Measurement of the chemotactic complement fragment C5a in rheumatoid synovial fluids by radioimmunoassay: role of C5a in the acute inflammatory phase

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Abstract

Evidence suggests that complement is activated in rheumatoid joints. A sensitive radioimmunoassay for the activation fragment of C5, C5a, which is a potent chemoattractant for neutrophils, was therefore developed. A mean C5a concentration in 22 rheumatoid joint fluids of about 2.5×10^{-9} mol/l was found. This concentration of C5a is sufficient to induce two of the characteristic features of the acute inflammatory phases of rheumatoid arthritis: neutrophil accumulation and microvascular plasma protein leakage. In animal models it has been shown that C5a is a potent inducer of inflammatory oedema by a neutrophil dependent mechanism. A striking feature of the acute inflammatory phases of rheumatoid arthritis is the appearance of high numbers of neutrophils in the synovial fluid. It is suggested that C5a might have a role in mediating neutrophil accumulation and, as a consequence, may be important in acute joint swelling and pain.

In addition to the chronic synovitis of rheumatoid arthritis (RA) are the acute flare-up reactions characterised by extravasation of neutrophils and plasma proteins. Evidence suggests that the complement system is activated in rheumatoid arthritis.¹ One product of complement activation is C5a, a potent mediator of neutrophil infiltration and oedema.²⁻¹⁰ The binding of C5a to receptors on leucocytes often renders it undetectable in the fluid phase even when complement activation has been clearly shown.¹¹ We developed a sensitive radioimmunoassay for the measurement of C5a in inflammatory exudate fluids, and in this paper we report for the first time as far as we know, the C5a concentrations in rheumatoid synovial fluid.

Activation of the complement system by either the classical or alternative pathway leads to the generation of C5a.¹² This 74 amino acid glycoprotein is rapidly converted to the physiologically more stable C5a *des Arg* by the action of carboxypeptidase N. C5a is a potent anaphylatoxin, releasing histamine from mast cells and basophils; C5a *des Arg* is virtually inactive in this respect (in man, but not in all species). In contrast, both forms of C5a stimulate leucocyte locomotion in vitro and emigration in vivo. The rabbit skin provides a convenient system to test the biological activity of C5a in vivo. In this system both rabbit and human C5a induce increased microvascular permeability that is

independent of the carboxyl terminal arginine and histamine release.^{5 6} Plasma protein leakage induced by C5a injected intradermally is rapid in onset: radioisotopic studies showed leakage five to six minutes after injection.⁷ This response, however, is entirely dependent on circulating neutrophils: neutrophil depletion abolished leakage induced by C5a but had no effect on leakage induced by histamine and bradykinin.^{6 7} Thus a rapid interaction between neutrophils and microvascular endothelial cells triggered by C5a gives rise to increased endothelial permeability to plasma proteins.¹³ These observations can explain the dependence of oedema on the presence of circulating neutrophils in animal models of immune vasculitis.¹⁴ In one of these models (an Arthus-type reaction in rabbits) we have shown that oedema is associated with the extravascular generation of C5a¹⁵ and that the response is inhibited by anti-C5a antibodies.¹⁶

Cleavage of the third and fourth components of complement leads to the formation of C3a and C4a. Although structurally similar to C5a, these proteins interact with cells at a site which is distinct from the C5a receptor and promote mast cell or basophil histamine release, but not leucocyte activation. Human C3a is about a thousand times less active than human C5a in increasing microvascular permeability when tested in rabbit skin.⁶

Synovial fluid contains complement,^{17 18} though at lower concentrations than is found in plasma. There is evidence for both classical and alternative pathway activation in rheumatoid arthritis, though activation of the classical pathway by IgG aggregates and immune complexes is likely to be more important.¹⁹⁻²¹ Concentrations of several C3 fragments are increased in rheumatoid synovial fluids.²²⁻²⁴ Based on their in vitro activity, none of these C3 fragments is likely to mediate neutrophil accumulation and neutrophil dependent oedema formation.

Ward and Zvaifler reported chemotactic activity for neutrophils in 38 of 54 RA synovial fluids tested.²⁵ Anti-C5 antibodies inhibited the activity (usually by 80-90%) in 14 out of 15 chemotactic rheumatoid synovial fluids. Inhibition by anti-C3 antibodies was detected in only seven of the 15 fluids and was much weaker than the inhibition by anti-C5 antibodies. These authors attributed chemotactic activity to the presence of C567 and C5a,²⁵ although subsequent work showed that the activity ascribed to C5a had a very different electrophoretic mobility to the purified protein.²⁶ In

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view of its potent biological activity there have been surprisingly few attempts to measure C5a in synovial fluids. Wagner and Hugli studied a total of 21 patients with gout, RA, and osteoarthritis: C3a and C4a were detected but C5a was not.²⁷ Moxley and Ruddy, who found much higher C3a concentrations in 41 patients with RA than in those with degenerative joint disease (osteoarthritis), reported the detection of C5a in only one patient with RA.²⁴ We developed a more sensitive radioimmunoassay for C5a than that available commercially, and in this paper we report the measurement of biologically active concentrations of C5a in rheumatoid joint fluids; there was no measurable C5a in the plasma of these patients. A small number of synovial fluid samples from patients with systemic lupus erythematosus, osteoarthritis, Reiter's disease, and mechanical joint trauma were also investigated. We also measured concentrations of the more abundant, but much less inflammatory C3a.

Patients and methods

The patients in this study fulfilled American Rheumatism Association criteria for definite or classical rheumatoid arthritis²⁸ (20 women aged 26–69, two men aged 40 and 57) and systemic lupus erythematosus²⁹ (five women aged 20–36). Osteoarthritis was diagnosed radiologically (three patients). The four patients with Reiter's syndrome were all men whose arthritis was associated with recent non-specific urethritis. 'Traumatic' synovial fluids were obtained from patients with mechanical injuries (two women aged 21 and 47, two men aged 21 and 58). All fluids were derived by arthrocentesis of knees carried out for relief of symptoms.

To prevent complement activation after collection blood or joint fluid was collected in tubes containing sufficient dry sodium EDTA to give a minimum final concentration of 20 mmol/l. Samples were centrifuged (1500 *g* for 10 minutes) as soon as possible after collection. Care was taken not to disturb the cell pellet when removing the supernatant, which was then stored frozen in aliquots while awaiting assay.

RADIOIMMUNOASSAY

The stable *des Arg* metabolites of C5a and C3a were purified from human plasma activated by zymosan using methods described by Fernandez and Hugli³⁰ followed by chromatofocusing (in the case of C5a *des Arg* only)¹⁵ and cation exchange high performance liquid chromatography (HPLC). Immunisation with these proteins was used to obtain goat anti-human C5a and rabbit antihuman C3a. The goat antiserum reacted equally with C5a and C5a *des Arg* but did not cross react with C3a or C3a *des Arg* (<0.03%). The rabbit antiserum recognised C3a and C3a *des Arg* but not C5a or C5a *des Arg* (<0.1%). As has been reported by others,^{31, 32} C5 cross reacted with anti-C5a (23% on a molar basis) and C3 cross reacted with the anti-C3a (42%). For this reason, it was essential to remove the C5 and C3 precursors before measuring the concentrations of their respective

split products of activation. A commercially available precipitating agent supplied with radioimmunoassay kits³² did not completely remove these precursors from samples containing varying protein concentrations such as those found in inflammatory exudate fluids. We therefore developed a system using a combination of polyethylene glycol 6000 (PEG) and protamine sulphate which effectively removes C5 and C3 under these conditions (fig 1).

Samples of plasma or joint fluids (0.2 ml undiluted for C5a assays, and diluted twofold for C3a assay) (fig 1) were mixed with an equal volume of a mixture of 22% PEG and 1% protamine sulphate in 10 mM sodium phosphate buffered saline (PBS), pH 7.4, containing 0.1% sodium azide and 10 mM EDTA. After incubation at 4°C for one hour and centrifugation at 5300 *g* for 10 minutes the supernatants were removed for assay. Under these conditions the recovery of C5a *des Arg* and C3a *des Arg* in the supernatants was at least 80% while no precursor C5 and C3 was detectable (<1%) (fig 1). Furthermore, recovery of IgG was far too low (1–2%) to interfere with the protein A separation step described below.

The competitive binding radioimmunoassay procedure was modified from that described previously for rabbit C5a.¹⁵ Radiolabelled tracer ligands were prepared by incubating 10 µg C5a *des Arg* (or 20 µg C3a *des Arg*) with 37 MBq Na¹²⁵I in the presence of a solid phase glucose oxidase-lactoperoxidase system (Enzymobeads; Rio-Rad Laboratories). The specific activities obtained were 590–780 kBq/µg C5a *des Arg* and 480–630 kBq/µg C3a *des Arg*. Using the procedure described below, binding of radiolabel in the presence of excess antiserum was 93% for C5a *des Arg* and 97% for C3a *des Arg*. Standards and, where necessary, sample supernatants were diluted with a mixture of 11% PEG and 0.5% protamine sulphate in PBS, 0.1% azide, 10 mM EDTA. ¹²⁵I labelled ligands, antisera, and protein A bacterial adsorbent (PABA; Miles Scientific) were diluted with PBS containing 0.5% protamine sulphate, 0.2% gelatin, 0.1% azide, and 10 mM EDTA. Sample supernatants (100 µl) were mixed with 100 µl radiolabelled ligand (0.5 ng ¹²⁵I-C5a *des Arg* or 2.0 ng ¹²⁵I-C3a *des Arg*) and 100 µl antiserum (goat anti-C5a diluted 1/34 000 or rabbit anti-C3a diluted 1/22 000). After incubation for 40 hours at 20–24°C an excess of PABA (25 µl of a 5% suspension) was added. After a further one hour 1 ml of PBS (0.1% azide) was added, the tubes were centrifuged (5300 *g* for 10 minutes) and the supernatants were removed by suction. The antibody bound radioactivity in the pellets was counted in a multiwell gammacounter (LKB Wallac 1260, Multigamma II) for a time sufficient to register at least 10 000 counts in samples to which no unlabelled ligand was added. Concentrations were determined from standard curves constructed by use of a computer assisted spline fit programme.

The limits (mean (SEM)) of adequate measurements (defined as the concentration required to give 20% inhibition of binding of the radiolabelled ligand) were 6.0 (0.2) ng C5a/ml plasma or joint fluid and 22.1 (1.4) ng

Figure 1 Effects of protein concentration on the precipitation of C5 and C3 and the recovery of C5a and C3a. Recovery of ^{125}I tracer in the supernatant of plasma samples treated with PEG/protamine sulphate (●) and, for comparison, the precipitation agent supplied with the Upjohn radioimmunoassay kits (○). The left hand panel shows results for C5a des Arg and C5; the right hand panel shows results for C3a des Arg and C3. Samples for C5a radioimmunoassay were used undiluted and samples for C3a assay were diluted twofold before addition of the PEG/protamine sulphate precipitating agent to ensure that recovery of the split fragments was at least 80%. Each point is the mean of three determinations.

C3a/ml ($n=15$ assays). These assays were about twice as sensitive as the commercial kits. The interassay coefficients of variation in the C5a assays were 21% for a rheumatoid joint fluid and 9% for a partially activated plasma sample. In the C3a assays these values were 7% for the rheumatoid joint fluid and 14% for the activated plasma sample. Intra-assay coefficients of variation for rheumatoid joint fluid were 4% in both radioimmunoassays.

IN VITRO STUDIES

Peripheral venous blood was withdrawn into heparin (10 U/ml) for preparation of plasma. Plasma was activated at 37°C for 30 minutes using a dose range of activator suspensions in saline. Sodium EDTA (pH 7.4) and magnesium EGTA (pH 7.4) were added to a final concentration of 10 mmol/l, as appropriate. Incubations were terminated by the addition of sodium EDTA, cooling in ice-cold water, and centrifugation at 2°C. The activators used were heat aggregated gammaglobulin (HAGG: human IgG (Sigma) heated to 63°C for one hour) and yeast cell walls (zymosan A, Sigma).

HPLC ANALYSIS

For HPLC analysis of immunoreactivity in rheumatoid joint fluid and activated plasma samples, PEG/protamine sulphate supernatants were mixed with an equal volume of 25 mM sodium phosphate buffer, pH 5.8, and applied to a TSK 535 CM column (7.5×150 mm). Proteins were eluted using a 100–900 mM sodium chloride gradient in the pH 5.8 buffer at a flow of 1 ml/minute. Fractions (1 ml) were collected into 0.2 ml of a solution designed to adjust the protamine sulphate concentration to

0.5% and the pH to about 7.4 for subsequent radioimmunoassay.

Results

GENERATION OF C5a AND C3a DURING ACTIVATION OF CLASSICAL AND ALTERNATIVE PATHWAYS IN VITRO

To investigate complement activation in vitro, plasma was incubated with varying doses of HAGG and zymosan. Generation of immunoreactive C5a and C3a was related to the dose of activator used (fig 2). Magnesium EGTA, which is known to chelate calcium ions and inhibit classical pathway activation, considerably reduced activation by HAGG but had no inhibitory effect on activation by zymosan. Sodium EDTA, which is known to chelate magnesium as well as calcium ions and therefore to inhibit both pathways of complement, completely inhibited activation by HAGG and by zymosan. These results show that the principal effect of HAGG was activation of the classical pathway while zymosan was effective in activating the alternative pathway of complement, and that activation of either pathway leads to the generation of C5a and C3a immunoreactivity.

Figure 2 Complement activation in vitro. Both heat aggregated gammaglobulin (HAGG) and zymosan produced a dose related generation of immunoreactive C5a (upper panel) and C3a (middle panel). Magnesium EGTA (open symbols) inhibited activation by HAGG but not by zymosan. The lower panel shows that HAGG produces less C5a relative to C3a than does zymosan. This may reflect differences in the physical nature of the activator or differences in the ability of classical and alternative pathway enzymes to cleave C3 and C5. Each point is the mean of duplicate incubations.

The results shown in fig 2 also show that the ratio of C5a:C3a generated depends on the activator used as zymosan produced a higher ratio than HAGG. Thus measurements of C3a cannot be used to predict accurately the concentrations of C5a when the nature of the stimulus is unknown.

C5a AND C3a CONCENTRATIONS IN JOINT FLUIDS
Joint fluids from patients with RA, systemic lupus erythematosus (SLE), osteoarthritis, Reiter's disease, and mechanical trauma were analysed for immunoreactive C5a and C3a (fig 3). For comparison, plasma samples from 11 of the 22 patients with RA were also analysed. C5a concentrations were well below the limit of adequate measurement (6 ng/ml) in the rheumatoid plasma samples. Joint fluids from patients with RA contained 4–86 ng C5a/ml (mean=21). Fluids taken from mechanically traumatised joints contained low C5a concentrations. Although the sample numbers were low, the concentrations of C5a in synovial fluid samples from patients with SLE, osteoarthritis, and Reiter's disease were increased but not to the degree seen in RA. Rheumatoid plasma samples contained C3a (0.26 (0.07) µg/ml). C3a concentrations in joint fluid were greatly increased in RA (mean 4.06 µg/ml); the other conditions showed a range of C3a concentrations above that in plasma.

Figure 3 Concentrations of immunoreactive C5a (upper panel) and C3a (lower panel) in joint fluids and plasma. Joint fluids from 22 patients with rheumatoid arthritis (RA) contained a wide range of C5a concentrations with a mean value of 21 ng/ml. In contrast, plasma samples (shown as smaller symbols) taken from 11 of these patients had virtually undetectable concentrations of C5a (1.3 (0.3) ng/ml). The mean values of C3a in rheumatoid joint fluids was 4.06 µg/ml, which is much higher than concentrations found in rheumatoid plasma samples (0.26 (0.07) µg/ml).

The concentrations of C5a and C3a in the 22 individual rheumatoid joint fluids are correlated in fig 4. Although there is a positive correlation, measurement of C3a was a very poor predictor of the concentration of C5a in individual joint fluids.

The two patients with RA with the highest concentrations of synovial C5a had normal concentrations of circulating C3, C4, and IgG. One of these patients, whose joint fluid contained 86 ng C5a/ml and 7.46 µg C3a/ml, was strongly seropositive for rheumatoid factor and was on a repeat course of gold treatment at the time of sample collection. The other, whose joint fluid contained 69 ng C5a/ml and 7.76 µg C3a/ml, was seronegative and was being treated with indomethacin and prednisolone at the time of sample collection. Both samples were taken during acute flare-up reactions.

HPLC OF IMMUNOREACTIVITY IN RHEUMATOID JOINT FLUID COMPARED WITH ACTIVATED PLASMA
A sample of rheumatoid joint fluid (1.8 ml containing 30 ng C5a and 14.5 µg C3a) was mixed with the PEG/protamine sulphate precipitation agent. The supernatant was then mixed with column buffer and applied to a carboxymethyl cation exchange HPLC column. The column was eluted with a salt gradient and fractions collected for radioimmunoassay. To compare the elution profiles this was followed by injection of the C5a *des Arg* and C3a *des Arg* standards.

The profile of immunoreactivity in the rheumatoid joint fluid is shown in fig 5. Immunoreactive C5a eluted as a discrete peak at fraction 18, corresponding to the position of standard C5a *des Arg*. Some apparent immunoreactivity

Figure 4 Correlation of C5a and C3a concentrations in individual rheumatoid joint fluids. Linear regression analysis of log transformed data showed a positive correlation ($r=0.70$) between C5a and C3a concentrations. The large deviation of data points from the predicted line shows that immunoreactive C3a measurement is a poor predictor of the concentration of the biologically more relevant C5a in individual samples.

Figure 5 Radioimmunoassays for C5a and C3a after cation exchange high performance liquid chromatography. Immunoreactive C5a (●) in rheumatoid joint fluid eluted as a single peak, corresponding to the retention time of the C5a des Arg standard (upper panel). C3a immunoreactivity (○) eluted as two peaks, the major one corresponding to the retention time of C3a des Arg. For comparison, plasma was activated *in vitro* by incubation with heat aggregated gammaglobulin and chromatographed (lower panel). Immunoreactive C3a eluted as a single peak corresponding to the C3a des Arg standard. In contrast with the joint fluid, the plasma sample did not contain the peak of unidentified C3a immunoreactivity. Immunoreactive C5a showed a similar elution profile to that seen with the joint fluid.

was seen in later fractions; this was below the limit of adequate measurement and was shown to be a reflection of the high salt concentrations (up to 900 mmol/l NaCl) in these fractions. Fractions 17–19 contained 83% of the immunoreactivity applied to the column, which is a high recovery for such a low C5a concentration. The major peak of immunoreactive C3a eluted in fraction 25, corresponding to the position of standard C3a des Arg. There was, however, a smaller peak of immunoreactivity in fraction 23. This profile was confirmed in a subsequent assay and both peaks gave complete displacement of radiolabelled ligand at low dilutions and showed parallels with the standard when tested in serial dilutions. The early eluting peak of C3a immunoreactivity may represent a breakdown product of C3a or C3a des Arg or a slightly different cleavage product of C3, perhaps generated by proteases released from tissue cells or the accumulating leucocytes.

When plasma samples activated by HAGG (fig 5) and zymosan (data not shown) were analysed, immunoreactive C3a eluted with the same retention time as the standard C3a des Arg. There was no evidence, however, for the smaller early eluting peak of immunoreactive C3a previously seen in the rheumatoid joint fluid. As was found with the joint fluid, immunoreactive C5a in the activated plasma samples eluted as a single peak corresponding to the mobility of C5a des Arg (fig 5).

Discussion

Complement activation has been well documented in the inflamed joints of rheumatoid arthritis.¹ Of the products of complement activation, C5a and its more stable des Arg metabolite have potent pro-inflammatory activities which might account for some of the features of the acute flare-up phases of rheumatoid arthritis, characterised by plasma protein and neutrophil extravasation.

Moxley and Ruddy, who used commercial radioimmunoassay kits and found high concentrations of C3a in rheumatoid joint fluids, concluded that the concentrations of C5a were either below or barely above the limit of detection of the C5a kit.²⁴ We developed a C5a radioimmunoassay of greater sensitivity and with more reliable elimination of the crossreacting C5 precursor, especially when using samples of variable protein concentrations. The results show that C5a was present in rheumatoid joint fluids, but not in the plasma of these patients (fig 3). The immunoreactive C5a in joint fluids is assumed to be C5a des Arg owing to the action of endogenous carboxypeptidase N. Moreover, the immunoreactivity chromatographed as a single peak, corresponding to the position of C5a des Arg on cation exchange HPLC (fig 5). The system used will distinguish between C5a and C5a des Arg (data not shown). The mean value found in rheumatoid joint fluids was 21 ng/ml (about 2.5×10^{-9} mol/l) and this concentration of C5a/C5a des Arg is sufficient to promote neutrophil chemotaxis *in vitro*,³³ oedema formation, and neutrophil accumulation in rabbit skin^{5–7 10} and wheal and flare reactions in human skin.³⁴

The detection of high concentrations of immunoreactive C3a agrees with the results of Moxley and Ruddy.²⁴ C3a is not a mediator of neutrophil accumulation and associated oedema formation,⁶ although it can cause mast cell degranulation and a wheal and flare reaction in human skin, provided that the carboxyl terminal arginine is present. Rheumatoid synovial tissues contain mast cells which can be stimulated to release histamine.³⁵ It is doubtful, however, that histamine release by anaphylatoxins is important in rheumatoid arthritis because treatment with antihistamines is ineffective and C3a is even more susceptible to conversion to its des Arg metabolite than C5a.³⁶ In this context it is interesting to note that oedema formation and neutrophil accumulation induced by injection of C5a and C5a des Arg in rabbit and human skin are largely resistant to antihistamines.^{5 6 34 37 38}

The correlation between C5a and C3a concentrations in individual fluids was poor (fig 4). This may be explained by *in vitro* studies. There is general agreement that various types of leucocytes bind C5a but not C3a.^{11 39 40} When complement was activated *in vitro* in the absence of cells the ratio of immunoreactive C5a:C3a was also found to vary with the stimulus (fig 2). Heat aggregated gammaglobulin, used as a model for rheumatoid factor complexes with IgG, generated a lower C5a:C3a ratio than zymosan. This may reflect differences in the physical nature of the activator as well as differences in the relative efficiencies of the C3 and C5 convertases generated during activation of the classical and alternative pathways. Furthermore, Ng *et al* found that immune complexes containing IgM $\times$ rheumatoid factor activate C3 in the fluid phase yet fix C3 poorly⁴¹; this would be expected to generate lower than normal

C5 convertase activity and thus to yield low C5a:C3a ratios. Taken together, the variable C5a:C3a ratios generated during activation and the subsequent removal of C5a by leucocytes might account for the variable C5a:C3a ratios observed in the rheumatoid joint fluids.

Chemotactic activity related to C5 was found in rheumatoid joint fluid in another study: this activity was attributed to the presence of the C567 complex and C5a.²⁶ Subsequent work, however, cast doubt on the identification of C5a because the chemotactic activity ascribed to it migrated anodally on Pevikon block electrophoresis.²⁶ The generation of C5a can be inferred from the detection of increased concentrations of the fluid phase terminal complement complex in rheumatoid joint fluids,⁴² as cleavage of C5 with the liberation of C5a is an essential step in assembly of this complex. Previous attempts to measure C5a in synovial fluids have been unsuccessful.^{24, 27} Using a more sensitive radioimmunoassay, we have now shown that rheumatoid joint fluids contain biologically active concentrations of C5a.

In animal models we have shown that C5a (and its *des Arg* metabolite) is a potent inducer of oedema by a neutrophil dependent mechanism.^{6, 7} A striking feature of the acute inflammatory phases of rheumatoid arthritis is the appearance of high numbers of neutrophils in synovial fluid. We suggest that C5a might have an important role in mediating neutrophil accumulation and, as a consequence, may be important in acute joint swelling.

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