Relation between synovial fluid C3 degradation products and local joint inflammation in rheumatoid arthritis, osteoarthritis, and crystal associated arthropathy

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SUMMARY C3 degradation products (C3dg/d) were estimated in 288 synovial fluid (SF) samples (rheumatoid arthritis (RA) 93, osteoarthritis (OA) 68, chronic pyrophosphate arthropathy 80, acute pseudogout 20, others 27) from knees of 138 patients (bilateral 67, serial sampling on two to six occasions 40). At each aspiration knees were defined as 'active' or 'inactive' by single observer global assessment using six clinical parameters of inflammation. Lack of correlation between paired SF and plasma C3dg/d implied local C3 activation within joints. Raised SF C3d levels were found in active compared with inactive RA joints (mean (range) 51 (15-105) and 6 (0-15) units/ml respectively). Low SF C3dg/d levels were found in OA (mean (range) 0.8 (0-7) units/ml) and chronic pyrophosphate arthropathy (mean (range) 4 (0-16) units/ml), irrespective of clinical activity. In contrast, very high levels (mean (range) 61 (16-126) units/ml) were present in all cases of pseudogout. These differences remained after correction for SF C3 or albumin. This study is the first to show a positive correlation between SF C3dg/d and local inflammation in RA joints. It further suggests that C3 activation is a constant feature of pseudogout but not an accompaniment of inflammation associated with chronic crystal associated synovitis or OA.

Key words: complement, C3dg, C3d, clinical activity, crystal synovitis, pyrophosphate arthropathy.

Complement activation by both classical and alternative pathways is thought to play an important part in mediation of inflammation in a variety of rheumatic diseases.¹⁻⁷ Evidence to support such a role includes (a) in vitro activation of complement by factors such as immune complexes,⁸ rheumatoid factors,^{9 10} and monosodium urate and calcium pyrophosphate dihydrate (CPPD) crystals¹¹⁻¹³ and (b) direct demonstration of complement activation in blood and synovial fluid (SF) of patients with rheumatic disease.¹⁻⁷

Although complement activation in rheumatic diseases has been extensively investigated in plasma, there are relatively few substantial

studies of in vivo complement activation within joints.^{1-6 14-26} Such studies, however, using a variety of assay systems, have demonstrated SF complement activation particularly in rheumatoid arthritis,^{1-6 14-25} gout,^{1 6 14 16 18 24} pseudogout,^{6 14 15 18 24} systemic lupus erythematosus,^{6 16} and seronegative spondyloarthropathies,^{1 6 15 16} but not in osteoarthritis.^{1 3-6 16 18 20 21 24} Marked variability within each disease group and considerable overlap between diagnostic categories have uniformly been reported.^{3-6 14-25} Only two studies, however, have attempted to characterise the clinical activity of the joints aspirated, and in these reports, including 40 patients with rheumatoid arthritis²¹ and 10 with juvenile chronic arthritis,²⁶ no apparent correlation was observed between SF complement activation and the clinical state of the joint. The relation between complement activation and

local joint inflammation, therefore, remains in question.^{21 24}

In this study we estimated complement degradation products with C3d specificity (C3dg/d²⁷—hereafter abbreviated as C3d) together with C3 in paired SF and plasma samples from a large number of patients with well characterised joint disease. For comparative purposes all SF aspirations were from a single site (the knee). Unlike most previous studies we assessed the clinical state of each joint aspirated, performed frequent bilateral sampling (often from knees showing different inflammatory activities), and used serial sampling of the same knee during differing phases of clinical activity.

Patients and methods

Approval for this study was obtained from the local ethical committee.

SUBJECTS

One hundred and thirty eight patients attending the rheumatology unit of the City Hospital with clinically evident disease affecting at least one knee were included in the study. Minimum investigation in each case included full blood count, erythrocyte sedimentation rate, serum rheumatoid factor, plain

radiographs of hands, feet, and knees, and examination of fresh knee SF for birefringent crystals (compensated polarised light microscopy); further investigation was determined by the individual patient characteristics. The principal diagnostic categories were rheumatoid arthritis (RA), osteoarthritis (OA), and crystal associated arthropathy. All patients with RA had erosive disease and fulfilled American Rheumatism Association criteria for classic or definite disease²⁸; patients designated as seropositive had raised titres of serum IgG rheumatoid factor²⁹ recorded on at least one occasion. Patients with OA had symptomatic seronegative gonarthrosis with knee radiographs showing cartilage loss plus subchondral sclerosis or osteophyte, or both; none had radiographic chondrocalcinosis, SF CPPD crystals, or evidence of other primary joint disease. Patients with pseudogout had typical self limiting acute episodes of knee synovitis with SF CPPD crystals and no evidence of coexistent joint disease (e.g., sepsis). Chronic pyrophosphate arthropathy (CPA) was defined as persistent (>3 months) symptomatic gonarthrosis with SF CPPD crystals and x ray features of OA (often with predominant patellofemoral involvement, bi- or tricompartmental disease, or chondrocalcinosis). Gout was confirmed by demonstration

Table 1 *Diagnosis and details of patients*

Diagnosis	No	F:M	Age mean (range) (years)	Duration of knee symptoms mean (range)	SF samples
RA					
Seropositive	33	22:11	63 (42–82)	8 (1–34) yr	74
Seronegative	6	2:4	62 (45–76)	4 (1–14) yr	19
Total	39	24:15	63 (42–82)	7 (1–34) yr	93
OA	36	19:17	67 (45–89)	6 (1–25) yr	68
CPA	33	19:14	72 (31–88)	8 (1–31) yr	80
Pseudogout	10	8:2	74 (53–94)	12–72 h	20
Chronic gout	5	1:4	63 (54–77)	6 (2–51) yr	5
Acute gout	3	0:3	43 (35–50)	12–72 h	3
Psoriatic arthritis	5	2:3	32 (18–57)	1 (0.5–2) yr	8
Acute Reiter's disease/reactive	7	3:4	31 (20–62)	0.8 (0.1–2) yr	11

Table 2 *Clinical assessment of knees*

	Inactive	Active
Individual parameters		
Increased warmth	Absent	Present
Effusion	Absent-mild/not tense	Moderate-marked/tense
Synovial thickening	Absent	Present
Joint line tenderness (0–3)	0–1	2–3
Early morning stiffness	<1 hour	1 hour or more
Inactivity stiffness	<15 minutes	15 minutes or more
Global assessment	<3 parameters active	4–6 parameters active

of monosodium urate crystals in fresh knee SF. The diagnosis of other conditions was similarly based on clinical presentation/distribution of arthropathy together with appropriate serological, radiographic, and SF characteristics; no patient with indeterminate or 'probable' disease was included. Patient details are shown in Table 1.

METHODS

Two hundred and eighty eight knee aspirations were performed on these 138 patients (bilateral on the same occasion 58, serial sampling of the same knee on two to six occasions 40). At each aspiration knees were examined and assessed by a single observer for six clinical parameters of inflammation—namely, increased warmth, effusion (0–3), anterior joint line and suprapatellar synovial thickening, joint line tenderness (0–3), early morning stiffness, and inactivity stiffness (Table 2). The joint was globally assessed as 'active' if four to six parameters were active, and 'inactive' if less than three parameters were active; 23 additional knees scoring three active parameters, i.e., 'intermediate', were not included. For complement estimations paired blood and SF samples were taken immediately into edetic acid (10 mmol/l), samples were then centrifuged for five minutes, and the plasma and SF stored in vapour phase liquid nitrogen (–186°C) within three hours of collection.

C3d levels (units/ml) were measured by the double decker rocket immunoelectrophoresis method of Brandslund *et al.*²⁷ Essentially, 4 µl edetic acid plasma were electrophoresed overnight (2.5 V/cm), initially through a gel layer containing antihuman C3c (Dako) and then through a layer containing antihuman C3d (Dako) where the C3d rockets were formed. After drying and staining, the rocket heights of test samples and standards were measured and the C3d values calculated from a standard curve. The C3d content of the standard (normal serum incubated at 37°C for four days) had previously been assigned an arbitrary value of 100 units/ml, and the upper limit of normal for plasma was statistically determined to be 12 units/ml. There is no normal range for SF, very low SF levels (<5 units/ml) cannot be accurately estimated by this method and were counted as 0 for statistical analysis. Plasma and SF C3 levels (g/l) and SF albumin (g/l) were estimated by a turbidometric method on a centrifugal fast analyser.³⁰

Correlation between plasma and SF levels was determined by Deming's procedure. Comparison of SF findings between active and inactive joints and between different diagnostic categories was by the Wilcoxon test when the number of values did not exceed 25 in the smaller group and 50 in the larger

Table 3 Plasma and SF findings in the different disease categories*

	Plasma				SF			
	Number		C3d (units/ml)	C3d/C3	C3 (g/l)	Number	C3d (units/ml)	C3d/C3
RA	56	16.4 (8.5)	13.1 (8.8)	1.27 (0.20)	74	41 (29)	73 (58)	1.93 (1.55)
Seropositive	12	10.2 (3.7)	7.9 (2.4)	1.27 (0.22)	19	24 (32)	21 (27)	0.81 (1.48)
Seronegative	49	9.5 (3.9)	8.8 (4.5)	1.10 (0.19)	68	0.8 (2)	1 (3)	0.02 (0.08)
OA	66	9.8 (3.6)	8.2 (3.1)	1.21 (0.19)	80	4 (5)	6 (11)	0.18 (0.30)
CPA	16	11.7 (3.5)	8.6 (2.3)	1.36 (0.21)	20	61 (32)	86 (49)	3.21 (1.81)
Pseudogout	5	11 (2.9)	7.2 (0.7)	1.52 (0.33)	5	4 (5)	6 (8)	0.11 (0.21)
Chronic gout	3	7 (2)	5.7 (2.1)	1.27 (0.15)	3	14 (5)	34 (12)	0.88 (0.08)
Acute gout	6	10.8 (2.9)	8.1 (1.8)	1.33 (0.10)	8	11 (8)	11 (10)	0.52 (0.20)
Psoriatic arthritis	8	12 (3.8)	8.7 (3.5)	1.39 (0.22)	11	12 (8)	14 (8)	0.64 (0.34)
Reiter's disease/reactive								0.89 (0.39)

*Results are mean (SD).

group, and by Student's *t* test when any group contained larger numbers than this.

Results

COMPARISON BETWEEN PLASMA AND SF C3d LEVELS

Paired plasma and SF findings in the different disease categories are shown in Table 3; the lower number of plasma samples (221) relates to frequent bilateral knee sampling on the same occasion.

Comparison between groups showed raised plasma C3d levels only in seropositive RA patients ($p<0.01$). Correction for C3 (i.e., the plasma C3d/C3 quotient^{4 31}) more clearly delineated the raised level found in this group (Table 3).

Comparison between major groups for SF C3d showed markedly raised levels in seropositive RA ($p<0.001$), seronegative RA ($p<0.001$), and pseudogout ($p<0.001$) compared with the very low levels in OA (Table 3); a modest, but highly significant, increase was seen in CPA ($p<0.001$). The highest levels were seen in pseudogout (mean (range) 61 (16–126) units/ml). In the other, smaller groups a moderate increase of SF C3d was seen in acute Reiter's disease ($p<0.01$), psoriatic arthritis ($p<0.01$), and in the three patients with acute gout; levels in chronic gout were similar to those in CPA. Correction for SF C3 did not significantly alter these between group comparisons but emphasised the increases seen in seropositive RA, pseudogout, and acute gout (Table 3). Correction for SF albumin similarly did not alter the differences between groups but emphasised the increases seen in pseudogout and seropositive RA (Table 3).

No correlation was observed between plasma and SF C3d levels either overall ($r=0.44$) or within the seropositive RA group alone ($r=0.40$). The higher range of C3d and C3d/C3 values for SF (0–126, mean (SD) 19.1 (27.6) units/ml and 0–221, mean

(SD) 28.8 (45.4) respectively) compared with plasma (0–28, mean (SD) 11.6 (6.0) units/ml and 0–63, mean (SD) 9.5 (5.7) respectively) supported local activation of C3 within joints.

CORRELATION BETWEEN SF C3d AND CLINICAL ACTIVITY

Mean SF C3d, C3d/C3, and C3d/albumin values in different diagnostic groups divided according to clinical joint activity are shown in Table 4; comparable data for groups requiring no division (i.e., pseudogout, acute gout, Reiter's disease—all joints active; chronic gout—all joints inactive) are contained in Table 3. Individual SF C3d values are shown in Fig. 1.

Markedly raised SF C3d levels were found in active compared with inactive RA joints, irrespective of seropositivity ($p<0.001$ both groups). No difference in SF C3d, however, was observed between active or inactive OA, or between active and inactive CPA; compared with active OA, SF C3d remained significantly raised in both active ($p<0.001$) and inactive ($p<0.001$) CPA knees. In contrast with active CPA, markedly raised SF C3d levels were seen in all cases of pseudogout ($p<0.001$).

Compared with pyrophosphate arthropathy, a less impressive difference in SF C3d was observed between the small number of patients with acute (active) and chronic (inactive) gout (Fig. 1). SF C3d levels in active psoriatic arthropathy were comparable with those in acute Reiter's disease (Fig. 1).

SF C3d IN BILATERAL AND SERIAL SAMPLES

Twelve patients with RA underwent simultaneous bilateral sampling at a time when their knees showed contrasting clinical activities. Comparison between the two sides in these patients showed marked differences in SF C3d, with higher levels in the active knee (Fig. 2). Simultaneous bilateral sampling of 18 patients with RA and clinically

Table 4 SF C3d findings in different diagnostic groups divided according to clinical activity of the aspirated joint*

		Number	C3d (units/ml)	C3d/C3	C3d/albumin
RA					
Seropositive:	Active	57	51.2 (24)	96.4 (48)	2.27 (1.45)
	Inactive	17	3.8 (4.8)	10.8 (17)	0.21 (0.32)
Seronegative:	Active	9	49.1 (30)	39.3 (28)	2.82 (1.09)
	Inactive	10	0.8 (2.2)	1.0 (3.2)	0.04 (0.10)
OA	Active	31	0.9 (2.1)	1.0 (2.7)	0.04 (0.08)
	Inactive	37	0.6 (1.7)	1.5 (3.7)	0.23 (0.09)
CPA	Active	27	4.5 (5.2)	6.8 (10.8)	0.22 (0.36)
	Inactive	53	3.5 (4.2)	4.2 (9.8)	0.14 (0.21)
Psoriasis	Active	6	14.3 (4.1)	21.4 (12)	0.95 (1.11)
	Inactive	2	0 (0)	0 (0)	0 (0)

*Results are mean (SD).

Fig. 1 Synovial fluid C3d levels in active (●) and inactive (○) knees. (RF=rheumatoid factor.)

similar knees (Fig. 2) also showed frequent marked differences in SF C3d between the two active joints in the same individual (mean (SD) difference 19.7 (21.4), range 1–60 units/ml; $n=15$). For the three patients with bilateral inactive knees all values were <5 units/ml. Owing to the narrow range of SF C3d values, simultaneous bilateral sampling in patients with OA and CPA showed only minor differences between joints of the same individual, irrespective of clinical activity. Similar differences to those observed in RA were seen, however, in the few bilateral samples of patients with pseudogout (four), psoriatic arthritis (two), and Reiter's disease (three).

Five patients with RA underwent serial sampling of the same knee during different phases of clinical activity (aspirations were performed at varying intervals over a 12 month period). Marked changes

in SF C3d were observed, with raised levels occurring at times of clinical activity (Fig. 3). As with bilateral aspiration, serial sampling of OA and CPA knees showed only minor differences in SF C3d irrespective of clinical activity.

Discussion

Reduction in individual native complement components is an unreliable indicator of complement activation *in vivo*. Enhanced hepatic synthesis of complement factors as acute phase reactants³² and variable synthesis by extrahepatic tissue, including synovium,³³ may permit significant activation of C3 without concomitant decrease in C3 concentration outside the normal range.³⁴ Conversely, low C3 levels may reflect decreased synthesis rather than C3 consumption.³⁵ Evaluation of *in vivo* complement

Fig. 2 Synovial fluid C3d levels in patients with RA who underwent bilateral aspiration of knees showing differing (left) or similar (right) clinical inflammatory activity. ●=active; ○=inactive.

Fig. 3 Serial synovial fluid C3d levels in five patients with RA who showed varying clinical activity on different occasions during a 12 month period. ●=active; ○=inactive.

activation is therefore ideally based on detection of activation products or on turnover studies. In the present study evidence of complement activation, by either classical or alternative pathway, was sought by estimation of stable breakdown products of C3 with C3d specificity (C3dg/d) using an established rocket immunoelectrophoresis system.²⁷ As the C3d/C3 ratio may be a more reliable indicator of complement activation than C3d alone,^{4 31} this quotient was additionally determined.

As in previous studies^{4 5 23 25} a marked increase of SF C3d was shown in certain disease categories. That such an increase represents local activation of complement within joints^{24 25} was supported by the lack of correlation between plasma and SF C3d, the markedly higher C3d values observed in SF, and the frequently marked variation in SF C3d levels between different joints of the same individual. As the concentration of serum proteins in SF varies considerably the albumin concentration was used to correct absolute C3d to relative values, thereby permitting comparison of SF results between different groups. It was not necessary, however, to correct between different groups. It was also unnecessary to correct for protein content in order to show local complement activation in SF (owing to the frequently much higher values in SF than in plasma), and correction for SF albumin made little difference to intergroup or within group comparisons. Similarly, because no group showed marked depression of SF C3, comparison of the corrected C3d/C3 quotient,^{4 31} though to some extent sharpening division between categories, did not substantially alter findings derived from comparison of SF C3d alone.

The results of the between group comparisons are in substantial agreement with previous SF complement studies in showing no evidence for complement activation in OA,^{1 3-6 16 18 20 21 24} but significant activation in RA^{1-6 14-25} (particularly those with seropositive disease,^{1 3 6 15}) and acute pseudogout.^{6 14 15 18 24} This is the first study to examine CPA as a separate major diagnostic group, and the finding of a modest but significant increase of SF C3d in CPA compared with OA has not previously been reported. SF complement activation has previously been demonstrated in psoriatic arthropathy^{15 16 21 25} and acute Reiter's disease,^{1 6 16 21 25} and the only unexpected finding was the comparatively modest increase of SF C3d in the three cases of acute gout.^{6 14 16 18 24} Although possibly explained by the small sample numbers or inappropriate timing of aspiration after onset of the acute attack, this finding is consistent with the suggested minor role for complement activation in acute gouty inflammation.^{16 21 36}

The major findings of this study, however, relate to division within each diagnostic group according to the local clinical inflammatory activity of the aspirated joint. There is no generally agreed system for clinical assessment of 'joint inflammation', and any division into active or inactive is necessarily arbitrary and an inevitable compromise. The six clinical parameters employed, however, may reasonably be expected to reflect predominantly inflammatory activity, rather than mechanical damage or periarticular injury, in the disease states examined, and for the purposes of the present study a single observer and a summated score were used with exclusion of joints that were intermediate between the two extremes of the scoring range. Further grading, other than into active or inactive, was not attempted. Such clinical characterisation would seem essential to any study that investigates mediators of inflammation in joint tissues. Surprisingly, however, only two SF complement studies have previously addressed this issue and attempted to define aspirated joints in both clinical and diagnostic terms. Sheppard *et al* graded clinical activity of knees as mild, moderate, or severe according to the degree of pain, swelling, warmth, and tenderness,²¹ whereas Mollnes and Paus used a functional knee score (0–100) devised for outcome assessment of RA knees after synovectomy.²⁶ Pain, unspecified tenderness, restriction of movement, deformity, and instability, however, do not necessarily reflect the presence of synovitis, and inappropriate clinical assessment may in part explain the failure of either study to show correlation between SF complement activation and clinical activity.

In the present study markedly raised SF C3d levels were seen in active compared with inactive RA joints, and serial sampling showed that SF C3d levels vary in concert with inflammatory activity. To our knowledge this is the first study to show a positive correlation between SF complement activation and local, clinically assessed inflammation in RA joints. A similar correlation was suggested in the two smaller seronegative spondyloarthritis groups in this study.

Seropositive and seronegative RA patients were considered separately as rheumatoid factors have been shown to activate complement *in vitro*,^{9 10} and positive correlation between rheumatoid factor and complement activation has been shown in plasma³⁷ and in certain SF studies^{1 2 6} of patients with RA. The present finding of a higher mean level of SF C3d in seropositive patients as a group appears to support a possible role for rheumatoid factor in complement activation.^{1 10} Comparison of SF C3d in active joints alone, however, as in certain previous reports,^{5 21 25} showed no clear relation

between seropositivity and complement activation, thus emphasising the importance of clinical and diagnostic characterisation. The cause(s) or pathways of complement activation in RA, however, were not the subject of the present study and cannot be deduced from the data.

Comparison of SF C3d findings in OA, CPA, and pseudogout was of special interest. Although OA joints are commonly used as 'non-inflammatory' disease controls in SF studies,^{1 3–6 16 18 20 21} many OA knees in the present study were clinically active, being associated with marked inflammatory stiffness and showing warm, large effusions, with joint line/capsular tenderness. Notwithstanding the presence of obvious clinical inflammation in such joints, no activation of SF C3 could be shown. CPA is a common subset of OA characterised by a frequent inflammatory component, atypical distribution, characteristic radiographic features, and the presence of SF CPPD crystals.³⁸ Although CPPD crystals may activate complement^{12 13} and other mediators of inflammation *in vitro*,^{36 39} a causal role for these crystals in chronic disease has been questioned.³⁸ Compared with uncomplicated OA, the modest but significant increase of SF C3d in active and inactive CPA and the very high levels in pseudogout support a role for CPPD crystals in complement activation *in vivo*. No significant difference in SF C3d was observed, however, between active and inactive CPA, suggesting that, as with OA, SF complement activation is not an accompaniment of the clinical inflammation associated with chronic CPPD crystal associated arthritis. These observations may be explained by gross 'shedding' of naked crystals (with surface characteristics capable of activating complement) during episodes of acute pseudogout,^{38 39} but less dramatic release of crystals (with subsequent masking of the active crystal surface by protein coating) during CPA.^{38–40} The role of CPPD crystals in joint disease and the nature of the inflammation associated with OA and chronic crystal associated synovitis, however, remain unresolved.

In the present study considerable additional information was obtained by further clinical division within diagnostic groups. We suggest that future SF studies relating to inflammation or tissue degradation take into account the clinical characterisation as well as the diagnosis of joints at time of aspiration. Improved systems of clinical activity assessment must necessarily be agreed and validated if the results of such studies are to be correctly interpreted and compared between centres.

We would like to thank the Arthritis and Rheumatism Council for financial support, Gillian Hull and April Powell-Richards

skilled technical assistance, and Caroline Bloomfield for excellent secretarial work.

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