRF binding and the serum IgG but not with other clinical and laboratory variables (Table 3).

Synovial fluid (Fig. 5). Five of the 10 synovial fluids had raised MCRF binding with the mean synovial fluid MCRF binding (32 µg/ml HAGG equivalents) being higher than that of the corresponding serum (25 µg/ml HAGG equivalents). This difference was not significant. MCRF binding in synovial fluid correlated significantly with MCRF binding in serum and with IgG and C1q binding in synovial fluid (Table 4).

Ultracentrifugation studies. (Fig. 6). Ultracentrifugal analysis of MCRF binding in RA sera has been previously described.⁵ A further 4 sera and 2 synovial fluids were analysed in the present study. MCRF binding in all 4 sera was observed in the region

Fig. 6 Sucrose gradient ultracentrifugal analysis of serum and synovial fluid from an illustrative RA patient. The optical density, IgM, IgG, and MCRF binding profiles are shown (the scales are the same for serum and synovial fluid). Note the considerable MCRF binding between the IgG and IgM position in the synovial fluid. The IgM profile is skewed owing to the presence of 7S IgM.

sedimenting with uncomplexed native IgG (as previously described). However, in the 2 synovial fluids MCRF binding occurred both in the region sedimenting between the IgM and IgG markers and also in the IgG region (Fig. 6). This suggested that MCRF was binding with IgG-containing intermediate complexes present in the synovial fluid but not in the corresponding serum.

Discussion

We have compared the C1q binding assay and a nephelometric MCRF assay to other clinical and laboratory variables in assessing the serum and synovial fluid from a group of 42 patients with RA.

The C1q binding assay in RA has been extensively studied by Zubler and colleagues.³ This group found that the C1q binding was raised in the serum and synovial fluid of 66% and 76% respectively of RA patients when compared with patients with degenerative and post-traumatic joint diseases. In addition they found a significant correlation between serum C1q binding and disease activity as measured by a joint score, duration of morning stiffness, and the sedimentation rate. In the present study we have confirmed the high incidence of C1q binding in RA, particularly in those patients with active disease, and have noted high levels of C1q binding in all patients with rheumatoid vasculitis. This latter observation suggests that the C1q BA may be of value in detecting those patients at risk of developing this complication. However, in contrast to Zubler and colleagues we have found a significant correlation between C1q binding and the rheumatoid factor titre, and this observation has also been reported elsewhere.^{2,6,7} Indeed Erhardt *et al.*,⁶ using insolubilised IgG columns, found that C1q binding was associated with material with rheumatoid factor activity.

The material which binds C1q has been shown by Zubler *et al.*³ to have the properties of soluble immune complexes. However, from our ultracentrifugal studies it appears that smaller non-immune complex material also binds C1q. Furthermore, we were also unable to show any major difference in the size of C1q binding material between different patients, and serial studies in 2 patients showed little variation in peak size or position despite changing serum C1q levels and disease activity. Whether this is indeed a true finding or reflects a lack of sensitivity of the sedimentation studies is unclear at this stage.

Several authors have found the MCRF assay to be of value in distinguishing RA patients from healthy controls. Winchester *et al.*,⁸ using a simple immunodiffusion technique, found that their MCRF would

precipitate with 57% of RA sera and 70% of RA synovial fluids. This group also showed that the precipitating material had the characteristics of IgG-containing immune complexes and were of high molecular weight. Similarly, Gabriel and Agnello,⁹ using a competitive radioimmunoassay, found that 56% of their RA sera gave positive results, but there was no correlation between MCRF reactivity and serum IgG (in contrast to the results in the present study) or with the rheumatoid factor titre or C1q binding. They also reported that MCRF reacted with material sedimenting between the 7S and 19S marker or in heavier fractions in RA sera, while in RA synovial fluid reactivity was only found in the heavy region. Luthra and colleagues,⁴ using MCRF in a solid phase radioimmunoassay, found that 26% of RA sera gave increased results compared with a control group. Levels in synovial fluids were generally higher than corresponding serum levels, and significant correlations were seen between serum MCRF reactivity and the severity of the disease as measured by anatomical stage or functional class and inversely with the C4 level. However, these authors found no correlation with the ESR or rheumatoid factor titre, and sucrose density gradient ultracentrifugation of serum revealed that MCRF reactivity occurred with material sedimenting at the 11S position or in heavier regions (19S or larger).

We have previously reported the isolation and characterisation of the MCRF used in the present study.⁵ It was shown to react with 59% of RA sera but surprisingly appeared to bind with material sedimenting in the 7S IgG position in 7 RA sera studied. In the present study these results have been closely duplicated, but analysis of synovial fluid has revealed that this reagent binds with material sedimenting in the IgG region and in the heavier regions between IgG and IgM. The interpretation of this latest finding is unclear, but it is possible that our MCRF binds with either conformationally altered monomeric IgG as previously described in RA¹⁰ and/or with IgG anti-IgG which are forming small stable 'intermediate' complexes particularly in synovial fluid due to self-association, as previously described by Pope *et al.*¹¹ It is clear, however, that our MCRF is reacting with material which is quite distinct from that binding with C1q.

A significant correlation was found between C1q binding and with other indices of disease activity including the ESR and 7S IgM. We have previously shown that low molecular weight or 7S IgM is found in up to 75% of RA patients, being particularly prominent in those with active disease and with high rheumatoid factor titres.¹² Stage and Mannik¹³ and Theofilopoulos *et al.*¹⁴ both reported its frequent occurrence in rheumatoid vasculitis and suggested

that it may be involved in the pathogenesis of this complication. This suggestion is further supported by the finding that 7S IgM appears to function as a univalent antibody which could form small soluble complexes.¹⁵ Additionally 7S IgM has been shown to have rheumatoid factor activity,¹³ but in our previous study, although patients with rheumatoid vasculitis had 7S IgM, we were unable to show any significant difference in 7S IgM levels between patients with or without vasculitis.¹² Nevertheless the significant correlation shown between 7S IgM and C1q binding in the present study supports the suggestion that some of the material binding C1q could be immune complexes involving 7S IgM and that this material might be involved in the immunopathogenesis of rheumatoid vasculitis.

Both the C1q and MCRF assays in the present study were found to discriminate RA patients from healthy blood donors. Furthermore, from the significant correlations described it appears that the C1q binding assay was of some value in the assessment of the severity and activity of the rheumatoid process and appears to relate to the presence of extra-articular manifestations such as rheumatoid vasculitis. In addition, we have unpublished data suggesting that serial C1q measurement is of value in monitoring disease activity and response to treatment. In contrast, the value of the nephelometric MCRF assay is less clear. The results from the present study indicate that this assay has limited value in assessing disease activity and no value in detecting rheumatoid vasculitis, but this could be very dependent on the properties of the individual MCRF used. The technique is both simple and rapid, and therefore further comparative studies using different MCRFs are required, as these reagents may provide further insight into the nature and immunopathogenesis of RA.

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