Reflection of disease activity in rheumatoid arthritis by indices of activation of the classical complement pathway

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SUMMARY Levels of C4d, a fragment of C4 generated during activation of the classical complement pathway, were measured in the plasma of 77 patients with rheumatoid arthritis and 30 healthy subjects. Disease activity was judged according to Ritchie's articular index to be mildly active in 31 (group 1), moderately active in 29 (group 2), and severely active in 17 patients (group 3). Plasma levels of C3d, a fragment of C3, and serum levels of C4, C3, and immune complexes were also measured. The ratios C4d/C4 and C3d/C3 were calculated. The C4d/C4 and C3d/C3 ratios and the levels of circulating immune complexes correlated with the degree of disease activity without significantly departing from linear trend and discriminated between patients with different grades of disease activity. C4d, C3d, C4, and C3 also correlated with disease activity but in a non-linear relationship. A significant correlation was found between C4d and C3d, and between C4d/C4 and C3d/C3. C4d and C4d/C4 also correlated with circulating immune complexes. These results indicate that indices of C4 and C3 activation, in particular the ratios C3d/C3 and C4d/C4, provide a sensitive assessment of disease activity in rheumatoid arthritis, and confirm the major part played by the classical complement pathway in the pathogenesis of this disease.

Activation of the complement system represents a major mechanism of inflammation in a variety of connective tissue disorders.[1] Complement involvement in rheumatoid arthritis has been demonstrated by the finding of depressed levels of complement factors in the synovial fluid of patients with active disease.[2 3] In the peripheral blood of these patients, however, the levels of complement factors are normal or increased,[4-7] owing to their behaviour as acute phase reactants.[8] Thus an increased synthesis compensates for the accelerated catabolism of these factors, explaining why their levels do not reliably reflect complement consumption in rheumatoid arthritis.[9]

Unequivocal evidence of complement activation can be obtained by the measure of complement fragments which are generated during complement activation.[10] Levels of the fragment C3d are found to be raised in rheumatoid arthritis, and their increase is not confined to the synovial fluid but is also detectable in the plasma.[7 11 12] Moreover, the levels of C3d tend to correlate with the severity of the disease.[6 13]

Using a recently developed technique[14] we measured plasma levels of C4d, a fragment produced as a consequence of the early activation events of the classical complement pathway,[15] in patients with rheumatoid arthritis with different degrees of disease activity. In these patients we also determined the circulating levels of C3d, C4, C3, and immune complexes and calculated the ratios C4d/C4 and C3d/C3, indices of complement activation independent of the initial concentration of intact complement components.

Subjects and methods

SUBJECTS

Seventy seven patients (60 female, mean age 53 years, range 30–80 years) with classical or definite

rheumatoid arthritis according to the American Rheumatism Association criteria[16] were studied. They were consecutive patients attending an established outpatient clinic at the department of rheumatology of King's College Hospital, London. Their mean disease duration was 9·2 years and ranged from 1·5 to 31 years. Ritchie's articular index, a simple scoring system of joint tenderness,[17] was used for the clinical assessment of disease activity. Each patient underwent clinical examination by the same observer (ASMJ) at the time that blood was taken. Thirty one patients with an articular index between 1 and 7 were assigned to disease activity group 1 (mildly active group), 29 patients with an articular index between 8 and 17 to group 2 (moderately active group), and 17 patients with an articular index exceeding 17 to group 3 (severely active group). Thirty healthy individuals (12 female, mean age 36 years, range 24–46 years), members of laboratory staff at King's College Hospital, were studied as controls.

BLOOD COLLECTION

Blood (5 ml) collected by venepuncture in a final concentration of edetic acid of 10 mmol/l was immediately separated by centrifugation at 1000 g at 4°C for 15 minutes and the plasma stored at −70°C. Another 5 ml of blood was allowed to clot at room temperature for two hours; after centrifugation serum was stored at −70°C. All laboratory investigations were performed by an operator unaware of clinical data.

C4d AND C3d MEASUREMENT

C4d levels were determined by a laser nephelometric technique.[14] Briefly, plasma was brought to 12% final concentration of polyethylene glycol 6000 and centrifuged at 1500 g for 30 minutes at 4°C. This manoeuvre precipitates C4 and its larger fragments, leaving C4d in the supernatant. The concentration of C4d was then measured by laser nephelometry, using anti-C4 intact antiserum (Behring). C3d plasma levels were similarly measured using anti-C3d antiserum (Dakopatts).[18] C4d and C3d results were expressed as percentages of a 100% C4d and C3d standard, obtained by exhaustive activation of complement through the classical pathway. Serial dilutions of the standard provided the C4d and C3d reference curves against which test samples were read.

C4 AND C3 MEASUREMENT

Serum concentrations of C4 and C3 were determined by laser nephelometry, using specific antisera (Behring), according to the manufacturer's instructions. Results were expressed in grams per litre,

using a protein calibrator distributed in the United Kingdom, consisting of pooled normal human serum (SPSO1).

MEASUREMENT OF CIRCULATING IMMUNE COMPLEXES

Serum levels of circulating immune complexes were determined by the ^{125}I C1q binding assay in liquid phase.[19] Results were expressed in micrograms per millilitre, using a WHO standard reference preparation containing tetanus toxoid-antitetanus toxoid complexes.

STATISTICAL ANALYSIS

Mean values of complement indices and circulating immune complexes of patients and controls were compared by Student's t test. The mean values of the three patient subgroups and controls were compared using an analysis of variance method, and a test for linear trend was performed over the four groups. Correlation between complement indices and circulating immune complexes was evaluated using Pearson's analysis.

To lessen the problem of unequal variances logarithmic transformations of all the above parameters, except C4 and C3, were used throughout the study.

Statistical computations were performed using the statistical package for the social sciences (SPSS) on an Amdahl 5980/300 at the University of London Computer Centre.

Table 1 *Comparison of complement indices between subgroups of patients and controls*

	Analysis of variance		
	Overall analysis of variance ($F_{3.103}$)	*Test for linear trend* ($F_{1.103}$)	*Departure from linear trend* ($F_{2.103}$)
C4	9·4**	13·2**	7·5**
C4d	29·5**	62·7**	12·8**
C4d/C4	7·0**	17·6**	1·7†
C3	10·9**	18·9**	6·8*
C3d	15·7**	35·9**	5·6*
C3d/C3	6·0**	15·2**	1·4†
CIC‡	28·3**	82·9**	1·0†

*0·001<p<0·01; **p<0·001.
†Not significant.
‡CIC=circulating immune complexes.
$F_{3.103}$ statistic summarises the overall analysis of the groups. The larger its value, the greater the evidence against the null hypothesis that the group means are equivalent. $F_{1.103}$ and $F_{2.103}$ statistics should be considered together in assessing the type of trend (linear or non-linear) across the groups. Low values of both suggest no trend, whereas high values for $F_{1.103}$ and low values for $F_{2.103}$ support a linear trend.
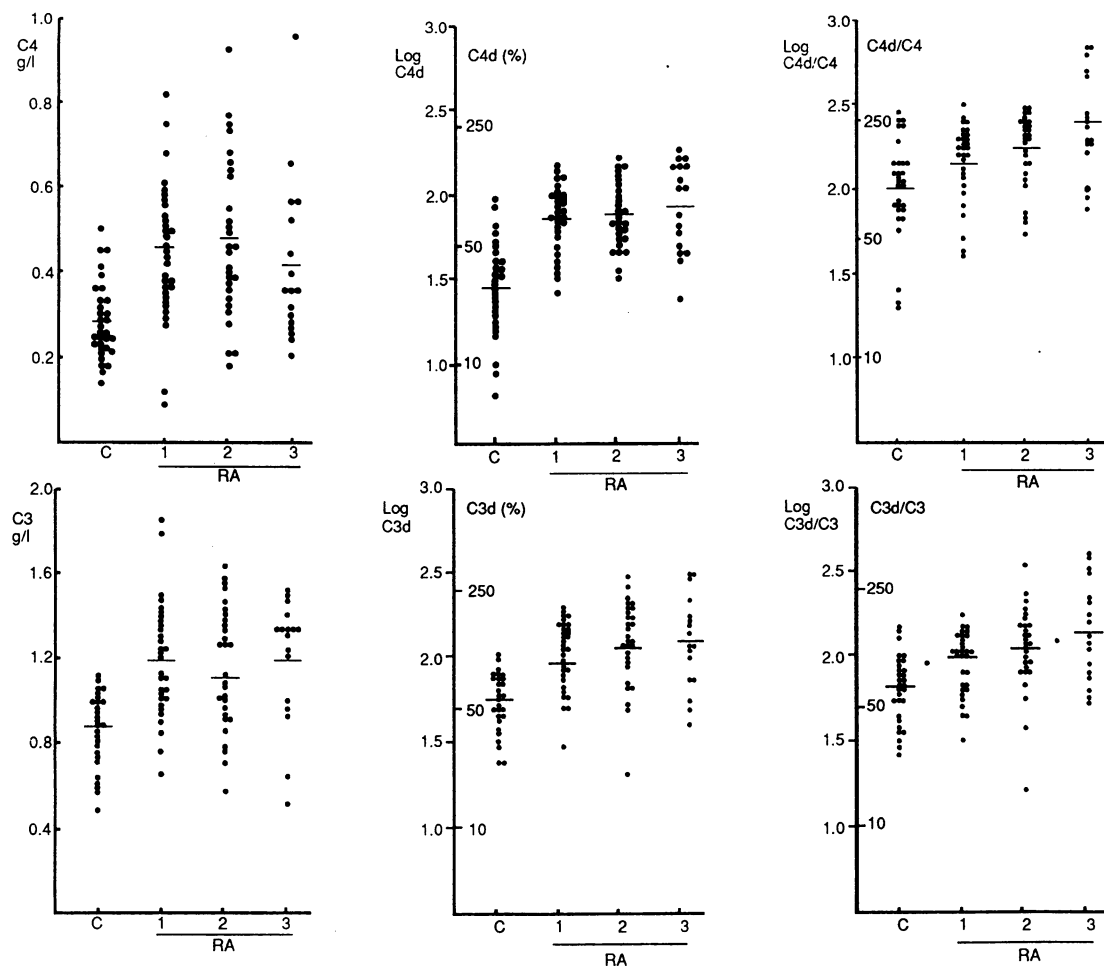
Fig. 1 *Complement indices (C4, C4d, C4d/C4, C3, C3d, C3d/C3) in subgroups of patients and controls. Both log transformed and raw values are given on the vertical axis for C4d, C4d/C4, C3d, and C3d/C3. Horizontal bars represent the mean values. C=controls; RA 1; RA 2; RA 3=subgroups of patients with increasing grades of disease activity (see text).*

Results

Table 1 and Fig. 1 show the results obtained. All complement indices were significantly higher in patients with rheumatoid arthritis than in controls (C4: t=5·18; C4d: t=9·39; C4d/C4: t=4·32; C3: t=5·71; C3d: t=6·77; C3d/C3: t=3·98; for all p<0·001) and they were significantly correlated with disease activity (Table 1). Only the ratios C4d/C4 and C3d/C3 increased in proportion to the grade of disease activity without significant deviation from

linear trend. That is, C4d/C4 and C3d/C3 values were higher in the most active group (group 3) than in group 2, in group 2 they were higher than in group 1, and in group 1 they were higher than in controls (Fig. 1).

Circulating immune complexes were significantly higher in patients than in controls. They also correlated with disease activity without significant deviation from linear trend (Table 1).

A correlation was found between C4d and C3d (r=0·29, p<0·01) and between C4d/C4 and C3d/C3

(r=0·25, p<0·02). C4d and C4d/C4 also correlated with circulating immune complexes (r=0·23, p<0·03 and r=0·28, p<0·01 respectively).

Discussion

The present study demonstrates the value of measuring the complement fragments C4d and C3d and calculating their ratios to the parent molecule in the assessment of disease activity in rheumatoid arthritis. This is of interest, as an improvement in objective testing of disease severity would aid the management of patients with this condition.

In rheumatoid arthritis tissue injury and clinical manifestations result, at least in part, from formation and deposition of antigen-antibody complexes.[20] Levels of immune complexes, which reflect this central pathogenic mechanism, would be expected to be reliable markers of disease activity, therefore. High levels of circulating immune complexes are a common finding in patients with rheumatoid arthritis,[21] [22] especially in those with the most severe disease.[6] Our data corroborate these observations and show that levels of circulating immune complexes increase linearly with the degree of disease activity.

Immune complexes incite tissue damage by virtue of their ability to activate complement and so initiate an inflammatory reaction.[23] It is by fixing C1q that immune complexes trigger the complement cascade through the classical pathway with production of the fragments C4d and C3d.[15] In keeping with this pathogenetic sequence, the measurement of C4d and C3d and the calculation of their ratios to the parent molecule not only provided evidence of complement involvement in our patients but also gave an indication of the grade of disease activity. Furthermore, a significant correlation was observed between C4d and C4d/C4 values and the levels of circulating immune complexes, between the levels of C4d and C3d, and between the ratios C4d/C4 and C3d/C3, showing that in rheumatoid arthritis complement activation proceeds through the classical pathway, immune complexes having a major role in initiating it. No correlation was observed between C3d and C3d/C3 and circulating immune complexes. This could be explained by the fact that cleavage of C3 is a relatively late event in the sequence of complement activation, also influenced by alternative pathway function, while cleavage of C4 represents an early event in classical pathway activation, immediately following immune complex mediated C1qrs conversion.[15]

An interesting finding of this study is that among the indices of complement activation only the ratios C4d/C4 and C3d/C3 could discriminate between patients grouped according to the severity of their condition. This may be because levels of the fragments C4d and C3d are activation indices affected by the initial levels of their parent molecules and not exclusively reflecting the catabolic turnover of C4 and C3, but the fragment to parent molecule ratios are indices of activation independent of the concentration of intact components. Of relevance in this context are the reports showing that the C4d/C4 and C3d/C3 ratios best reflect the in vivo fractional catabolic rate of C4 and C3.[24] [25]

In agreement with previous studies[4–7] levels of C4 and C3 were not reduced in any of our patients, indicating that the measurement of intact complement factors fails to identify complement activation in rheumatoid arthritis. Nevertheless, C4 and C3 were found to correlate with disease activity, though in a non-linear relationship. This loose association with disease activity could be explained by the fact that in rheumatoid arthritis levels of C4 and C3 mirror the reactive increase[8] in the production of these proteins rather than their consumption, which is the phenomenon more closely related to the pathogenesis of the disease.

In conclusion, our data show that indices of activation of the classical complement pathway, and in particular the ratios C4d/C4 and C3d/C3, provide a sensitive measurement of disease activity in rheumatoid arthritis. Additionally, the close correlation observed between levels of circulating immune complexes and indices of C4 activation, and between the latter and grades of disease activity, confirms the major part played by the classical complement pathway in the pathogenesis of this disorder.

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