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The complement fixing ability of putative circulating immune complexes in rheumatoid arthritis and its relationship to extra-articular disease

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SUMMARY

The hypothesis that the pathogenicity of putative circulating immune complexes (CIC) in rheumatoid arthritis (RA) is related to their ability to fix complement was investigated. Three assays for CIC were employed; (a) the ^{125}I C1q binding assay (C1q BA), (b) the C1q solid phase assay (C1q SP) and (c) the Raji cell assay (RCA). Evidence for hypercatabolism of complement was obtained by using a highly sensitive quantitative assay for C3d (a breakdown product of C3) by rocket immunoelectrophoresis. One hundred and fifty-two patients with classical or definite RA were studied; 54 had clinical evidence of extra-articular disease including vasculitis, nodules, scleritis, neuropathy and lung disease; 98 patients had clinical evidence of joint disease alone. Plasma levels of C3d were significantly elevated in the RA group as a whole $16{\cdot}7 \pm 4{\cdot}4$ mg/l (mean $\pm$ 1 s.d.) compared with $13{\cdot}1 \pm 3{\cdot}15$ mg/l in a group of 55 normal controls ($P < 0{\cdot}01$). Elevated levels of C3d were found in 26% of all patients but occurred significantly more often in the extra-articular disease group ($P < 0{\cdot}05$). Fifty-four percent of patients had at least one positive assay for CIC although no individual assay was positive in more than 36% of the group as a whole. The prevalence of positive CIC was significantly greater in those patients with extra-articular disease than in those with joint disease alone ($P < 0{\cdot}005$). Of the total of 82 patients with putative CIC, 30 (37%) had a raised C3d level. The coincident finding of positive tests for CIC and an elevated C3d level was very significantly correlated with the presence of extra-articular disease ($\chi^2 = 12{\cdot}7$ $P = 10^{-3}$). Whilst putative CIC are frequent in RA (54%) these findings in contrast to previous work, suggest that the majority are not associated with abnormal complement activation and may account for the relative infrequency of clinically detectable active extra-articular disease.

INTRODUCTION

It is well established that depressed levels of complement components and increased levels of complement breakdown products may be found in the joint cavity of patients with rheumatoid arthritis (RA) (Ruddy & Austen, 1970; Hedburgh, Lundh & Laurell, 1970; Perrin *et al.*, 1977). The concurrent presence of immune complexes and pattern of changes in complement indicates that complement activation is chiefly mediated via the classical pathway (Winchester, Agnello & Kunkel, 1970; Lambert *et al.*, 1975; Zubler *et al.*, 1976b) although evidence for alternative pathway activation has also been documented (Ruddy, Fearon & Austen, 1971; Hunder, McDuffie & Clark,

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1979; El-Ghobarey & Whaley, 1980). In addition many studies have shown that a high proportion of RA patients may have putative circulating immune complexes (CIC) in the blood although their prevalence may depend as much upon patient selection as upon the actual immune complex assay employed and this may account for the differences reported (Winchester, Kunkel & Agnello, 1971; Zubler *et al.*, 1976b). Complement activation in the blood has been demonstrated in immune complex-mediated diseases by the finding of circulating complement breakdown products (Perrin, Lambert & Meischer, 1975) especially C3d, the relatively stable low molecular weight breakdown product of C3. C3d had been measured quantitatively and has been found to be raised in 79% of patients with RA (Nydegger *et al.*, 1977). Mean plasma C3d levels were also found to be significantly higher in patients with extra-articular than articular disease alone and the levels of C3d were related to levels of CIC detected by the C1q binding assay. Levels of C1q binding have been found to be higher in patients with extra-articular disease than articular disease alone and cryoglobulinaemia also appears to correlate well with the presence of extra-articular features of disease (Erhardt, Mumford & Maini, 1979).

We have explored the hypothesis that the pathogenicity of CIC is related to their ability to fix complement and that this process is related to the development of extra-articular disease features. Measurement of complement activation was assessed by a highly sensitive assay for C3d using a rocket immunoelectrophoresis technique and three assays for the detection of CIC (a) the ^{125}I-C1q binding assay (Zubler *et al.*, 1976b), (b) the C1q solid phase assay (Hay, Nineham & Roitt, 1976a,b) and (c) the Raji Cell Assay (Theophilopoulos, Wilson & Dixon, 1976). These techniques have been applied to a large number of patients with RA of varying disease duration, activity and including patients with extra-articular disease in order to seek correlations which may support the role of intravascular complement activation by immune complexes in the pathogenesis of extra-articular manifestations.

PATIENTS AND METHODS

One hundred and fifty-two patients with classical or definite rheumatoid arthritis classified according to the American Rheumatism Association (1959) criteria were studied. Their ages ranged from 17 to 78 years (mean age 54 years) and duration of disease varied from 6 months to 30 years. Fifty-four patients had clinical evidence of one or more extra-articular disease manifestations which had been present for a variable period at the time of study. These features included cutaneous vasculitis (biopsy proven), peripheral neuropathy, occular scleritis, subcutaneous rheumatoid nodules and lung disease (fibrosing alveolitis, nodules or pleural effusions). The remaining 98 patients had clinical evidence of joint disease alone. Disease activity and treatment regimes varied in the patients and were not analysed in relation to results. All patients were assessed clinically at the time of blood sampling which was performed mid-morning and serum and plasma samples were separated and stored at -70°C until assayed. Control samples from healthy volunteers were collected and stored in the same way. The numbers of controls for each assay were as described below.

C3d assay. Measurements of C3d were carried out according to a method already described (Bourke, Moss & Maini, 1981). Essentially, blood samples were collected in 10 mM disodium ethylene tetra-acetic acid (EDTA) and the plasma separated and stored at -70°C. Fractionation of the low molecular weight C3d was performed by incubating the plasma with an equal volume of 22% polyethylene glycol (PEG) at 4°C for 2·5 hr followed by high speed centrifugation. Supernatants were then subjected to rocket immunoelectrophoresis using a 1% concentration of anti-C3d antiserum (Netherlands Red Cross) in a 1% agarose gel. Quantitative results expressed in milligrams per litre were obtained from a standard curve of rocket height versus C3d concentration using three dilutions of a standard incorporated into every electrophoretic assay. Two other normal controls of known C3d concentration were also incorporated into every electrophoretic plate. The normal range for this assay based on 55 normal human plasma samples was 6·8–19·4 mg/l.

Immune complex assays

^{125}I-C1q binding assay. The binding assay of Zubler *et al.* (1976a) was used with the following

modifications: (a) an aliquot of ^{125}I-C1q was made up to 3 ml with veronal-buffered saline containing 10% bovine serum albumin (VBS-BSA) and after centrifugation the upper 2·5 ml were used, (b) the assay was carried out in polystyrene tubes (LP3, Luckhams), (c) the PEG borate buffer contained 0·05% Tween and the supernatants were aspirated. Results were expressed as a percentage of the trichloroacetic acid precipitatable counts, the normal range being 0–10%. Each assay included three normal sera, a standard curve of aggregated human gammaglobulin and a pathological reference serum to check reproducibility.

C1q solid phase assay. The solid phase assay was based on that described by Hay *et al.* (1976a). Flexible vinyl microtitre plates (Linbro) were coated with 200 μl/well of C1q at a concentration of 10 μg/ml in phosphate-buffered saline (PBS), for 72 hr at 4°C. After six washes with PBS unoccupied sites on the solid phase were blocked with 0·01% gelatine in PBS at room temperature for 2 hr. The plates were washed with PBS before use. Sera to be assayed were diluted 1 to 3 with 0·2 M EDTA pH 7·5 and incubated at 37°C for 30 min, then transferred to an ice bath. The diluted sera (20 μl) were added to duplicate wells followed by 180 μl of PBS containing 0·05% Tween (PBS-Tween). The plates were incubated at 37°C for 1 hr and at 4°C for 30 min. After washing with PBS, 50 ng of ^{125}I-protein A in 200 μl of PBS-Tween was added to each well and the plates incubated as before. After washing, individual wells were counted in a gamma counter and the results expressed as pg of protein A bound (the normal range in this assay being 0–650 pg). Controls in each assay included three normal sera, a standard curve of aggregated human gammaglobulin and a pathological reference serum to check reproducibility.

Raji cell assay. The assay was modified from the method described by Theophilopoulos *et al.* (1976). Briefly, 25 μl of serum diluted 1:8 was incubated with 2×10^6 Raji cells in duplicate in U-well microtitre plates (Cooke). After washing, the cells were incubated with ^{125}I-protein A, washed and counted. Results were expressed as test/control ratio, i.e. mean duplicate c.p.m. test serum:geometric mean c.p.m. normal human serum (NHS). The upper limit of normal was derived by dividing the duplicate mean for each of the NHS by their geometric mean. The arithmetic mean plus two standard deviations of these six ratios was taken as the upper limit of normal.

Statistical analysis. The differences in values found in the CIC assay and C3d levels in RA patients subdivided into articular and extra-articular disease were analysed by the Student's *t*-test and the Mann–Whitney *U*-test. The prevalence of positive results in all four assays was compared by Yates' Chi-square test.

RESULTS

C3d

The mean level of C3d in the 152 RA patients of 16·7 ± 4·4 mg/L (mean ± s.d.) was significantly raised above the level in normals of 13·1 ± 3·15 mg/l (Mann–Whitney *U*-test $P < 0{\cdot}01$). The mean level in the 54 patients with extra-articular (EA) disease was 18·0 ± 7·1 mg/l and was marginally

Table 1. Frequency of elevated plasma C3d levels and positive CIC tests

	Total patients ($n = 152$)	Articular disease alone ($n = 98$)	Extra-articular disease ($n = 54$)	χ^2 A v EA
C3d ↑	26%	19%	37%	$P < 0{\cdot}05$
RCA +ve	30%	25%	39%	n.s.
C1q BA +ve	36%	25%	56%	$P < 0{\cdot}002$
C1q SP +ve	30%	24%	44%	$P < 0{\cdot}05$
Any one or more CIC assay +ve	54%	41%	78%	$P < 0{\cdot}005$

higher than the mean of 16·0 ± 6·2 mg/l in the 98 patients with articular (A) disease alone although these levels were not significantly different from one another. Using a level of 19·4 mg/l (2 s.d. above the mean) as a criterion for raised levels of C3d, 39 patients (26%) of the total of 152 patients had elevated levels. Twenty (37%) of the 54 patients with extra-articular disease were found to have raised levels compared to 19 (20%) of the 98 patients with articular disease alone (Table 1). Using a non-parametric test (Yates Chi-square analysis), the difference between the two groups was statistically significant ($P < 0·05$), an analysis contrasting with the lack of significance using Student's *t*-test. Mean levels of C3d in a number of other rheumatic conditions were not significantly elevated from normal with the exception of patients with systemic lupus erythematosus (Table 2).

Circulating immune complex assays

Examining the total RA group initially, 45 of 150 patients' sera tested (30%) were positive in the Raji cell assay (RCA), 55 of 152 (36%) in the C1q binding assay (C1q BA) and 41 of 138 (30%) in the C1q solid phase assay (C1q SP). Taking all three assays in combination, 82 patients' sera (54%) were positive in at least one assay (Table 2). Dividing the patients into those with extra-articular features and those with articular disease alone, by the RCA 39% were positive in the EA group compared with 25% in the articular disease group although this difference was not statistically significant ($P < 0·1$). However by the C1q BA and the C1q-SP the differences between the groups was highly significant (C1q BA, $P = 0·002$; C1q SP, $P < 0·05$). Taking all three assays in combination there was a highly significant difference in the prevalence of positivity in one or more CIC assays between those patients with EA disease (42 of 54) and those with articular disease alone (40 of 98) ($P < 0·005$).

Examining for the presence or absence of EA disease in those patients with and without CIC a more striking difference was seen. Of the 82 patients positive in at least one CIC assay, 42 (51%) had EA disease whereas in the 72 patients with negative CIC assays only 12 (17%) had EA disease.

When the presence of CIC and complement activation (i.e. raised C3d levels) were examined together a different picture emerged (see Fig. 1). Of the 82 patients with positive CIC (one or more assays), 30 (37%) had elevated C3d levels and 20 of these 30 had EA disease. In the remaining 52 with normal C3d levels only 23 had EA disease. On the other hand in the 70 patients without CIC only nine (12%) had a raised C3d level and interestingly all these nine patients had articular disease alone. The remaining 63 patients had normal C3d levels and only 12 (19%) had EA disease. Linear

Table 2. Plasma C3d levels in patients with rheumatoid arthritis (RA) other rheumatic conditions and in controls

	Number of patients	C3d (mg/l)	Standard deviation (mg/l)
RA	152	16·7	4·4
RA (Extra-articular)	54	18·0	7·1
RA (Articular)	98	16·0	6·2
SLE*	40	18·8	6·1
Gout	16	13·4	4·3
O*A.†	11	15·0	3·2
Miscellaneous‡	15	13·1	4·3
Normal controls	55	13·1	3·15

*Systemic lupus erythematosus.

†Osteoarthrosis.

‡2 patients with scleroderma, 3 with psoriatic arthritis, 4 with ankylosing spondylitis, 3 with systemic vasculitis and 3 with polymyositis.

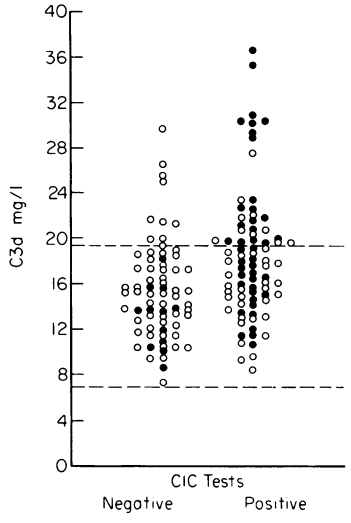

Fig. 1. Correlation of levels of plasma C3d and tests for circulating immune complexes. (● = Extra-articular disease; ○ = articular disease only).

Table 3. Correlation of plasma C3d levels with CIC tests

	Total patients ($n=152$)	Articular disease alone ($n=98$)	Extra-articular disease ($n=54$)
C3d v RCA	$r=0{\cdot}30$ $P<10^{-3}$	$r=0{\cdot}14$ n.s.	$r=0{\cdot}47$ $P<10^{-3}$
C3d v C1q-BA	$r=0{\cdot}43$ $P<10^{-7}$	$r=0{\cdot}28$ $P<10^{-2}$	$r=0{\cdot}47$ $P<10^{-3}$
C3d v C1q-SP	$r=0{\cdot}53$ $P<10^{-9}$	$r=0{\cdot}08$ n.s.	$r=0{\cdot}65$ $P<10^{-7}$

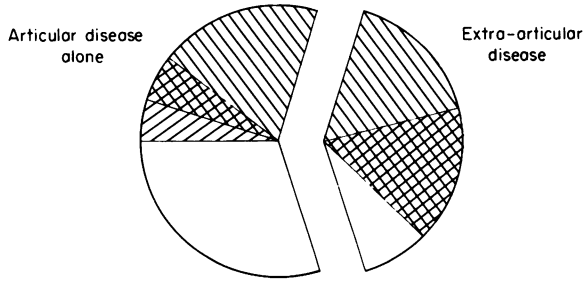

Fig. 2. Schematic representation of the coincident occurrence of positive tests for circulating immune complexes (CIC) and raised C3d levels in RA patients ($n=152$) with and without extra-articular disease (Chi-square analysis: $\chi^2=12{\cdot}7$, $P<10^{-3}$). (▨ = patients with +ve CIC test; ▩ = patients with ↑ C3d; ▩ = patients with −ve CIC test).

regression analysis between C3d levels and levels in the individual CIC assays showed good correlations when the total RA patient group was examined (C3d vs RCA, $r=0{\cdot}30$, $P<10^{-3}$; C3d vs C1q, BA $r=0{\cdot}43$, $P<10^{-7}$; C3d vs C1q-SP, $r=0{\cdot}53$, $P<10^{-9}$). In patients with articular disease alone, C3d did not correlate with CIC levels in the RCA and C1q-SP although there was a weak correlation with the C1q-BA levels. On the other hand there were strong correlations between C3d levels and levels of CIC in the patients with EA diseases especially in the C1q SP assay (Table 3). This suggests that the C1q SP assay and C3d taken together discriminate best for the presence of EA disease.

Chi-square analysis of the coincident finding of a raised C3d and positive CIC assays between patients with and without EA disease showed a highly significant association with EA disease ($\chi^2=12{\cdot}7$; $P<10^{-3}$) (see Fig. 2).

DISCUSSION

We have shown a relatively modest prevalence of CIC in RA patients (54%) overall as compared with other studies (Lambert *et al.*, 1978; Zubler *et al.*, 1976b) especially since only one third of our patients were positive in each individual assay. However it is noteworthy that the prevalence did not vary much between the individual assays (30–36%) suggesting that this prevalence was probably due to case selection rather than the assay employed. These patients were all of varying disease duration and activity and were therefore undergoing a variety of treatment regimes and represent a relatively heterogenous population. There was however a striking difference in the prevalence of CIC between patients with and without extra-articular disease features using the three CIC assays together ($P<0{\cdot}005$) and this is similar to findings of other workers (Zubler *et al.* 1976a). However the C1q-BA proved to be the best assay at discriminating extra-articular disease and the RCA the worst. When patients were divided on the basis of positivity or negativity in the CIC assays, 51% of those with CIC had extra-articular disease as compared to 17% of those without CIC detectable by any one of the three assays. This finding adds support to our previous studies which implicated CIC in extra-articular disease in RA (Erhardt *et al.*, 1979).

Twenty-six per cent of the total RA patients had a raised C3d level although the mean C3d level was significantly higher in these patients compared with a normal control group. Levels were higher in patients with extra-articular disease than in those with articular disease alone, but not significantly so. However, the prevalence of raised C3d levels in patients with extra-articular disease was approximately double that observed in patients with joint disease alone. Our prevalence rates are still well under half of that found by other workers (Nydegger *et al.*, 1977). At least two explanations for these differences may exist. Firstly, our study was on a larger group of patients and included patients at various stages of disease activity. Secondly, our assay for C3d is up to six times more sensitive than a radial immunodiffusion (RID) technique being able to measure as little as 3 mg/l of C3d and therefore includes a distribution of values amongst normal subjects which overlaps with RA patients more distinctly than in RID assays.

Correlations between C3d levels and levels in the individual CIC assays were good but were not significant in patients with articular disease alone with the exception of the C1q-BA. However, in extra-articular disease the correlations were very significant and this difference would support the idea that extra-articular rheumatoid disease is a circulating immune complex disease characterized by complement consumption but that articular rheumatoid in most instances is not.

However, there were nine patients with articular disease alone in whom there was evidence of complement activation in the absence of detectable CIC in the intravascular compartment. A number of explanations may be responsible, for example: (a) complement breakdown products may be spilling over into the circulation in these patients with very active joint disease; (b) these patients may have CIC which are undetectable by our assays (e.g. IgA containing complexes); (c) alternative pathway activation is predominant in this group; (d) effective solubilization of complexes is occurring and thereby rendering CIC undetectable in these assays; (e) reticulo-endothelial clearance of C3d is impaired more than that for CIC. Of these explanations the first seems the most likely on the basis of current evidence available.

The findings of this study suggest that about half of the patients with RA (54%) have circulating immune complexes in their blood. Although in contrast to previous work, only a small proportion (20%) are associated with complement activation as judged by raised C3d levels. However, when immune complexes and complement activation are found together there is a very strong association ($P < 10^{-3}$) with the presence of extra-articular disease features in RA.

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