

Circulating complement breakdown products in patients with rheumatoid arthritis. Correlation between plasma C3d, circulating immune complexes, and clinical activity.

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Research Article

Quantitative determination of the small C3 breakdown product, C3d, was used to investigate complement activation in 45 plasma samples from 30 patients with rheumatoid arthritis (RA). The mean plasma C3e level in these samples (3.0 +/- 1.3 mg/100 ml) was significantly increased (P less than 0.001) as compared to patients with degenerative joint disease (0.9 +/- 0.4 mg/100 ml) and healthy blood donors (0.8 +/- 0.5 mg/100 ml). C3d levels were increased by more than s SD in 79% of RA samples. Plasma C3d levels were compared with C3d concentrations in synovial fluid. In most RA patients, the C3d levels were higher in synovial fluid than in plasma. A very significant correlation between plasma C3d levels and circulating immune complexes, as measured by determination of Clq binding activity (Clq BA), was observed (P less than 0.001). C3d levels were more elevated in RA patients with extra-articular disease manifestations (3.8 +/- 1.2 mg/100 ml) as compared to patients with joint disease alone (2.2 +/- 1.0 mg/100 ml). C3d levels and Clq BA were also significantly correlated (P less than 0.001) with the RA disease activity expressed by an index derived from sedimentation rate, joint score, and duration of morning stiffness. A close relationship between C3d levels, Clq BA, and the clinical activity further appeared during follow-up studies. The present observations suggest [...]

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Circulating Complement Breakdown Products in Patients with Rheumatoid Arthritis

CORRELATION BETWEEN PLASMA C3d, CIRCULATING IMMUNE COMPLEXES, AND CLINICAL ACTIVITY

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ABSTRACT Quantitative determination of the small C3 breakdown product, C3d, was used to investigate complement activation in 45 plasma samples from 30 patients with rheumatoid arthritis (RA). The mean plasma C3d level in these samples (3.0 ± 1.3 mg/100 ml) was significantly increased ($P < 0.001$) as compared to patients with degenerative joint disease (0.9 ± 0.4 mg/100 ml) and healthy blood donors (0.8 ± 0.5 mg/100 ml). C3d levels were increased by more than 2 SD in 79% of RA samples. Plasma C3d levels were compared with C3d concentrations in synovial fluid. In most RA patients, the C3d levels were higher in synovial fluid than in plasma.

A very significant correlation between plasma C3d levels and circulating immune complexes, as measured by determination of Clq binding activity (Clq BA), was observed ($P < 0.001$). C3d levels were more elevated in RA patients with extra-articular disease manifestations (3.8 ± 1.2 mg/100 ml) as compared to patients with joint disease alone (2.2 ± 1.0 mg/100 ml). C3d levels and Clq BA were also significantly correlated ($P < 0.001$) with the RA disease activity expressed by an index derived from sedimentation rate, joint score, and duration of morning stiffness. A close relationship between C3d levels, Clq BA, and the clinical activity further appeared during follow-up studies.

The present observations suggest that a parallel but rather independent activation of the complement

system may be induced by immune complexes in circulating blood and in the joint spaces during the course of rheumatoid arthritis.

INTRODUCTION

The formation of immune complexes and the activation of the complement system are known features of rheumatoid arthritis (RA).¹ Evidence of the involvement of these two factors in RA is mainly derived from studies of the joint spaces with demonstration of depressed synovial fluid complement levels and presence of immune complexes in the synovium and in synovial fluid (1-4). Moreover, patients with RA show signs of systemic disease such as nodules and vasculitis, and some serological findings further reflect the systemic character of this disease. Indeed, immune complexes have been detected by a variety of methods in the circulation of RA patients and their levels often correlated with extra-articular disease manifestations (4-6). However, the level of complement components is generally normal or increased in plasma from RA patients (1, 7, 8). This finding has been explained by an increased synthesis of complement components associated with the inflammatory syndrome in RA and masking a possibly increased catabolism (8, 9). An increased catabolism of C3 in synovial fluid and plasma during RA has been suggested in turnover studies with radiolabeled C3 and in immunochemical analysis of C3 breakdown with semiquantitative methods (9-13).

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¹Abbreviations used in this paper: Clq BA, ¹²⁵I-Clq binding activity; DJD, degenerative joint disease; ESR, erythrocyte sedimentation rate; RA, rheumatoid arthritis.

C3 is the central component of the complement system and may be activated by both the classical and the alternative pathway of complement activation. During this process, C3 is first cleaved into C3a + C3b by C3 convertase, then C3b is further cleaved by C3b inactivator. Cleavage of C3b by C3b inactivator was shown to involve two steps, probably with participation of another trypsin-like plasma enzyme, leading finally to the generation of C3c, C3d, and a so far unnamed small fragment derived from the alpha-chain of the C3 molecule (14, 15). It has been shown previously that the level of C3d in biological fluids largely reflects the extent of C3 activation (16). Thus, the quantitation of C3d levels has allowed for an estimation of complement activation in patients with systemic lupus erythematosus or glomerulonephritis (16) as well as in synovial fluids from RA patients.²

In the present study, C3d levels were measured in plasma from RA patients and from patients with degenerative joint disease; plasma levels were compared to synovial fluid levels in paired samples from RA patients. These serological studies were combined with the estimations of soluble immune complexes through the measurement of C1q binding activity, (C1q BA) (17), allowing for correlation studies between C3d and C1q BA levels in blood as well as in synovial fluid. Data were also analyzed in relation to the disease activity and to some anatomical features.

METHODS

Patients. The subjects included in this study were hospitalized or outpatients at the Geneva University Hospital. 45 serum and plasma samples and 17 synovial fluids were collected from 30 patients with RA. 15 blood and 8 synovial fluid samples were obtained sequentially from 4 of the 30 patients. Together with the synovial fluid aspirations, a paired blood specimen from the same patient was obtained within less than 1 h. For comparison, 18 paired blood and synovial fluid samples were studied from 18 patients with degenerative joint disease as defined by clinical, physical, and X-ray observation.

Patients with RA satisfied the American Rheumatism Association's criteria for classical or definite RA (18). 7 were males and 23 were females. The average age was 58 yr (range 38–77) and the mean disease duration was 8 ± 4 yr (mean \pm 1SD). Mean synovial fluid protein content as determined by the biuret method was 4.2 ± 1.8 g/liter. 21 patients had a positive serum latex fixation test for rheumatoid factor (RA+, titer equal or higher than 1/64) and 9 had a negative test (RA-). For numerical evaluation of disease activity, the percentage equivalent method was chosen (19). Activity indices were calculated considering duration of morning stiffness, joint scores, and Westergren erythrocyte sedimentation rate which were determined at the day of

² Perrin, L. H., U. E. Nydegger, R. H. Zubler, P. H. Lambert, and P. A. Miescher. 1976. Correlation between the levels of breakdown products of C3, C4, and properdin factor B in synovial fluids from patients with rheumatoid arthritis. *Arthritis Rheum.* In press.

sampling of 25 blood specimens from the patients. Anatomical staging of the disease was performed according to Steinbrocker et al. (20). To avoid any subjective interference in this investigation, the clinical team and the staff of the immunology laboratory worked independently.

Collection and storage of serum, plasma, and synovial fluids. To obtain serum, blood was allowed to clot at room temperature for 60 min. Serum was separated by centrifugation at 1,500 g for 15 min and stored at -70°C . To obtain plasma, blood was collected in tubes containing EDTA (20 mM, final concentration), centrifuged, and the supernate was immediately stored at -70°C . Synovial fluids were aspirated mostly from the knee joints and only in some cases from the shoulder or elbow joints. The samples were immediately mixed with EDTA (20 mM final concentration), centrifuged at 1500 g for 15 min, and stored at -70°C in portions of 0.5 ml.

Complement studies. These were carried out on EDTA plasma or EDTA synovial fluids. Hemolytic activity was measured in a continuous flow system (21). C1q, C4, and native C3 levels were quantitated by single radial immunodiffusion as described in (22). For measurement of native C3 and C3d, antisera were raised in rabbits by injections of purified C3 in complete Freund's adjuvant. Antisera were made specific against the main antigens of native C3 and C3d by selective absorptions with aged (10 days at 37°C) and heated (60 min at 60°C) human serum as described in detail in reference 16.

The C3 breakdown product C3d levels were quantitated in a two-step procedure (16). In the first step, native C3 and the high molecular weight fragments C3b and C3c were precipitated with polyethylene glycol. In the second step, the C3d was measured in the polyethylene glycol supernate by single radial immunodiffusion with anti-C3d antiserum. The values for CH50, C1q, and C4 were expressed in per cent of a normal plasma pool. The values of native C3 and C3d were expressed in mg/100 ml.

The ^{125}I -C1q binding test. The ^{125}I -C1q binding test was used for detection of soluble immune complexes in patients' sera and synovial fluids (5, 17). Briefly, C1q was isolated from normal human serum and labeled with ^{125}I . This preparation was then mixed with unheated test serum or EDTA synovial fluids from the patients. Free ^{125}I -C1q was separated from ^{125}I -C1q bound to complexes by precipitation with polyethylene glycol. Results were expressed as per cent ^{125}I -C1q precipitated with respect to protein-bound radioactivity (precipitable by 10% trichloroacetic acid) and are referred to as C1q BA.

Statistical evaluations. These were carried out according to Student's *t* test and Spearman's rank correlation test.

RESULTS

Complement activation in plasma and synovial fluids. The levels of the C3 breakdown product C3d were first measured in plasma samples. 45 samples came from 30 patients with RA, 18 samples from 18 patients with degenerative joint disease (DJD), and 30 samples from 30 healthy blood donors. A significant increase ($P < 0.001$) of the C3d levels in RA plasma was observed when compared with DJD and blood donors, and the mean values are listed in Table I. In 79% of the 45 RA plasma samples the C3d concentrations were increased by more than 2 SD above the mean value observed in plasma from patients

TABLE I
Circulating C3d and Immune Complexes in Patients with RA and in Controls

	Patients studied	Samples studied	C3d*	Clq
				Binding activity†
RA (total)	No. 30	No. 45	mg/100 ml 3.0±1.3§	% 22±13§
RA seropositive	21	34	3.1±1.3	24±14
RA seronegative	9	11	2.5±1.4	18±8
RA with extra-articular disease manifestations	14	23	3.8±1.2	29±13
RA without extra-articular disease manifestations	16	22	2.2±1.0	15±9
DJD	18	18	0.9±0.4	8±4
Healthy blood donors	30	30	0.8±0.5	7±2.5

* Plasma values.

† Serum values.

§ Mean±1 SD.

^{||} Subcutaneous nodules and in one patient pulmonary fibrosis.

with DJD and from blood donors. In those patients with RA, the mean plasma levels of complement hemolytic activity and of Clq were within the normal range, while the levels of native C3 were elevated (175±38 mg/100 ml, normal 135±40 mg/100 ml, mean±1 SD), and the levels of C4 slightly depressed (87±32%, normal 100±44%).

The C3d levels were also studied in 17 synovial fluid samples from RA patients and in 18 synovial fluid samples from patients with DJD. In agreement with our earlier studies (15), the C3d levels in RA synovial fluids were significantly increased as compared to DJD ($P < 0.001$). C3d levels were compared in the paired samples of plasma and synovial fluids. The C3d concentrations were higher in synovial fluids than in plasma, particularly when the C3d concentrations were expressed in milligram per gram total protein (Table

TABLE II
Comparative Analysis of C3d and Native C3 Levels in 17 Paired Plasma and Synovial Fluid Samples from RA Patients

	Plasma	Synovial fluid
C3d, mg/100 ml	3.1*±1.5	4.0±2.5
Native C3, mg/100 ml	172±24	40±16
C3d, mg/g total protein	0.45±0.21	0.95±0.51
Native C3, mg/g total protein	25±4	9.5±4.0
C3d/Native C3 ratio	0.018±0.013	0.100±0.173
Clq BA, %	29±14	38±15

* Mean±1 SD.

II). Also, the ratio C3d/native C3, which is independent of the protein concentration, was much higher in synovial fluid than in plasma. There was a significant but poor correlation between plasma and synovial fluid C3d levels ($r = 0.469, P > 0.05$) (Fig. 1). However, in three paired samples the absolute concentration of C3d was higher in plasma than in synovial fluid. Inversely, in four other paired samples low or normal C3d concentrations were observed in plasma in presence of an elevated concentration of C3d in synovial fluid.

In parallel with the complement studies the levels of immune complexes were also assessed with the ¹²⁵I-Clq binding test for determination of the Clq BA in serum and synovial fluid. Confirming our earlier observations (5), the mean Clq BA was significantly increased in both, serum and synovial fluid from RA patients ($P < 0.001$) (Table I), when compared to DJD and healthy blood donors. In 87% of the 45 RA serum samples the Clq binding activities were increased by more than 2 SD above the mean observed in blood donors.

The levels of C3d and Clq BA were compared in the blood and synovial fluid samples from RA patients (Fig. 2). With Spearman's rank correlation test, it appeared that C3d levels and Clq BA were significantly correlated in blood as well as in synovial fluids ($P < 0.001$). In the four paired samples in which low or normal C3d concentrations were observed in plasma, although the C3d levels in synovial fluid were elevated, the Clq BA was within the normal range in serum while it was increased in synovial fluid.

C3d levels and Clq BA compared to clinical and laboratory features of RA. Various laboratory and

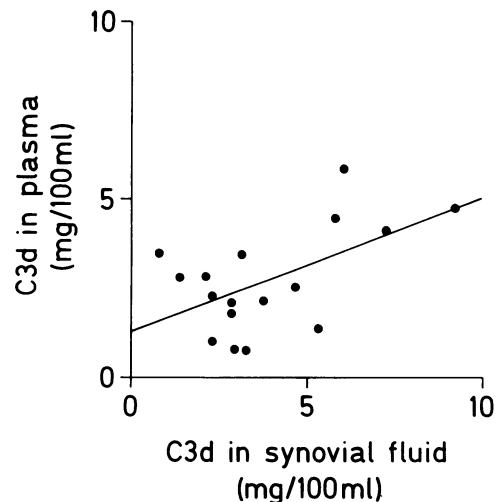


FIGURE 1 Correlation between C3d levels measured in simultaneously obtained paired plasma and synovial fluid samples from 17 patients with RA ($r = 0.469, P > 0.05$).

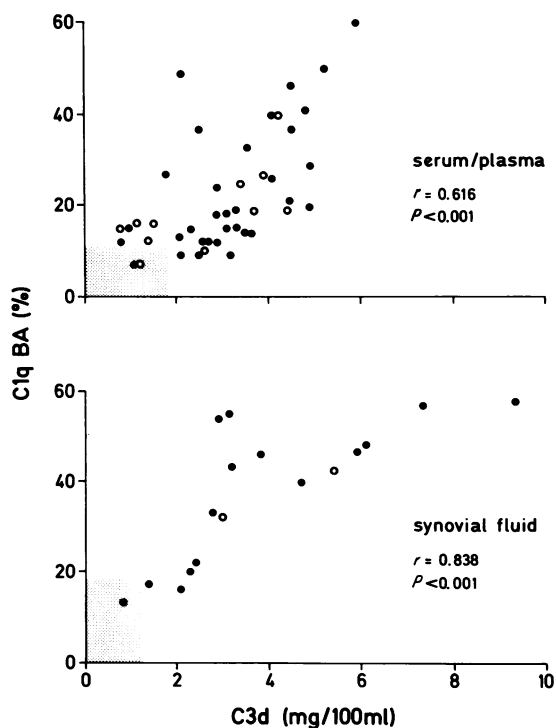


FIGURE 2 Upper part: correlation between C3d levels in plasma and ^{125}I -C1q BA in paired serum samples from patients with Seropositive RA (RA+) (●) and Seronegative RA (RA-) (○). Lower part: correlation between C3d levels and C1q-BA in individual synovial fluid samples. The shadowed areas indicate mean +2 SD limits of normal range as observed in patients with DJD and healthy blood donors.

clinical parameters of RA were used to define more precisely the patient's actual clinical condition at the time the samples for analysis of C3d and C1q BA levels were obtained (Table I). Whereas latex agglutination titer for seropositivity was not correlated with the increase of C3d and C1q BA levels ($P > 0.1$), it appeared that these levels were significantly increased ($P < 0.001$) in patients with extra-articular symptoms, mostly nodules, as compared to non-nodular RA. Disease staging was available in 24 patients with consideration of further anatomical features of RA. There was no significant difference in the mean levels of C3d and C1q BA according to the stage of the disease.

C3d and C1q BA levels were also compared to erythrocyte sedimentation rate, joint score, and duration of morning stiffness. These data were available on the day of collection of 25 blood samples from RA patients. Significant correlations were found by Spearman's rank correlation test when C3d concentrations and C1q BA were compared to joint score and morning stiffness ($r = 0.643$, $P < 0.01$ for C3d, $r = 0.602$, $P < 0.01$ for C1q BA), but not when compared to erythrocyte sedimentation rate ($r = 0.385$, $P > 0.05$

for C3d, $r = 0.400$, $P = 0.05$ for C1q BA). Correlations which were observed when C3d concentrations and C1q BA were compared to the activity index derived from all these three criteria for disease activity (19) were more significant than the correlations obtained by using the criteria separately ($r = 0.785$, $P < 0.001$ for C3d, $r = 0.695$, $P < 0.001$ for C1q BA) (Fig. 3).

The relationship of C3d levels and C1q BA to clinical activity also appeared in sequential studies performed in four patients with RA. One case report is illustrated on Fig. 4. This patient (M.H.) with nodular RA was followed, with serial bleedings and synovial fluid aspirations from the knee joint, during 5 mo. His disease was diagnosed in 1965 and was slowly progressive. This led to a particularly heavy inflammatory exacerbation at the end of February, beginning of March 1976. He was hospitalized until March 24 and immunosuppressive therapy (azathioprine, 2 mg/kg daily) was started. After leaving the hospital, he again presented a transient inflammatory exacerbation which was less intense than before and involved mainly carpal and tarsal joints. He had much improved by May 1976. C3d levels and C1q BA determinations were made at various moments of this evolution and it can be observed that these levels follow the clinical evolution with considerable parallelism.

DISCUSSION

There is increasing evidence of an involvement of immune complexes and complement activation in rheumatoid arthritis. This is supported by a variety of investigations with demonstration of immune complexes and depressed complement levels in synovial fluid. Immune complexes in the joint space have been

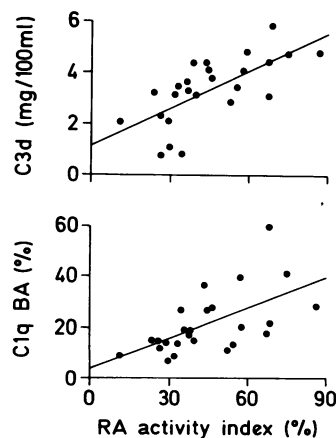


FIGURE 3 Correlation between rheumatoid activity index and C3d levels (upper part: $r = 0.785$, $P < 0.001$) and C1q-BA (lower part: $r = 0.695$, $P < 0.001$) in plasma and serum samples from RA patients.

M.H. 1914, ♂, Rheumatoid Arthritis

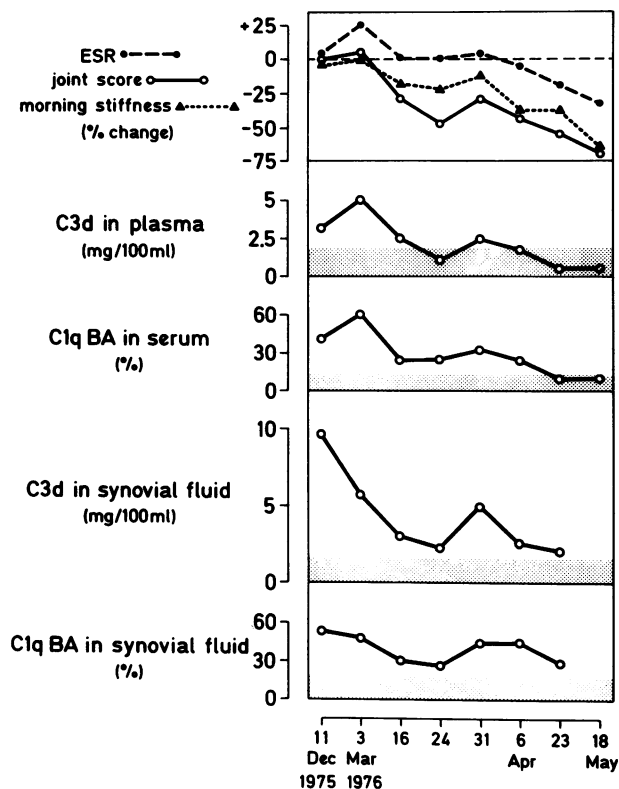


FIGURE 4 Disease course of a patient with RA during 6 mo. Periods with active disease are associated with high C3d and C1q-BA levels in plasma or serum and synovial fluid and remissions are followed by improvement of these parameters. Note inflammatory exacerbation on March 31. Shaded areas are normal range (± 2 SD) as evaluated in patients with DJD and healthy blood donors. ESR, erythrocyte sedimentation rate.

demonstrated in synovial tissue as well as in synovial fluids from patients with RA (2-4). The complement activation in RA is suggested by the finding of depressed levels and/or hemolytic activities of C1, C4, C2, C3, properdin factor B, and properdin in synovial fluids (1, 23). In the circulating blood, immune complexes have been demonstrated (4, 5) but an increased synthesis of certain complement components secondary to the inflammatory syndrome in RA often may mask a possible increase in their catabolism, therefore resulting in a normal or even augmented conventional complement profile (1, 7, 8). Indeed, with turnover studies, it was seen that complement was hypercatabolized not only in synovial fluid but also in blood from RA patients (9, 10). Further evidence of plasmatic complement utilization in RA has also come from demonstration of complement breakdown products with semiquantitative measurements (11, 13).

In the present investigation, increased C3d levels

were found in plasma from RA patients when compared to patients with DJD and healthy blood donors. The same quantitative two-step procedure has been used recently (16) for demonstration of C3 breakdown products in EDTA plasma from patients with various immunopathological diseases, and it was seen that increased C3d levels in material mixed with EDTA at the time of sampling reflects in vivo complement activation rather than an in vitro artefact. The technical procedure which was used for the quantitation of C3d does not lead to cleavage of native C3 as may occur, e.g. during two dimensional crossed immunoelectrophoresis. The present results also confirm our previous observation of an increased level of C3d in synovial fluid from RA patients.² Several hypotheses may be considered about the origin of the C3d detected in plasma from RA patients. This C3 breakdown product may be generated in the joint spaces, in the intravascular or the whole extravascular compartment, or in any combination of these three sites.

First, a local production of C3d in synovial fluid with further diffusion into the bloodstream may be suggested by the higher mean C3d level in synovial fluid as compared to the plasma concentration. This is not a general rule, since in three individual instances the synovial fluid C3d levels were lower than the plasma C3d levels. These discrepancies may result from the fact that the concentration of C3d in synovial fluid probably differs from one diseased joint to another in the same patient. Alternatively, it is possible that C3d diffusing from synovial fluid to plasma would be highly diluted in the vascular compartment and would not account for much of the measured C3d plasma level. This would agree with the observation that in four paired samples normal plasma C3d levels were observed in presence of increased synovial fluid C3d levels.

Secondly, one should consider the possibility that a complement activation occurring in circulating blood would lead to diffusion of C3d into the synovial fluid. Such a diffusion is not likely to be responsible for the increase of C3d levels in synovial fluid, since in most cases C3d levels were lower in plasma than in synovial fluid, and since the mean ratio C3d/native C3 was much higher in synovial fluid than in plasma.

Thirdly, a somewhat independent C3 activation occurring simultaneously in joint spaces and in circulating blood is also possible. This is suggested by the significant correlation between C3d levels and those of immune complexes in plasma as well as in synovial fluid which was better than the correlation between plasma and synovial fluid C3d concentrations. We think that such a parallel C3 activation seems to be the most probable mechanism. However, an extravascular activation of complement, mainly in rheuma-

tic nodules or in unrecognized lesions (6, 24) may also be an important source of C3d diffusing into plasma. Indeed, plasma C3d levels were particularly high in RA patients with extra-articular disease manifestations. Metabolic studies of C3 catabolism have shown that C3d may persist for some time in the organism and recirculate between extravascular and intravascular spaces (25). The contribution of various extravascular sites to the plasma pool of C3d is difficult to assess. The C3d concentration is hardly the same in all joints, and if the C3d were being generated in the synovial fluid or in some other extravascular site the contribution of the individual joints to the plasma pool would be expected to vary greatly from time to time and from patient to patient.

It was suggested earlier from the finding of complement depletion in rheumatoid synovial fluids and the pattern of complement utilization, that activation of the complement system in RA is in great part due to immune complexes (1). Although recent studies suggest that, in addition to the classical pathway, the alternative pathway may also be operative in joint spaces during RA (23), the importance of immune complexes in complement activation occurring in this disease is further supported by the correlation between C3d level and C1q BA in synovial fluid and in plasma. The latter finding would suggest that circulating immune complexes in RA may also trigger an activation of the complement system. In this respect our results differ from those obtained during a previous investigation which has shown that immune complexes in the serum from patients with RA do not precipitate with C1q in agarose plates (26). This may be due to a difference in the sensitivity between the C1q binding test and the C1q agarose precipitation test for the detection of soluble immune complexes.

The significance of the quantitations of C3 breakdown products and immune complexes in clinical investigation of RA patients is indicated by the analysis of the results in relation to various clinical data. There is indeed a good correlation of plasma C3d levels and C1q BA with the activity index and, more precisely, with the joint score and the duration of morning stiffness. Therefore, these new serological tests may be of some interest in the objective evaluation of the disease activity, independent of other biological criteria of RA such as erythrocyte sedimentation rate or rheumatoid factor titrations. This was also suggested in sequential studies where plasma C3d levels and C1q BA were closely associated with joint score and duration of morning stiffness. Seropositivity as evaluated by latex agglutination was not a prerequisite for C3 activation. This may be due to presence of IgG rheumatoid factor which has not been detected by this technique and confirms earlier findings showing C3 conversion in patients with seronegative RA (27).

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