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Terminal complement complexes and C1/C1 inhibitor complexes in rheumatoid arthritis and other arthritic conditions

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SUMMARY

Terminal complement complex (TCC) and C1r–C1s–C1 inhibitor complex (C1/C1 INH) concentrations were measured in plasma and synovial fluid from patients with arthritis and related to other measures of disease activity. Both TCC and C1/C1 INH concentrations were significantly increased in patients with rheumatoid arthritis (RA) compared with patients with osteoarthritis (plasma and synovial fluid, $P < 0{\cdot}05$) and normal subjects (plasma only, $P < 0{\cdot}001$). In the patients with RA, there was no correlation between plasma or synovial fluid TCC concentrations and IgM rheumatoid factor, immune complex or C1/C1 INH levels. However, in 10 patients with seronegative RA, C1/C1 INH and immune complex levels correlated significantly in synovial fluid ($r = 0{\cdot}69$, $P < 0{\cdot}05$) although not in plasma ($r = 0{\cdot}52$). Plasma and synovial fluid TCC and C1/C1 INH concentrations did not differ in rheumatoid patients with severe compared with mild joint disease (categorized by the Ritchie score). These results confirm a role for complement activation in RA but suggest that several mechanisms are involved in its pathogenesis.

Keywords complement terminal complement complex C1/C1 inhibitor complex rheumatoid arthritis

INTRODUCTION

Rheumatoid arthritis (RA) is an important inflammatory disease affecting joints, but the primary stimulus to the inflammatory process is unknown. One facet of this inflammation is the stimulation of an immune response with the production of both IgG and IgM anti-immunoglobulin autoantibodies which may activate complement (Hay *et al.*, 1979).

The role of complement in RA was first suggested by the demonstration of low total haemolytic complement activity in the synovial fluid of patients with RA (Hedberg, 1963) and confirmed by more recent studies involving the measurement of individual complement components (Ruddy & Austen, 1973; Rumfeld, Morgan & Campbell, 1986). However, it is preferable to assess complement activation by measurement of complement breakdown products, such as C3dg, or activation complexes (reviewed by Peakman, Senaldi & Vergani, 1989); levels of native complement components are affected by other factors including increased synthesis due to the acute-phase response.

Complement activation via the classical or the alternative pathway results in the formation of the membrane attack complex (MAC) from the terminal five complement components C5–9. Formation of the MAC and/or the inactive SC5b-9 complex, collectively termed terminal complement complexes (TCCs), can now be demonstrated in body fluids using assays that rely on antibodies specific for neoantigens expressed by the complexed, rather than free, terminal complement components (Mollnes *et al.*, 1985; Morgan, Daniels & Williams, 1988). Increased serum TCC levels have been reported in several autoimmune diseases, including systemic lupus erythematosus (SLE) and mesangioproliferative nephritis (reviewed by Morgan, 1989) and also Graves' disease (Weetman *et al.*, 1989), in the active phase of disease. Increased plasma and synovial fluid TCC levels have previously been reported in RA (Mollnes *et al.*, 1986; Morgan *et al.*, 1988), but have not been related to other clinical and immunological markers of disease activity (apart from C3dg) except in juvenile RA (Mollnes & Paus, 1986).

Assays that assess classical complement pathway activation specifically by measuring the inactive C1r–C1s–C1 inhibitor complex (C1/C1 INH), formed when C1 INH combines with and removes C1r and C1s from activated C1, have also been described (reviewed by Cooper, Nemerow & Mayes, 1983). Increased serum C1/C1 INH levels have been reported in SLE (Sturfelt, Sjoholm & Svensson, 1983; Waldo & West, 1987) and Graves' disease (Weetman *et al.*, 1989) in the active phase of disease, implicating classical pathway activation in the pathogenesis of these autoimmune diseases. Increased C1/C1 INH levels

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have also been reported in both serum and synovial fluid (Berglund *et al.*, 1980; Inman & Harpel, 1983) in RA and have been correlated with measures of disease activity, although not with TCC concentrations.

C1/C1 INH levels have been measured in other arthritides such as psoriatic arthropathy (Inman & Harpel, 1983), but only in a few patients.

In this study, we have extended our previous observations on complement activation in arthritis (Morgan *et al.*, 1988) by measuring plasma and synovial fluid TCC and C1/C1 INH concentrations in a larger group of patients with RA, osteoarthritis (OA) and psoriatic arthropathy and related these concentrations to the Ritchie score (Ritchie *et al.*, 1968), a clinical index of the severity of joint disease, and to rheumatoid factor (RF) and immune complex levels.

MATERIALS AND METHODS

Patients and sample collection

Plasma and synovial fluid samples were collected from 37 patients (14 men, 23 women) with RA, 17 patients (six men, 11 women) with OA, seven patients (four men, three women) with psoriatic arthropathy and one man with ankylosing spondylitis, attending the Rheumatology Outpatient Clinic at this hospital. Their mean ages were, respectively, 56 years (range 29–79), 68 years (range 37–93), 41 years (range 20–63) and 54 years. Plasma was also collected from 47 healthy blood donors (15 men, 32 women; mean age 41 years, range 19–60). Disease severity, in the knee joint from which synovial fluid was aspirated, was evaluated (independently of assay results) by the Ritchie score (Ritchie *et al.*, 1968).

All samples were collected into commercial glass tubes containing 48 μl of 0·34 M EDTA per 5 ml sample volume. Synovial fluid was centrifuged within 1 h of collection at 10 000 *g* for 5 min to remove cells, and stored in portions at −70°C. Plasma was similarly separated and stored. All assays were performed blind, i.e. without knowledge of the clinical diagnosis, Ritchie score or the results of other assays.

TCC assay

Plasma and synovial fluid TCC concentrations were measured by a two-site ELISA using polyclonal anti-neoantigen antibody as first antibody and anti-C9 monoclonal antibody MC-47 as second antibody (Morgan *et al.*, 1988). Microtitre plate wells were coated with anti-neoantigen antibody (2 μg/ml in NaCl/$NaHCO_3$ buffer, pH 9·3, 100 μl/well) for 24 h at 4°C. The wells were washed and all other steps were performed as previously described. TCCs were quantified in patient samples by comparing results with standard curves produced by addition of partially purified SC5b-9 to fresh EDTA plasma prior to measurement in the ELISA.

C1/C1 INH assay

Plasma and synovial fluid C1/C1 INH levels were measured by a two-site ELISA (Weetman *et al.*, 1989; modified from that described by Langlois & Gawryl, 1988) using a rabbit antibody to C1s (Calbiochem, Cambridge, UK) and a goat antibody to C1 INH (Atlantic Antibodies, Winnersh, UK). Standard (stored in portions at −70°C) was prepared by maximally activating normal human serum with an equal volume of heat-aggregated human gammaglobulin (5 g/*l* in PBS) for 1 h at 37°C and then adding EDTA to 10 mM. Patient samples were diluted 1/4 before assay and results calculated in terms of standard concentration expressed as percentage of neat activated serum (%AS).

The effect of antibody to IgM was evaluated in the assay by including rabbit antibody to human IgM (Dako, High Wycombe, UK) in the assay buffer at a final dilution of 1/33. The effect of IgM RF was assessed in the assay by adding IgM RF which had been purified by affinity chromatography on Sepharose (Pharmacia, Milton Keynes, UK) coupled to human IgG, followed by gel filtration under non-dissociating conditions on Sephacryl S-300 (Pharmacia).

Other assays

RF was assessed qualitatively in plasma and synovial fluid by latex agglutination at a 1/20 dilution of sample, using latex particles coated with human IgG (Wellcome Diagnostics, Dartford, UK). IgM RF was quantified (as absorbance) by ELISA, using rabbit IgG (10 μg/ml) as antigen and peroxidase-conjugated $F(ab')_2$ anti-human IgM antibody (Faith *et al.*, 1982). Patients with RA were considered positive for IgM RF, i.e. seropositive, if the latex agglutination test was positive and/or the ELISA absorbance was greater than 1·15 in either plasma or synovial fluid.

Plasma and synovial fluid levels of IgG-containing immune complexes were quantitated (as percentage of maximum precipitation) using a ^{125}I-labelled human monoclonal IgM RF (Barratt & Naish, 1979).

Statistical analysis

Differences between groups were compared by the Wilcoxon unpaired rank sum test (two-tailed). Results are quoted as the mean and range. Associations were tested by estimating the Spearman's rank correlation coefficient (r).

RESULTS

Plasma TCC concentrations were 852 (< 100–3200) ng/ml in 37 patients with RA, 970 (320–1650) ng/ml in seven patients with psoriatic arthropathy, 450 (< 100–1300) ng/ml in 17 patients with OA, and 77 (30 to 280) ng/ml in 47 normal subjects (Fig. 1a). Plasma TCC concentrations were significantly increased in all three patient groups compared with normal subjects ($P < 0·001$ for each) and also in the patients with RA ($P < 0·01$) and those with psoriatic arthropathy ($P < 0·05$) compared with those with OA. Plasma TCC concentrations were not significantly different in the patients with psoriatic arthropathy compared with those with RA.

Synovial fluid TCC concentrations were 1020 (< 100–3550) ng/ml in 37 patients with RA, 769 (240–1440) ng/ml in seven patients with psoriatic arthropathy, and 605 (< 100–2000) ng/ml in 17 patients with OA (Fig. 1b). Synovial fluid TCC concentrations were significantly increased in the patients with RA compared with those with OA ($P < 0·05$) but were not significantly different in the patients with psoriatic arthropathy compared with the other two patient groups.

RF was measured by both ELISA and latex agglutination in plasma and synovial fluid from 24 patients with RA, 14 patients with OA, five patients with psoriatic arthropathy and one patient with ankylosing spondylitis. Plasma and synovial fluid TCC concentrations were 681 (< 100–1560) ng/ml and 1109 (< 100–2020) ng/ml, respectively, in 11 patients with seroposi-

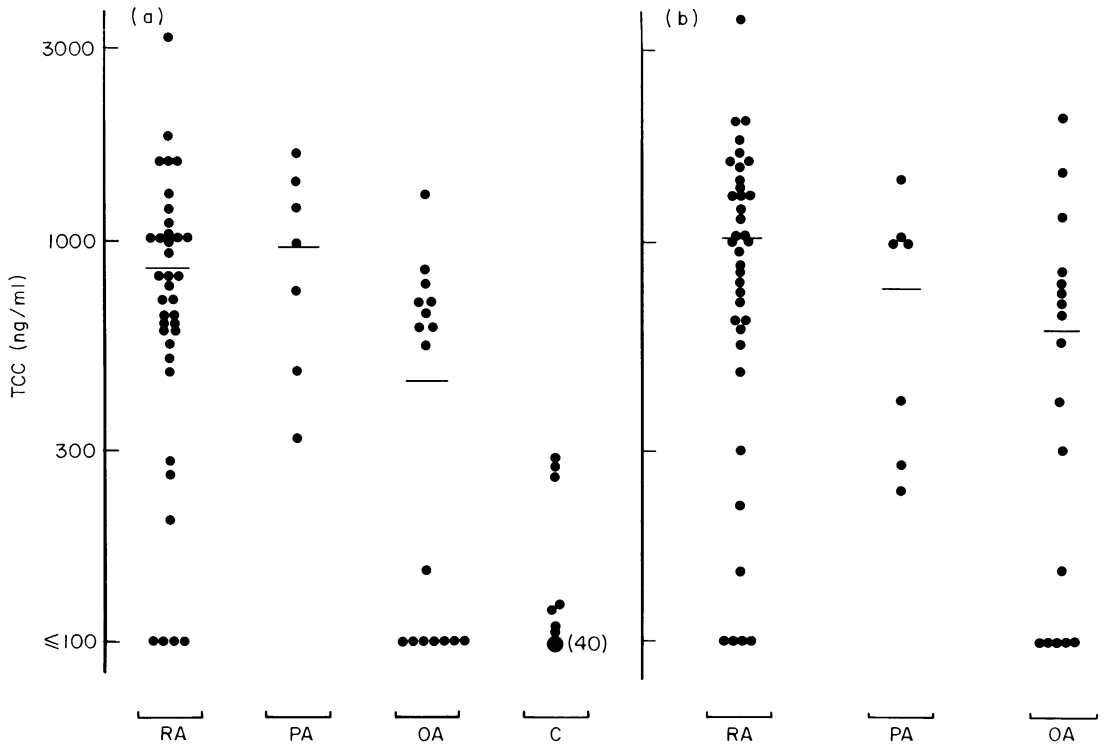

Fig. 1. TCC concentrations in plasma (a) and synovial fluid (b) from 37 patients with rheumatoid arthritis (RA), seven patients with psoriatic arthropathy (PA), 17 patients with osteoarthritis (OA) and 47 normal subjects (C). Horizontal bars represents the means.

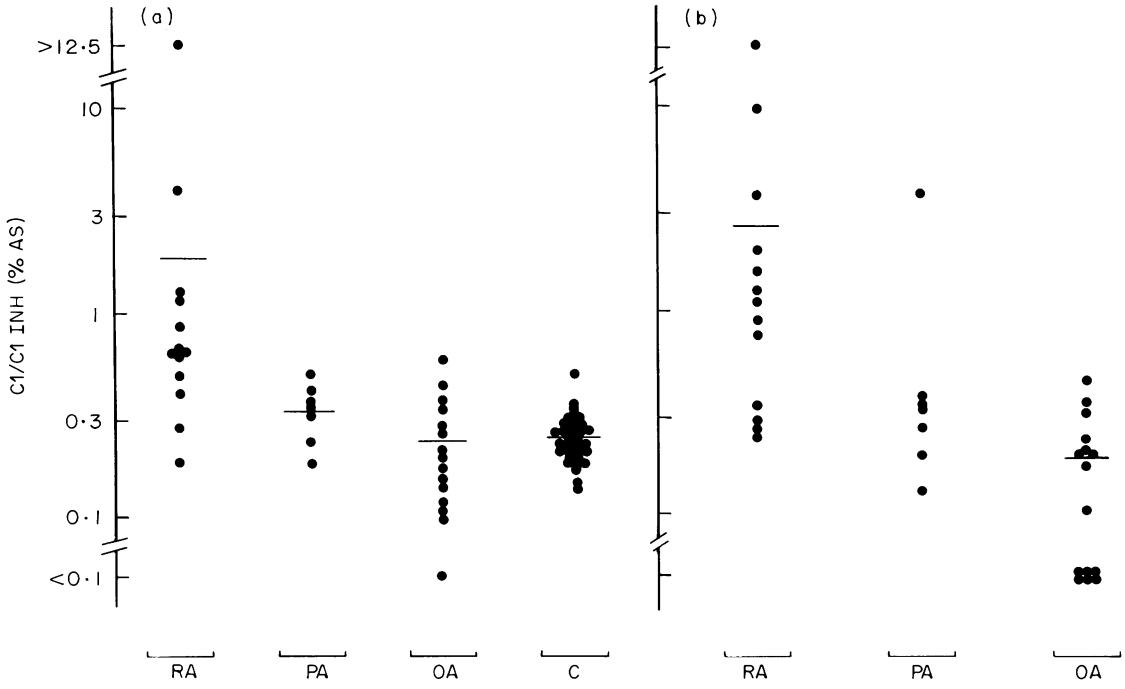

Fig. 2. C1/C1 INH levels as percentage of neat activated serum (%AS) in plasma (a) and synovial fluid (b) from 13 patients with seronegative rheumatoid arthritis (RA), seven patients with psoriatic arthropathy (PA), 15 patients with osteoarthritis (OA) and 47 normal subjects (C). Horizontal bars represents the means.

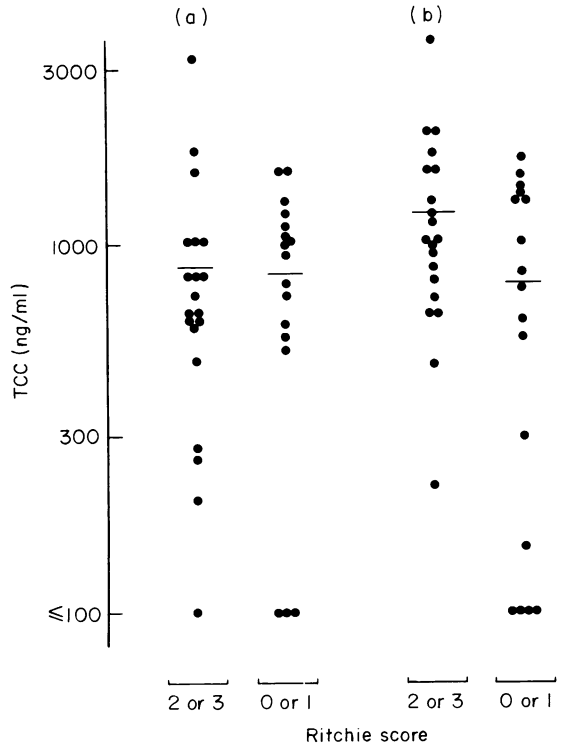

Fig. 3. TCC concentrations (ng/ml) in plasma (a) and synovial fluid (b) from 37 patients with rheumatoid arthritis (RA), of whom 20 had a Ritchie score of 2 or 3 (severe joint disease) and 17 had a Ritchie score of 0 or 1 (mild joint disease). Horizontal bars represents the means.

tive RA, compared with 753 (260–1120) ng/ml and 1003 (220–1600) ng/ml, respectively, in 13 patients with seronegative RA; these TCC concentrations did not differ significantly. RF was negative in all the patients with other diseases who were tested. In the patients with RA, there was no significant correlation between TCC concentrations and IgM RF level (ELISA absorbance) in either plasma ($r=-0{\cdot}08$) or synovial fluid ($r=0{\cdot}13$).

However, in the 24 patients with RA in whom RF was measured, there was a significant correlation ($P<0{\cdot}001$) between C1/C1 INH level and IgM RF level (ELISA absorbance) in both plasma ($r=0{\cdot}70$) and synovial fluid ($r=0{\cdot}71$). Synovial fluid C1/C1 INH levels in four patients with RA decreased by 4·8% (0–22·8) when 40 mg/*l* rabbit gammaglobulin was included in the assay buffer in addition to 2 mg/*l* bovine gammaglobulin. However, when rabbit antibody to human IgM was included in the assay buffer, synovial fluid C1/C1 INH levels decreased by 85·5% (from 3·43 to 0·50 %AS) and 92·9% (from >25·0 to 1·77 %AS), respectively, in the two samples considered positive for IgM RF compared with 6·0% (from 2·57 to 2·42 %AS) and 15·9% (from 0·96 to 0·81 %AS), respectively, in the two samples considered IgM RF negative. The amount of anti-IgM added was shown to be sufficient to substantially reduce measurable IgM RF levels (by at least 50%) in two other synovial fluid samples. Addition of high concentrations of purified IgM RF to a seronegative plasma sample caused a marked dose-dependent increase in the apparent C1/C1 INH level (from 0·45 to >12·5 %AS at the highest RF concentration used, which was equivalent to that in a plasma sample strongly positive for IgM RF). These results suggest that IgM RF may have caused interference in the C1/C1 INH assay. Therefore, C1/C1 INH levels have only been reported for those patients with RA considered negative for IgM RF. C1/C1 INH levels were also measured in all the other subjects studied, except for two patients with OA.

Plasma C1/C1 INH levels were 1·82 (0·18–>12·5) %AS in 13 patients with seronegative RA, 0·33 (0·19–0·49) %AS in seven patients with psoriatic arthropathy, 0·23 (<0·1–0·58) %AS in 15 patients with OA, and 0·25 (0·14–0·51) %AS in 47 normal subjects (Fig. 2a). Plasma C1/C1 INH levels were significantly increased in the patients with RA ($P<0{\cdot}001$) and those with psoriatic arthropathy ($P<0{\cdot}05$) compared with normal subjects, and also in the patients with RA compared with those with psoriatic arthropathy ($P<0{\cdot}02$) and OA ($P<0{\cdot}001$). Plasma C1/C1 INH levels were not significantly different in the patients with OA compared with either the patients with psoriatic arthropathy or the normal subjects.

Synovial fluid C1/C1 INH levels were 2·70 (0·25–>12·5) %AS in 13 patients with seronegative RA and 0·19 (<0·1–0·46) %AS in 15 patients with OA (Fig. 2b); these levels differed significantly ($P<0{\cdot}001$). Synovial fluid C1/C1 INH levels were 0·28 (0·13–0·38) %AS in six out of the seven patients with psoriatic arthropathy (Fig. 2b); however, in the seventh patient the C1/C1 INH level was much higher (3·89 %AS), and so no statistical comparisons have been reported for this group.

In the 13 patients with seronegative RA, the correlation between TCC concentration and C1/C1 INH level in plasma ($r=0{\cdot}44$) was not significant and there was no correlation in synovial fluid ($r=0{\cdot}01$).

Plasma and synovial fluid were collected from the patient with ankylosing spondylitis on two separate occasions. TCC and C1/C1 INH concentrations were increased in both plasma (means 1365 μg/ml and 2·76 %AS, respectively) and synovial fluid (means 1210 μg/ml and 6·85 %AS, respectively).

In 17 patients with RA in whom immune complex levels were measured, there was no significant correlation between TCC concentration and immune complex level in either plasma ($r=-0{\cdot}10$) or synovial fluid ($r=-0{\cdot}26$). Neither plasma nor synovial fluid TCC concentrations differed significantly in the 10 patients with increased immune complex levels (>16%) compared with those with normal levels. However, in the 10 patients with seronegative RA, there was a significant correlation between C1/C1 INH and immune complex levels in synovial fluid ($r=0{\cdot}69$, $P<0{\cdot}05$) although the correlation in plasma was not significant ($r=0{\cdot}52$). Immune complex levels were also increased in the patient with ankylosing spondylitis but raised levels were only found in a few of those with OA (one out of 14) or psoriatic arthropathy (one out of five).

Severity of the inflammatory response in the knee joint was assessed in the 37 patients with RA using the Ritchie score. Plasma and synovial fluid TCC concentrations were 865 (<100–3200) ng/ml and 1226 (220–3550) ng/ml, respectively, in 20 patients with the most severe inflammation (Ritchie score 2 or 3) compared with 836 (<100–1560) ng/ml and 778 (<100–1680) ng/ml, respectively, in 17 patients with mild inflammation (Ritchie score 0 or 1). These TCC concentrations did not differ significantly, although the synovial fluid TCC concentrations were slightly higher in patients with more severe disease (Fig. 3). Plasma and synovial fluid C1/C1 INH levels were 2·42 (0·18–>12·5) %AS and 2·78 (0·27–>12·5) %AS, respectively, in eight

patients with seronegative RA who had severe inflammation (Ritchie score 2 or 3) compared with 0·88 (0·48–1·24) %AS and 2·57 (0·25–9·95) %AS, respectively, in five patients with seronegative RA who had mild inflammation (Ritchie score 0 or 1); these levels did not differ significantly.

DISCUSSION

The role of complement activation in the pathogenesis of RA and other arthritides has been studied by measuring plasma and synovial fluid TCC and C1/C1 INH concentrations and relating them to disease severity and RF and immune complex levels. *In vitro* studies of human rheumatoid synovial cells have suggested that the release of inflammatory mediators (prostaglandin E_2, reactive oxygen metabolites and leukotriene B_4) following non-lethal complement attack on cells within the synovial membrane may be important in the pathogenesis of RA (Daniels *et al.*, 1990).

Plasma and synovial fluid TCC and C1/C1 INH concentrations were elevated in patients with RA, confirming previous findings for both TCC (Mollnes *et al.*, 1986; Morgan *et al.*, 1988) and C1/C1 INH (Berglund *et al.*, 1980; Inman & Harpel, 1983) concentrations, but did not differ significantly in patients considered clinically to have severe joint disease compared with those considered to have milder disease. Mollnes & Paus (1986) found no correlation between synovial fluid TCC or C3dg levels and the 'knee score' of the corresponding joint in 10 patients with juvenile RA, although levels did correlate with the synovial fluid C-reactive protein level and white blood cell count. By contrast, Berglund *et al.* (1980) found a significant correlation between the serum C1/C1 INH level and a Synovitis index in 16 patients with active RA. Correlations with disease severity indices are likely to vary as each index evaluates different clinical features.

Plasma and synovial fluid immune complex levels did not correlate with TCC concentrations in patients with RA, but there was evidence of a correlation with C1/C1 INH levels in patients with seronegative RA. In previous studies, the relationship between C1/C1 INH and immune complex levels has been variable. Berglund *et al.* (1980) reported a significant correlation in patients with RA between the serum C1/C1 INH and immune complex levels measured by the C1q deviation test but not by the C1q binding assay. Inman & Harpel (1983) reported a significant positive correlation between synovial fluid C1/C1 INH and immune complex levels (measured by a Staphylococcal protein A binding assay) in the patients with RA and other arthritides when considered together but not with the patients with RA alone; by contrast, they found a significant inverse correlation between plasma C1/C1 INH and immune complex levels in the patients with RA. In our study immune complex levels were measured using a human monoclonal IgM RF (Barratt & Naish, 1979). The discrepancies in these findings may reflect the diversity of different assays for immune complexes and the varying ability of immune complexes to activate C1. The TCC can be formed as a result of activation of the alternative pathway of complement as well as the classical pathway so the lack of any correlation between TCC concentrations and immune complex levels is not necessarily surprising.

Plasma and synovial fluid RF levels did not correlate with TCC concentrations but did correlate positively with C1/C1 INH levels in the 24 patients with RA who had RF levels measured. In contrast, Inman & Harpel (1983) did not find any correlation between RF titres and C1/C1 INH levels in serum or synovial fluid from patients with RA. While the correlation between IgM RF and C1/C1 INH levels found here may reflect activation of the classical pathway of complement by IgM RF, it is possible that IgM RF was causing interference in the C1/C1 INH assay. Two-site immunometric assays, including ELISAs, are potentially subject to interference by heterologous antibodies, especially IgM (John, Henley & Barron, 1989) resulting in an apparent increase in analyte concentration. This problem can be minimized by including non-specific immunoglobulin in the assay buffer; the C1/C1 INH assay buffer contained 2 mg/*l* bovine gammaglobulin but even the addition of 40 mg/*l* rabbit gammaglobulin had little effect on C1/C1 INH levels in four synovial fluid samples from patients with RA. However, inclusion of anti-human IgM in the assay buffer resulted in large decreases in apparent C1/C1 INH levels in the two synovial fluid samples studied from patients with RA considered positive for IgM RF, and addition of purified IgM RF to a seronegative plasma caused a marked increase in the apparent C1/C1 INH level. C1/C1 INH levels were therefore only reported in those patients in whom RF interference in the assay was unlikely. Reports of other C1/C1 INH ELISAs (Inman & Harpel, 1983; Langlois & Gawryl, 1988) have not mentioned the inclusion of any non-specific gammaglobulin in the assay buffer, although non-specific binding to rabbit IgG was subtracted when calculating the results for one of these ELISAs (Inman & Harpel, 1983).

While TCC and C1/C1 INH concentrations have been measured previously in RA and OA, only a few patients with other arthritides have been studied. The patient with ankylosing spondylitis reported here had increased plasma and synovial fluid TCC, C1/C1 INH and immune complex levels. There were also increases in TCC and C1/C1 INH concentrations in some of the patients with psoriatic arthropathy. Moderate increases in synovial fluid C1/C1 INH levels have been previously reported in one patient with ankylosing spondylitis and one patient with psoriatic arthritis (Inman & Harpel, 1983). These increases suggest complement activation via the classical pathway in these two diseases, although more patients should be studied to confirm this finding. TCC concentrations, but not those of C1/C1 INH, were elevated in some of the patients with OA, suggesting that complement activation occurs—but not via the classical pathway. This may reflect an inflammatory component in the disease process in some cases of OA.

The results obtained in this study confirm a role for complement activation in RA. However, it is likely that several mechanisms are involved in the pathogenesis of the inflammation as TCC concentrations were not related to other clinical and laboratory measures of disease activity (Ritchie score; RF and immune complex levels). Differences between the TCC and C1/C1 INH results suggest that in RA complement may be activated independently of antibody by routes other than the classical pathway.

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