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# Measurement of terminal complement complexes in rheumatoid arthritis

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## SUMMARY

Though complement activation is recognized as a central event in inflammation in the rheumatoid joint, little attention has been paid to the role of the cytolytic membrane attack complex of complement in the pathogenesis of this disease. The membrane attack complex causes a variety of non-lethal effects in nucleated cells, including stimulation of release of inflammatory mediators, and cell proliferation. Thus in the rheumatoid synovium, non-lethal effects of complement membrane attack may play a major role in disease pathology. In order to investigate this possibility, assays for the detection of terminal complement complexes in biological fluids have been established, and used to demonstrate membrane attack pathway activation in rheumatoid arthritis. Terminal complement complexes were present in increased levels in synovial fluid (mean, 1,334 ng/ml) and plasma (mean, 513 ng/ml) in 20 patients with rheumatoid arthritis when compared with controls (mean, 285 ng/ml and 129 ng/ml respectively). Using an assay specific for the SC5b-9 complex it was demonstrated that the raised levels of terminal complement complexes in rheumatoid synovial fluid consisted of a mixture of inactive SC5b-9 complexes and fluid-phase complement membrane attack complexes.

**Keywords** terminal complement complex rheumatoid arthritis synovial fluid

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic recurrent, systemic inflammatory disease primarily involving the joints. The disease affects all ages, though the maximum incidence is in the fifth and sixth decades, and women are affected four times more frequently than men (Glynn, 1968). The synovial membrane of affected joints undergoes marked villous hypertrophy with synovial cell proliferation and infiltration with plasma cells and lymphocytes. Subchondral erosion of bone and articular cartilage subsequently occurs resulting in irreversible joint damage (Catto, 1976).

The antigenic stimulus that initiates the immune response and subsequent inflammation in rheumatoid joints is unidentified, although a genetic susceptibility associated with certain HLA phenotypes has been demonstrated (Stastny, 1978). Whatever the initiating stimulus, IgG production within the joint stimulates an immune response, with production of anti-immunoglobulin autoantibodies of both IgG and IgM isotype (Hay *et al.*, 1979).

A role of complement as an effector of inflammation in the rheumatoid joint was first suggested over 25 years ago with the demonstration of low total haemolytic complement activity in the synovial fluid (SF) of patients with RA (Hedburg, 1963; Pekin & Zvaifler, 1964), though others have disputed the validity of these findings (Sheppeard, Lead & Ward, 1981). Recent studies utilising more specific indicators of complement activation, including the levels of individual components (Ruddy & Austen, 1973; Rumfeld, Morgan & Campbell, 1986) and of complement breakdown products, particularly products of C3 conversion (Mollnes, 1985; Peakman *et al.*, 1987) have confirmed ongoing complement activation in the rheumatoid joint.

Activation of complement results in the release of a number of biologically active products, including the anaphylatoxic and chemotactic peptides C3a and C5a, which stimulate influx of neutrophils and mononuclear cells into the joint and cause release of histamine from macrophages (Jose, 1987). Relatively little attention has been paid to the pathological role in RA of another important biologically active product of complement activation, the membrane attack complex (MAC). The MAC is formed from the terminal five complement components C5-C9 after activation via either the classical or alternative pathways, and may cause irreversible damage to cells by inserting into and disrupting the cell membrane (Esser, 1982). Cell death is not a major feature in the pathology of the rheumatoid synovium and this is perhaps a reason why the role of the MAC has not been more closely investigated. However, it is becoming increasingly

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clear that the MAC may cause more subtle, transient changes in nucleated cells without irreversibly damaging the cell. It is therefore possible that activation of complement within the rheumatoid joint will result in deposition of non-lethal amounts of the MAC within the rheumatoid synovium or on cells in the synovial fluid. This non-lethal complement membrane attack may then activate a variety of cellular processes, including recovery processes.

This paper will seek to demonstrate activation of the membrane attack pathway of complement in rheumatoid arthritis by measurement of fluid-phase (SC5b-9) and membrane-associated (MAC) terminal complement complexes (TCCs) in synovial fluid and plasma. To this end specific assay methods for terminal complement complexes in biological fluids have been developed utilising antibodies raised against unique epitopes expressed only on the formed terminal complex (neoantigens) (Mollnes *et al.*, 1983). These reagents will also provide a means of detecting terminal complexes in diseased tissue. The following paper will investigate the existence of recovery mechanisms and non-lytic effects of complement membrane attack on synovial cells both *in vivo* and *in vitro*.

## MATERIALS AND METHODS

*General materials*

Cyanogen bromide-activated Sepharose 4B, Protein A-Sepharose CL-4B and Sepharose 2B were from Pharmacia (Milton Keynes, Bucks). Tissue culture media, sera and plastic ware were from Gibco (Uxbridge, Middlesex). All other chemicals were from Sigma Fine Chemicals (Poole, Dorset) or BDH Ltd. (Poole, Dorset) and were of the best grade available. Krebs-hepes buffer contained 0·12 M NaCl, 0·0048 M KCl, 0·0012 M $KH_2PO_4$, 0·0012 M $MgSO_4$, 0·0013 M $CaCl_2$, 0·025 M hepes and 0·1% BSA, pH 7·4. Phosphate-buffered saline (PBS) tablets were from Oxoid (Basingstoke, Hampshire). Tris-buffered saline (TBS) contained 0·15 M NaCl, 0·01 M Tris hydroxymethylmethylamine, pH 7·4.

*Proteins and Sera*

Human C8 and C9 were isolated from plasma utilizing published methods (Abraha, Morgan & Luzio, 1988; Morgan *et al.*, 1984). The membrane attack complex (MAC) was partially purified from a detergent extract of complement-lysed sheep erythrocytes by the method of Bhakdi & Tranum-Jensen (1981). The SC5b-9 complex was isolated from inulin-activated human serum by a modification of the method of Bhakdi and Roth (1981), gel filtration on Sepharose 2B being substituted for the final sucrose density gradient step. Poly-C9 was produced from monomeric C9 by limited tryptic digestion (Dankert, Shiver & Esser, 1985) and chromatography on Sepharose 2B. Normal human serum (NHS) was obtained from healthy volunteers and stored in portions at −70°C. Serum depleted of C9 or C8 was prepared by passage of NHS over a column of the appropriate monoclonal antibody immobilized on Sepharose 4B (Abraha *et al.*, 1988; Morgan *et al.*, 1984).

*Antibodies*

Monoclonal antibodies against human C9 were produced as previously described (Morgan *et al.*, 1984). Monoclonal antibody against the human S-protein was purchased from Cytotech (San Diego, Cal.). Polyclonal antibody against human 5′-nucleotidase, an ectoenzyme present on most nucleated cells, was a kind gift from Dr. J.P. Luzio (University of Cambridge). Monospecific polyclonal antibodies against neo-antigens of the human MAC were produced as follows: Rabbits were bled about 10 ml per animal from the posterior marginal ear vein into a heparinized container. The erythrocytes were washed and resuspended in Krebs-hepes buffer at a cell concentration of $5 \times 10^8$/ml, and then incubated with human serum at a final dilution of 1:3 at 37°C for 60 min. Activation of human complement by rabbit erythrocytes via the alternative pathway (Mayer, 1961) resulted in the deposition of MACs and lysis of about 30% of the cells. The erythrocyte-ghost mixture was washed in Krebs-hepes to remove serum, resuspended in 5 ml Krebs-hepes without BSA and re-injected intravenously via the posterior marginal ear vein into the donor animal. This process was repeated three times at 2-weekly intervals, and the animals bled 10 days after the final immunization. Serum was separated, and IgG isolated by passage over a column of protein A-Sepharose. The purified IgG was then passaged over a Sepharose-4B column on which normal human serum had been immobilized, thereby removing any antibodies recognizing native serum components. Unretarded IgG was pooled, concentrated to 1 mg/ml in an Amicon ultrafiltration cell and stored in aliquots at −20°C.

*Characterization of anti-neoantigen antibody*

Binding of anti-neoantigen antibody to C9, C8, the MAC, the SC5b-9 complex and poly-C9 was assessed using an ELISA assay. Each of the above proteins at dilutions in the range 10 μg/ml-10 ng/ml in coating buffer ($NaCl/NaHCO_3$ pH 9·3) were placed in a 96 well polypropylene microtitre plate (100 ul/well) and incubated at 37°C for 30 min. The protein-containing solution was then removed, the wells blocked by incubation for 30 min at 37°C with 2% BSA in PBS, and repeatedly washed with PBS containing 0·1% BSA, 0·1% Tween 20. Anti-neoantigen antibody at a final concentration of 1 μg/ml in PBS was then added and incubated at 37°C for 30 min. The wells were again washed and peroxidase-labelled goat anti-rabbit IgG antibody (ICN Biomedicals, High Wycombe, Bucks.) at a final dilution of 1/1000 in PBS (100 μl/well) added and incubated for 30 min at 37°C. The wells were again washed repeatedly, peroxidase substrate (1,2-phenylenediamine dihydrochloride, OPD) added, and the colour allowed to develop for 15 min. The extinction at 492 nm in the wells was read using a microtitre plate ELISA reader (Biorad, Watford, Herts.). As a control, ELISA assays were developed in parallel using a monoclonal anti-C9 antibody (MC-47) and a peroxidase-labelled anti-mouse IgG antibody as first and second antibodies respectively.

*Detection of TCCs in Patient samples*

A two-site ELISA method based on the screening ELISA described above, and utilizing the anti-neoantigen antibody as first antibody and the anti-C9 monoclonal antibody MC-47 as second antibody was established. Anti-neoantigen antibody at a final concentration of 2 μg/ml in coating buffer was incubated in the microtitre plate wells at 37°C for 30 min. The wells were blocked with BSA and washed as detailed above. Various dilutions of serum, synovial fluid, or purified TCCs in PBS were then placed in the wells and incubated for 30 min at 37°C. After washing, the second antibody, MC47 was added at a final dilution of 4 μg/ml in PBS and incubated for 30 min at 37°C. The

plate was washed and peroxidase-labelled goat anti-mouse IgG antibody (ICN) at a final dilution of 1:1000 from stock added. After a further incubation for 30 min at 37°C, the plate was extensively washed, OPD substrate added and colour allowed to develop as detailed above. Quantification of TCCs in patient samples was achieved by comparison of results with standard curves produced by addition of various amounts of partially purified SC5b-9 to fresh EDTA plasma prior to measurement in the above assay. To differentiate TCCs containing the S-protein (SC5b-9) from those not containing this protein (MAC), an identical two-site ELISA was established in which monoclonal anti-S-protein at a concentration of 2 μg/ml was substituted for the anti-C9 monoclonal as second antibody.

*Patients, sample collection and storage*

Plasma and synovial fluid samples were collected from 20 patients with rheumatoid arthritis (RA) and from 12 patients with non-rheumatoid synovial effusions (8 osteoarthritis (OA), 4 traumatic) attending Rheumatology Outpatients Clinic at this hospital. Plasma was also collected from 12 healthy volunteers for use as controls. All samples were collected into commercial glass tubes containing EDTA (48 μl of a 0·34 M solution of $K_3$-EDTA, 5 ml sample volume). Synovial fluid was centrifuged at 10,000 *g* for 5 min to remove cells and either assayed immediately or stored in portions at −70°C. Plasma was separated and similarly assayed or stored. TCCs were measured using the sandwich ELISA in plasma and synovial fluid from each of the 32 patients, and in plasma from each of the 12 controls.

## RESULTS

*Characterization of anti-neoantigen antibody*

Binding of anti-neoantigen antibody to C9, C8, the MAC, the SC5b-9 complex and poly-C9 was assessed in an ELISA assay. For comparison, binding of anti-C9 monoclonal antibody MC-47 was also determined for each antigen. The results are summarized in Table 1. The anti-neoantigen antibody reacted strongly with the MAC, SC5b-9 complex and poly-C9, the apparent order of affinity being MAC > SC5b-9 > poly C9, though these differences may be a result of different amounts of protein binding to the microtitre plate. No reaction above background levels was seen with pure C8, and only a very weak reaction was seen with pure C9. In contrast, the anti-C9 monoclonal antibody reacted most strongly with native C9, with each of the protein complexes with an affinity ranking of poly-C9 > MAC > SC5b-9, and not at all with C8. The small amount of reactivity of the anti-neoantigen antibody against native C9 was thought to be the result of traces of polymerized C9 which were always present in the purified protein.

*Measurement of TCCs in biological fluids*

A two-site ELISA utilizing the anti-neoantigen antibody was first antibody and anti-C9 antibody as second antibody, was established and used to measure TCCs in samples of plasma and SF. To enable quantification of TCC levels, a standard curve was developed using fresh normal plasma supplemented with known amounts of purified SC5b-9. A typical standard curve is shown in Fig. 1. The detection limit of the assay was 10 ng SC5b-9/ml. Using this assay, TCCs were quantified in plasma and SF from 20 patients with RA and 12 patients with non-inflamma-

**Table 1.** Characterization of anti-neoantigen antibody by ELISA

| | A 492 | |
|---|---|---|
| ANALYTE | Anti-neoantigen | Anti-C9 |
| C9 | 0·09 (0·02) | 0·93 (0·10) |
| C8 | 0·03 (0·01) | 0·04 (0·01) |
| MAC | 0·85 (0·10) | 0·75 (0·09) |
| SC5b-9 | 0·72 (0·09) | 0·60 (0·07) |
| Poly-C9 | 0·65 (0·05) | 0·81 (0·08) |
| Blank | 0·04 (0·01) | 0·04 (0·01) |

Each of the five analytes was immobilized on wells of polypropylene plates under identical conditions. After blocking non-specific protein binding sites with albumin, either rabbit anti-neoantigen antibody or monoclonal anti-C9 antibody were added as detailed in methods. After incubation and washing, the appropriate peroxidase-labelled second antibody was added, and the colour developed using OPD as substrate. The results are expressed as absorbance units and are the means of triplicate determinations. The standard deviation of the three determinations is displayed in parentheses.

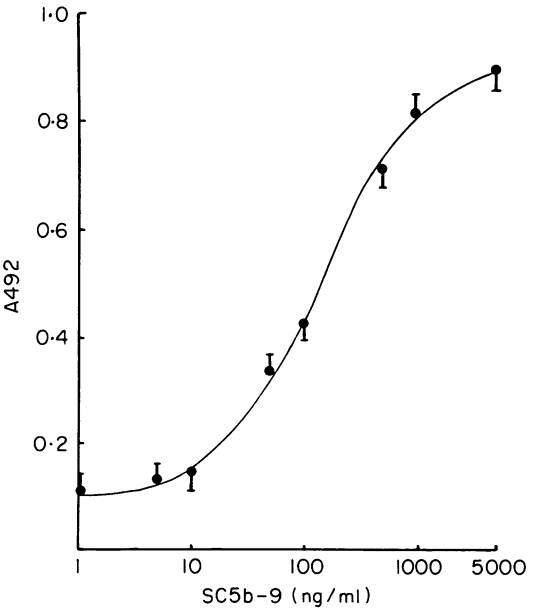


**Fig. 1.** Standard curve for TCC ELISA. The standard curve was developed by adding known amounts of purified SC5b-9 to fresh human plasma, and measuring the samples in the two-site ELISA as detailed in methods. Each point is the mean of four determinations, and the vertical bars represent standard deviation of the four measurements.

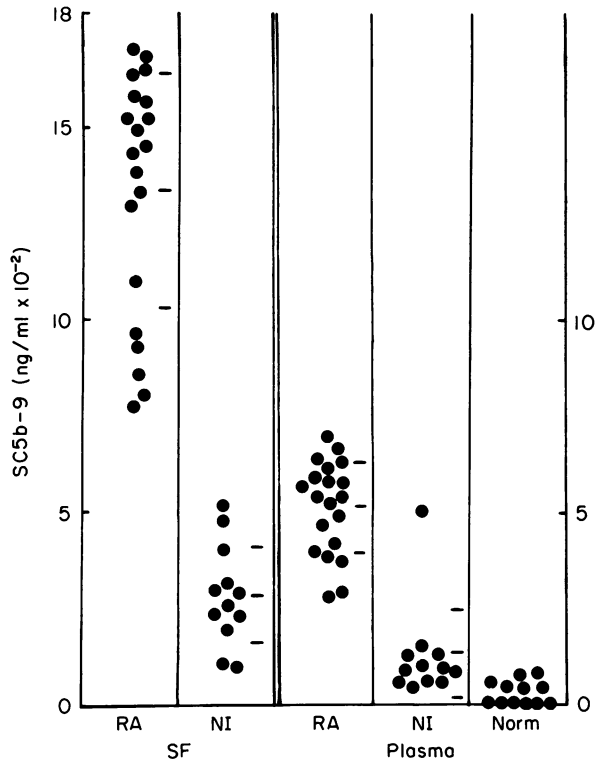


**Fig. 2.** Measurement of TCCs in plasma and SF. TCC concentrations were measured using the two-site ELISA in SF and plasma from 20 patients with RA, and from 12 patients with non-inflammatory joint pathology (NI) as detailed in methods. Plasma TCC concentrations were also measured in 12 normal individuals (Norm). The results are expressed in terms of ng SC5b-9/ml by comparison with the standard curve shown in Fig. 1. Each point represents a single sample (mean of duplicate determinations) and the horizontal lines represent the mean ± one standard deviation.

**Table 2.** Measurement of TCCs in plasma and SF (ng SC5b-9/ml)

| | No. | Mean | Range | s.d. | Significance of difference |
|---|---|---|---|---|---|
| Rheumatoid SF | 20 | 1334 | 770–1705 | 307 | $P<0.001$ |
| non-inflammatory SF | 12 | 285 | 95–510 | 124 | — |
| Rheumatoid plasma | 20 | 513 | 285–690 | 117 | $P<0.001$ |
| non-inflammatory plasma | 12 | 129 | 55–500 | 114 | — |
| Normal plasma | 12 | 30 | 0–85 | 32 | |

Statistical analysis was by the Mann–Whitney U-test (Wilcoxon rank sum test for two samples). The TCC concentration in rheumatoid SF is significantly different from that in non-inflammatory SF, and TCC concentration in rheumatoid plasma is also significantly elevated when compared with non-inflammatory or normal control plasma.

tory synovial effusions. TCCs were also quantified in plasma from 12 healthy volunteers. The results are displayed in Fig. 2.

Concentrations of TCCs, expressed in terms of ng SC5b-9/ml, were elevated in SF from patients with RA (mean 1334 ng/ml) compared with non-inflammatory SF (mean 285 ng/ml).

**Table 3.** TCC levels in biological fluids using anti-C9 or anti-S-protein as second antibody

| Sample | Anti-Neo/Anti-C9 | Anti-Neo/Anti-S | % decrease |
|---|---|---|---|
| $SF_1$ | 1520 | 1340 | 12 |
| $SF_2$ | 850 | 675 | 21 |
| $SF_3$ | 770 | 690 | 10 |
| $Pl_1$ | 640 | 490 | 13 |
| $Pl_2$ | 390 | 300 | 13 |
| $Pl_3$ | 375 | 350 | 7 |
| SC5b-9 | 350 | 359 | +1 |
| MAC | 425 | 10 | 100 |
| Poly-C9 | 520 | 10 | 100 |

ELISA methods were established using the anti-neoantigen antibody as first antibody, and either the anti-C9 monoclonal antibody or a monoclonal antibody against the S-protein as second antibody. Standard curves were constructed for each ELISA using purified SC5b-9, and samples of SF, plasma (Pl), purified TCCs or poly C9 measured in each assay in triplicate. The results are the means of triplicate measurements expressed in ng SC5b-9/ml. The final column expresses the percentage decrease in measured TCC for each sample when the anti-Neo/anti-S-protein assay was used.

Similarly, plasma levels of TCCs were elevated in patients with RA (mean 513 ng/ml) in comparison with patients with non-inflammatory joint disease (mean 129 ng/ml). Plasma levels of TCCs in twelve normal controls were all close to the baseline of the standard curve and never exceeded 100 ng/ml. The elevated levels of TCCs in RA plasma and SF were both statistically highly significant ($P<0.001$) using the Mann–Whitney U-test for unpaired samples. The data are summarized in Table 2. Modifying the ELISA assay to detect only SC5b-9 complexes by substituting the anti-S protein antibody for the anti-C9 antibody resulted in lower measured levels of TCCs in SF and, to a lesser extent, in plasma from patients with RA (Table 3), suggesting that non-S-protein-containing TCCs (i.e. MACs) were also present in these samples.

## DISCUSSION

The results presented in this paper provide a substantial framework for the hypothesis that the membrane attack pathway of complement is involved in the pathogenesis of RA. Measurement of terminal complement complexes (TCCs) in plasma and SF demonstrates membrane attack pathway activation in RA.

Measurement of TCCs required the initial production of reagents specific for the TCC—antibodies to neoantigenic determinants. Though others have recently reported the production of both polyclonal (Curd *et al.*, 1978; Sanders *et al.*, 1985) and monoclonal (Mollnes *et al.*, 1983; Hugo, Jenne & Bhakdi, 1985) antibodies to TCC neoantigens using purified TCCs as immunogen, these methods require extensive purification of the complex, followed by careful screening to eliminate antibodies recognising native complement components. A novel and simple method was therefore devised utilizing the inherent capacity of rabbit erythrocytes to activate human complement in the absence of antibody. After multiple intravenous injections

of autologous erythrocytes bearing human MACs, the rabbits were bled, immunoglobulin purified and antibodies against native complement components removed by adsorption with serum proteins immobilised on Sepharose. The resultant antibody was highly specific for TCCs and poly-C9 formed *in vitro*. This antibody formed the basis of a sensitive and specific ELISA for TCCs in biological samples. Several reports of ELISA methods for TCCs have recently appeared, either utilizing combinations of antibodies against native terminal complement components (Gawryl *et al.*, 1986), or anti-neoantigen antibody in combination with an antibody against a native TCC component protein (Mollnes *et al.*, 1983; Sanders *et al.*, 1985). The former type of assay has the inherent problem that the immobilized antibody may be saturated with the native component from the sample, limiting its capacity to bind TCCs. The latter assays overcome this problem, as the immobilized antibody will only bind TCCs, and the assay developed here utilizes this principle. The published assays vary widely in their sensitivity, and their capacity to recognise the MAC in addition to the SC5b-9 complex. The assay described here has a detection limit for SC5b-9 of 10 ng/ml, using fresh normal plasma, which is assumed to have insignificant levels of TCCs, as a baseline. Others have suggested that normal plasma may contain small amounts of TCCs (Hugo *et al.*, 1987), however the use of purified SC5b-9 in buffer to produce calibration curves in their study may lead to erroneous plasma measurement because of matrix effects. In the present study, TCC levels in twelve fresh normal plasma samples were distributed close to the baseline, and never exceeded 100 ng SC5b-9/ml. Using this assay, highly significant elevations of TCC concentrations were detected in plasma and SF from patients with RA, the mean plasma level being 513 ng/ml (control mean 129 ng/ml) and SF level 1334 ng/ml (control mean 285 ng/ml), all expressed in terms in ng SC5b-9/ml. Correlation between TCC level and presence or absence of rheumatoid factor or severity of disease were not investigated in this study but will be examined in a larger study now underway.

While this work was in progress another group, utilizing a similar assay system reported increased levels of TCCs in SF and plasma in juvenile and adult onset RA (Mollnes & Paus, 1986; Mollnes *et al.*, 1986). However, neither of these studies provided a quantitative assessment of TCC levels, nor did they attempt to distinguish the type of TCC produced. An important feature of the ELISA methods developed here is their ability to distinguish SC5b-9 complexes from the MAC, by substituting an antibody to the S-protein for the anti-C9 second antibody. Using the combination of ELISA methods developed here, it was demonstrated that the increased levels of TCCs in rheumatoid plasma and SF consisted of both SC5b-9 complexes and fluid-phase MACs. The importance of this finding will be explored in the following paper.

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