

# Breakdown Products of C'3 in Human Synovial Fluids

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**ABSTRACT** Activation of the complement sequence results in conversion of the third component of complement (C'3) to an inactive product (C'3i) and the elaboration of additional fragments of smaller molecular weights and faster electrophoretic mobilities. Immunoelectrophoretic analysis of fresh synovial fluids with an anti-human C'3 antiserum disclosed in some a variable degree of conversion of C'3 to C'3i, but a more striking finding was an additional line in the  $\alpha$ -globulin region. This faster migrating protein gave a reaction of partial identity with C'3/C'3i. With this antiserum a similar pattern developed when fresh human serum was incubated with immune complexes, or aggregated  $\gamma$ -globulin. The same breakdown product of C'3 was obtained by treatment of fresh human serum with Zymosan, ammonium, hydrazine, agar, or dextran. Heating serum at 56°C for 1 hr destroys the breakdown product; aging of serum produces it.

Breakdown products of C'3 were looked for in 49 synovial fluids from patients with a variety of joint diseases. A significant correlation was found between the demonstration of the fast migrating breakdown product of C'3 and the diagnosis of rheumatoid arthritis and the presence of rheumatoid factor. A similar immunoelectrophoretic pattern was not found in the serum of any of the patients studied.

When human  $\gamma$ -globulin, which has been reduced and alkylated, is heat aggregated it loses the ability to fix human complement but still reacts with rheumatoid factor. Addition of reduced, aggregated  $\gamma$ -globulin to fresh normal human serum produced no conversion of C'3, but when incubated with serum containing a high titer of rheumatoid factor, there was conversion of C'3 and the appearance of a breakdown product. Quantitative complement fixation studies with fresh serum from normal subjects and patients with rheumatoid arthritis disclosed complement fixation by reduced, aggregated  $\gamma$ -globulin.

This study was presented in part to the Southern Society for Clinical Investigation, New Orleans, La., on 26 January 1968.

*Received for publication 25 July 1968 and in revised form 6 November 1968.*

The per cent of complement fixation was proportional to the titer of rheumatoid factor present in the test serum. These findings were interpreted as showing that rheumatoid factor can fix complement.

The possibility is discussed that the presence of breakdown products of C'3 in the synovial effusions of most patients with seropositive rheumatoid arthritis and the ability of rheumatoid factor to fix complement are related phenomena.

## INTRODUCTION

Müller-Eberhard and Nilsson have reported the presence of a protein in fresh human serum known as  $\beta$ 1C-globulin (1). They have identified it as the third component of complement (C'3). When serum is reacted with Zymosan or immune precipitates, the C'3 is converted to another protein designated as C'3i. The C'3i has a slightly faster electrophoretic mobility, slightly smaller molecular weight, and is antigenically deficient when compared with C'3. Another breakdown product of C'3 has been described by West et al. (2). It has an antigenic determinant distinct from either C'3 or C'3i and migrates as a fast  $\alpha$ -globulin. He called it  $\alpha$ 2d. This breakdown product of C'3 has been demonstrated in plasma from patients with hypocomplementemic glomerulonephritis and has been cited as evidence that C'3 participates in an in vivo immune reaction in that disease (3). The present study reports the finding of breakdown products of C'3 in the synovial fluids of patients with rheumatoid arthritis.

Hemolytic complement activity is significantly depressed in synovial effusions from most patients with rheumatoid arthritis (4). It is tempting to speculate that the low levels are due to complement consumption by an antigen-antibody reaction. A variety of substances with complement-fixing capabilities, aggregated  $\gamma$ -globulin (5), Feulgen-positive deoxyribonucleic acid ([DNA]) particles (6), antinuclear factors (7), and an unidentified cryoprecipitable protein (8) have been identified in fluids from some patients with rheumatoid arthritis. Polymorphonuclear leukocytes, the predominant cell in

rheumatoid effusions, have inclusions containing  $\gamma$ G-,  $\gamma$ M-, and  $\gamma$ A-globulins when studied by immunofluorescent techniques (9). Anti- $\gamma$ -globulin activity (presumably rheumatoid factor) has been demonstrated in the synovial fluid white blood cells from patients with seropositive rheumatoid arthritis. A correlation has been noted between the presence of inclusions and lowered joint fluid complement activity (10). This is of interest because, until recently, it has been inferred that the reaction of  $\gamma$ -globulin and rheumatoid factor does not fix complement. Direct testing of this hypothesis has not been possible, however, because aggregated  $\gamma$ -globulin is, by itself, too anticomplementary (11). This problem can be circumvented by the use of  $\gamma$ -globulin which has been reduced and alkylated before aggregation, since this form of  $\gamma$ -globulin loses the ability to fix complement while retaining the capacity to react with rheumatoid factor (12).

By use of this reagent it has been demonstrated that preformed complexes of rheumatoid factor and reduced, aggregated  $\gamma$ -globulin can fix complement at 37°C (13). Further evidence for complement fixation by rheumatoid factor is presented in this paper, and its possible role in the generation of the breakdown products of C'3 is discussed.

## METHODS

Studies were done with synovial fluids and serum or plasma samples from 49 patients. The clinical diagnosis included 24 patients with definite or classical adult rheumatoid arthritis (14), two with juvenile rheumatoid arthritis, seven with rheumatoid variants (ankylosing spondylitis, Reiter's Syndrome, or the arthritis of ulcerative colitis), two with infectious arthritis, two with crystal-induced synovitis, and six with degenerative joint disease. Immediately after joint aspiration, the fluid samples were placed on ice and centrifuged in the cold to remove leukocytes. All specimens, either serum, plasma, or synovial fluid were examined within 3 hr after collection. Immunoelectrophoresis was performed according to the micromethod of Scheidegger (15). Electrophoresis was carried out in 1% ion agar containing a barbital buffer of pH 8.6 and 0.005 M ethylenediaminetetraacetate (EDTA). For better visualization of the synovial fluid specimen, the diameter of the antigen well was increased to 3 mm, from the usual diameter of 2 mm, as suggested by West et al. (2).

*Preparation of rabbit anti-human  $\beta$ 1C-antisera.* Antibody to  $\beta$ 1C-globulin was prepared according to the method of Mardiney and Müller-Eberhard (16). 500 mg of Zymosan (Fleischmann-0B298, Standard Brands, Incorporated, New York) was boiled for 30 min in 50 ml of 0.15 M saline, centrifuged, and the pellet obtained was mixed with 11 ml of fresh normal human serum for 60 min at 37°C. The pellet was then washed six times with 0.15 M buffered saline (0.05 M phosphate, pH 7.2) and resuspended at a concentration of 10 mg of Zymosan per ml. Aliquots of 5 ml were mixed with an equal volume of incomplete Freund's adjuvant and used to immunize four rabbits. Initial injections were given into the footpads at weekly intervals for 4 wk, and then intramuscularly every other week for six additional

doses. Bleedings were taken from the marginal ear veins, pooled, and absorbed three times with human  $\gamma$ -globulin Cohn fraction II (FII), E. R. Squibb & Sons, New Brunswick, N. J.), 20 mg/ml, and then three times with human serum albumin (Pentex, Inc., Kankakee, Ill.) at 10 mg/ml. After absorption, the antisera gave a single precipitin line with fresh human serum. (When immunoelectrophoresis slides were photographed an additional line was present in the  $\gamma$ -globulin region.)

*Conversion of  $\beta$ 1C-globulin.* Conversion of  $\beta$ 1C-globulin was accomplished by reacting fresh human serum with hydrazine, ammonia, Zymosan, agar, or immune precipitates. The final concentration of  $\text{NH}_4\text{OH}$  in the serum was 0.03 moles/liter and of hydrazine 0.015 moles/liter. The specimen treated with  $\text{NH}_4\text{OH}$  was neutralized with hydrochloric acid after incubation for 90 min at 37°C. The hydrazine-treated sample was maintained at 37°C for 60 min. As a control fresh serum was treated with 0.15 M NaCl in a volume equal to that of the reagents. Zymosan was prepared as described above and suspended in saline at a concentration of 1 mg/ml. 4.05 ml of the stock solution was centrifuged, the supernatant discarded, and the pellet was resuspended into 3 ml of fresh human serum. This mixture was incubated at 37°C for 60 min. 6 mg of agar (ion agar No. 2, Oxoid, Consolidated Laboratories, Inc., Chicago Heights, Ill.) was incubated with 3 ml of serum at 37°C for 30 min. Equal amounts of sheep anti-bovine serum albumin, (generously provided by J. Barbaro, Walter Reed Army Institute of Research, Washington, D. C.) bovine serum albumin (BSA) (Pentex, Inc., Kankakee, Ill.), at 1 mg/ml, and phosphate saline were mixed. These proportions had previously been shown to be at or near equivalence for this antiserum. The mixture was maintained at 37°C for 60 min and then for 48 hr at 4°C. The precipitates which formed were washed three times with iced saline and suspended in 1 ml of saline before use. Precipitates formed in antigen excess were made in an identical manner, except that BSA was at 10 mg/ml concentration. To form antigen-antibody complexes solubilized in antigen excess, 2 ml of BSA (100 mg/ml) was added to 0.75 ml of the washed, resuspended precipitates. This mixture was incubated at 37°C for 60 min, centrifuged, and the supernatant removed and saved for further use. The preparation of rheumatoid factor-aggregated  $\gamma$ -globulin complexes and rheumatoid factor-reduced, aggregated  $\gamma$ -globulin complexes have been described (13).

In all experiments with immune precipitates or aggregated  $\gamma$ -globulin 0.25 ml of the test material was incubated with 2.0 ml of fresh human serum for 2 hr at 37°C. Controls were treated in a similar manner except that 0.15 M saline replaced the test material.

*Complement-fixation studies.* Human  $\gamma$ -globulin (Cohn fraction II, American Red Cross) at a concentration of 10 mg/ml in phosphate-buffered saline was dialysed for 3 hr at room temperature against 100 ml of 0.1 M 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, N. Y.). The reduced  $\gamma$ -globulin solution was dialysed for 4 hr against a large volume of 0.01 M iodoacetamide, (J. T. Baker Chemical Co., North Phillipsburg, N. J.) and then overnight in the cold against several changes of phosphate-buffered saline. Aggregation of  $\gamma$ -globulin, either reduced or nonreduced, was accomplished by heating at 63°C for 15-20 min. The concentration of  $\gamma$ -globulin was always adjusted to 10 mg/ml before aggregation, and in experiments utilizing lesser amounts of  $\gamma$ -globulin dilutions were made of the stock solution. After aggregation had been accomplished, proteins were measured by the Lowry method utilizing a  $\gamma$ -globulin standard with a known protein concentration

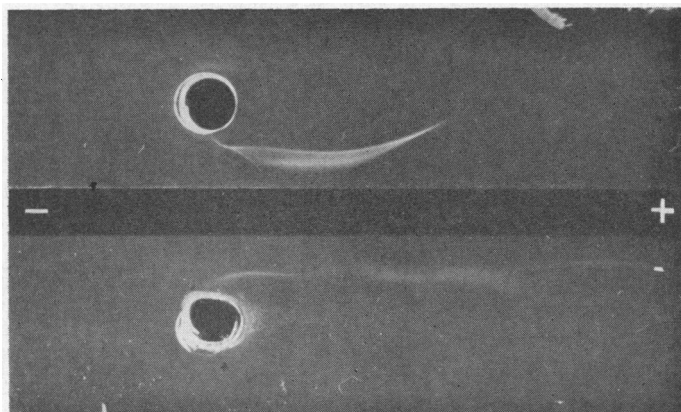


FIGURE 1 Immunoelectrophoretic patterns of fresh ethylenediaminetetraacetate (EDTA) plasma from a patient with rheumatoid arthritis (top well) and an EDTA synovial fluid from the same subject (bottom well). The trough contains an antiserum against human C<sub>3</sub>. There is partial conversion of the C<sub>3</sub> to C<sub>3</sub>i in the synovial fluid, and in addition, a more anodal precipitin arc is present. These changes are not seen in the fresh plasma obtained simultaneously.

determined by micro-Kjeldahl analysis. Quantitative complement fixation was performed by the method of Barbaro and Becker (17). Hemolysis was measured spectrophotometrically. The reaction mixture consisted of 0.5 ml of fresh human serum, 0.5 ml of aggregated  $\gamma$ -globulin, either reduced or nonreduced, and 1.0 ml of triethanolamine-buffered saline (TBS) containing optimum concentrations of metals (17). A control tube containing fresh serum, TBS, and BSA at an equal concentration to the  $\gamma$ -globulin was included with each experiment. The tubes were incubated at 37°C for 60 min. The reaction was stopped by the addition of ice-cold TBS, and the test and control tubes were centrifuged in the cold at 2000 rpm for 15 min. Residual total hemolytic complement activity was determined in the supernatants. The per cent of total complement fixed was calculated as:

$$\text{calculated as: } \frac{C'H_{50} \text{ fixed by } \gamma\text{-globulin}}{C'H_{50} \text{ fixed by bovine albumin}} \times 100.$$

Synovial fluid white blood cell counts, microscopic examination for crystalline material, and total hemolytic complement were performed as previously described (4). Quantitative analysis of  $\beta$ 1C-globulin was by radial diffusion utilizing anti- $\beta$ 1C-globulin antisera immunoplates (Hyland Laboratories, Los Angeles, Calif.). Rheumatoid factor titers were determined by the bentonite flocculation test (18). Statistical analysis of the synovial fluid results employed a fourfold ( $2 \times 2$ ) contingency table utilizing the Yates equation for 1° of freedom.

## RESULTS

Immunoelectrophoretic analysis of synovial fluids from patients with rheumatoid arthritis with an anti-human C<sub>3</sub> antiserum revealed variable degrees of conversion of C<sub>3</sub> to C<sub>3</sub>i, but a more striking finding was an additional line in the  $\alpha$ -globulin region (Fig. 1). Initially we considered this faster migrating line to be an impurity in our antiserum, but our attention was directed to the findings of West et al. which showed a similar

conversion product of  $\beta$ 1C-globulin in the serum of patients with hypocomplementemic nephritis (3). Studies were designed to demonstrate that the anti-C<sub>3</sub> antiserum used was detecting a breakdown product of C<sub>3</sub>. With this antiserum a fast migrating line developed when fresh human serum was allowed to age or when treated with antigen-antibody complexes (Fig. 2). Conversion of C<sub>3</sub> and generation of the fast fraction was also produced by incubation of fresh human serum with heat-aggregated  $\gamma$ -globulin, rheumatoid factor  $\gamma$ -globulin precipitates, agar, dextran, or Zymosan. When serum containing the fast migrating fraction was heated at 56°C for 1 hr, it was no longer detectable. These findings support the contention that the fast migrating fraction identified in the synovial effusions is a breakdown product of the third component of human complement. It differs from the  $\alpha$ 2d breakdown product described by West et al. in two particulars. In our experience hydrazine treatment caused a fast migrating arc, whereas West et al. found that when fresh serum was reacted with hydrazine or ammonia, the  $\alpha$ 2d protein had approximately the same migration as that of C<sub>3</sub>i. West et al. also found that the arcs of  $\alpha$ 2d and  $\beta$ 1A-globulin always crossed (2). This reaction of nonidentity was not seen with the antiserum used in this study.

Synovial effusions were examined from 49 patients with a variety of rheumatic disorders. These were analyzed for a correlation between the presence of a breakdown product of C<sub>3</sub> and (a) the type of joint disease; (b) occurrence of rheumatoid factor; (c) synovial fluid C<sub>3</sub> globulin concentration; and (d) total hemolytic complement activity of the effusion (Table I).

The results of the occurrence of the fast migrating fragment when compared with the diagnosis of the joint disease is shown in Table II. 16 of the 20 fluids which showed the fast migrating fragment were from patients with rheumatoid arthritis. Of the four positives listed as nonrheumatoid arthritis, one was a child with monoarticular arthritis, one with an acute infectious arthritis, another a patient with ankylosing spondylitis, and the fourth, a fluid from a patient with arthritis accompanying ulcerative colitis. Of the eight patients with rheumatoid arthritis who were negative, one was a seronegative, probable rheumatoid arthritis. Five others, with classical rheumatoid arthritis, had very low levels of complement in the synovial fluid, and C'3 lines were barely detectable. One of these patients had no fast moving fragment on two separate occasions when the synovial complement values were 9 C'H<sub>50</sub> units and less than 5 C'H<sub>50</sub> units, but the fragment was present in a third examination when the fluid complement had risen to 23 C'H<sub>50</sub> units.

Six patients with systemic lupus were studied; none had significant renal disease, and only one had a positive test for rheumatoid factor. A fast migrating fragment was not identified in any of the six effusions from the lupus patients. If the six subjects with osteoarthritis are removed from the analysis, so that rheumatoid arthritis is only compared with other forms of inflammatory arthritis, the *P* value is still < 0.01.

Fig. 3 compares the  $\beta$ 1C-globulin concentration and hemolytic complement levels from 31 synovial effusions. It is apparent that the fast moving fragment is not seen when complement levels are less than 20 C'H<sub>50</sub> units or when  $\beta$ 1C-globulin concentration is less than 0.45 mg/ml. These data suggest that a sufficient amount of complement must be present for the fast migrating fragment to be demonstrated.

An analysis of rheumatoid factor in the subject's serum and the presence of the fast migrating fragment in the synovial fluid discloses a high degree of correlation (Table III). 12 of 16 patients with a positive test for rheumatoid factor (bentonite flocculation titer = to or > 1:32) showed the fragment in their synovial effusions; four patients with seronegative rheumatoid arthritis also showed the fragment. If the six patients with degenerative joint disease are removed from the analysis, the *P* value is reduced from < 0.001 to < 0.025, still a high degree of correlation.

*Complement fixation by reduced, aggregated  $\gamma$ -globulin and serum containing rheumatoid factor.* Recently it has been shown that preformed complexes of rheumatoid factor and aggregated  $\gamma$ -globulin can fix complement (13). The suggestion of a correlation between the presence of rheumatoid factor and the demonstration of a breakdown product of  $\beta$ 1C-globulin in synovial fluids

prompted further study of this relationship. Reduced, aggregated human  $\gamma$ -globulin does not fix complement by itself but does react with rheumatoid factor (12). When aggregated, reduced  $\gamma$ -globulin is added to fresh human serum there is no generation of a fast migrating fragment, but when incubated with serum containing a

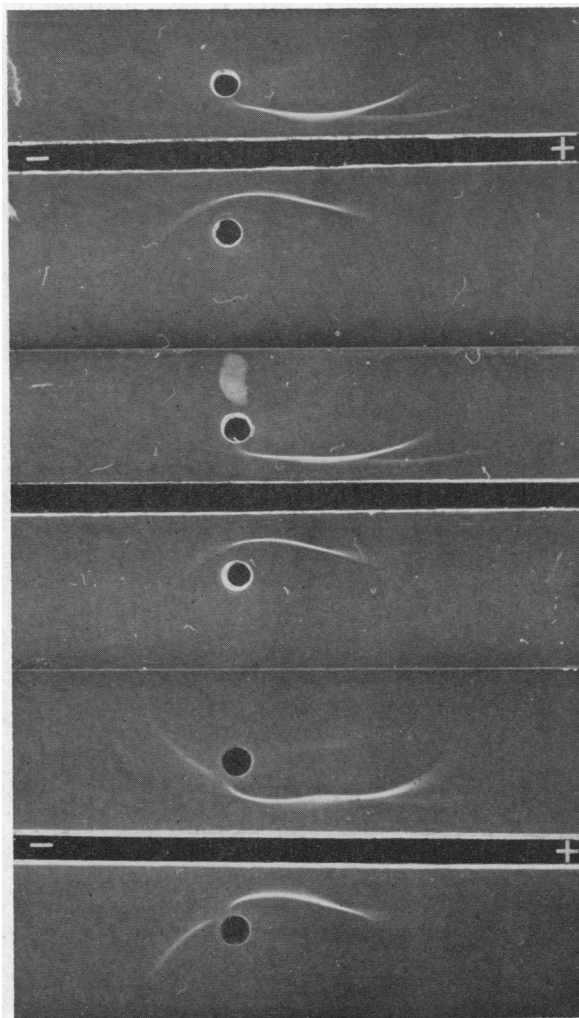


FIGURE 2 Immunoelectrophoretic patterns of fresh normal human serum treated with antigen-antibody precipitates. In each trough is an antiserum to human C'3. The well above each trough contains fresh human serum which has been incubated with the complexes, and the well below contains the same fresh human serum incubated with saline. The top pattern compares bovine serum albumin (BSA)-anti-BSA complexes made at equivalence to the control. There is conversion of C'3 to C'3i and an additional faster migrating precipitin band. The middle pattern is similar and compares BSA-anti-BSA complexes made at 10 times equivalence to the saline control. The bottom pattern is the reaction of fresh human serum incubated with rheumatoid factor-aggregated gamma globulin precipitates compared with the control.

TABLE I  
Clinical and Laboratory Data

Patient	Diagnosis*	WBC‡	C'H <sub>50</sub> ‡	β1C‡	Conversion product of C'3‡	Rheumatoid factor§
		mm <sup>3</sup>	U/ml	mg/ml		
E. P.	RA	—	45	0.50	+	128
F. McD.	RA	—	—	—	+	128
D. S.	RA	11,500	43	0.65	+	>1024
A. E.	RA	68,400	29	0.80	+	>1024
C. M.	RA	20,500	9	<0.20	0	>1024
R. S.	RA	31,400	22	0.30	0	16
M. P.	RA	14,000	22	0.57	+	0
E. G.	RA	8,500	5	<0.20	0	>1024
E. T.	RA	11,300	38	0.60	+	1024
F. W.	RA	24,600	117	1.4	+	0
C. H.	RA	—	35	0.70	+	0
J. M.	RA	34,500	53	0.70	+	>1024
V. F.	RA	7,500	10	<0.20	0	16
G. G.	RA	26,300	62	0.95	+	0
M. A.	RA	24,500	18	<0.20	0	0
B. L.	RA	41,200	<5	—	+	1024
W. Y.	RA	—	30	—	+	1024
E. G.	RA	3,150	40	—	0	1024
G. A.	RA	55,200	36	—	+	1024
W. C.	RA	20,700	44	—	+	1024
H. F.	RA	12,000	—	—	+	16
M. S.	RA	16,500	64	—	+	32
A. U.	RA	—	52	—	0	4
A. H.	RA	6,000	49	—	0	0
J. K.	JRA	8,400	57	0.65	+	0
C. H.	JRA	12,100	51	—	+	0
W. J.	SLE	—	14	0.17	0	0
T. B.	SLE	1,150	70	0.50	0	—
M. L.	SLE	5,400	50	1.1	0	0
W. P.	SLE	750	43	0.30	0	0
M. P.	SLE	2,100	22	—	0	0
E. J.	SLE	750	40	—	0	512
N. P.	AS	2,200	71	0.50	+	4
V. D.	AS	1,500	53	—	0	0
G. W.	CUC	6,800	117	0.68	+	0
R. R.	RS	9,500	86	1.2	0	0
R. B.	RS	20,000	148	—	0	0
W. McC.	Inf	12,500	90	0.80	0	0
S. E.	Inf	63,000	214	1.3	+	0
J. S.	Sarc	1,000	18	<0.20	0	64
T. S.	Gout	5,500	48	0.48	0	0
R. G.	CC	—	77	0.80	0	—
A. H.	PAN	2,650	24	—	0	0
C. M.	DJD	<100	29	0.26	0	0
A. H.	DJD	<500	65	—	0	0
E. G.	DJD	<400	81	—	0	0
J. L.	DJD	<500	59	—	0	0
L. S.	CM	1,750	53	—	0	0
D. P.	VS	7,000	69	1.0	0	0

\* RA, rheumatoid arthritis; JRA, juvenile rheumatoid arthritis; SLE, systemic lupus erythematosus; AS, ankylosing spondylitis; CUC, chronic ulcerative colitis; RS, Reiter's syndrome; Inf, infectious arthritis; Sarc, sarcoid arthritis; CC, chondrocalcinosis; PAN, periarteritis nodosa; DJD, degenerative joint disease; CM, chondromalacia; VS, villonodular synovitis.

‡ Determined in synovial fluid.

§ Determined in serum by the bentonite flocculation method (positive test is a titer of 1:32 or greater).

TABLE II  
Relation of a Conversion Product of C'3 in Synovial Fluid to the Type of Joint Disease

Source of synovial fluid	Rheumatoid arthritis	Conversion product of C'3		Fourfold contingency	
		Present	Absent	× 2	P
All joint diseases (49)	Present	16	8	11.08	<0.001
	Absent	4	21		
Only inflammatory joint diseases (43)	Present	16	8	7.7	<0.01
	Absent	4	15		

Number in parenthesis indicates number of joint fluid (also patients) studied.

high titer of rheumatoid factor, there is partial conversion of  $\beta$ 1C- to  $\beta$ 1A-globulin, and the fast moving fragment appears (Fig. 4). These findings were interpreted as showing that the interaction of reduced, aggregated  $\gamma$ -globulin with rheumatoid factor was able to activate the third component of complement.

To investigate this reaction further, total hemolytic complement fixation studies were performed. Reduced, aggregated  $\gamma$ -globulin (10 mg/ml) was added to fresh sera from a group of patients with rheumatoid arthritis. The residual hemolytic complement activity of 0.5 ml of serum was measured after a 60 min incubation with 0.5 ml of reduced, aggregated  $\gamma$ -globulin. This was compared with the residual C'H<sub>50</sub> after similar treatment with BSA (10 mg/ml). It can be seen (Fig. 5) that the per cent of complement fixed by the reduced, aggregated

$\gamma$ -globulin was proportional to the titer of the rheumatoid factor present in the serum. Little or no fixation was observed in fresh serum from normal subjects or rheumatoid patients with minimal amounts of rheumatoid factor.

Quantitative complement fixation reactions were carried out with the four rheumatoid sera which gave the greatest complement fixation. The results are shown in Fig. 6. With increasing amounts of reduced, aggregated  $\gamma$ -globulin, a greater per cent of the available complement was fixed. Fixation of 50% of the complement occurred with 0.20, 0.33, 0.62, and 0.70 mg of reduced, aggregated  $\gamma$ -globulin. An idea of the efficiency of this complement fixation reaction can be appreciated by the fact that 0.12 mg of nonreduced, aggregated  $\gamma$ -globulin is required to fix 50% of the complement in 0.5 ml of

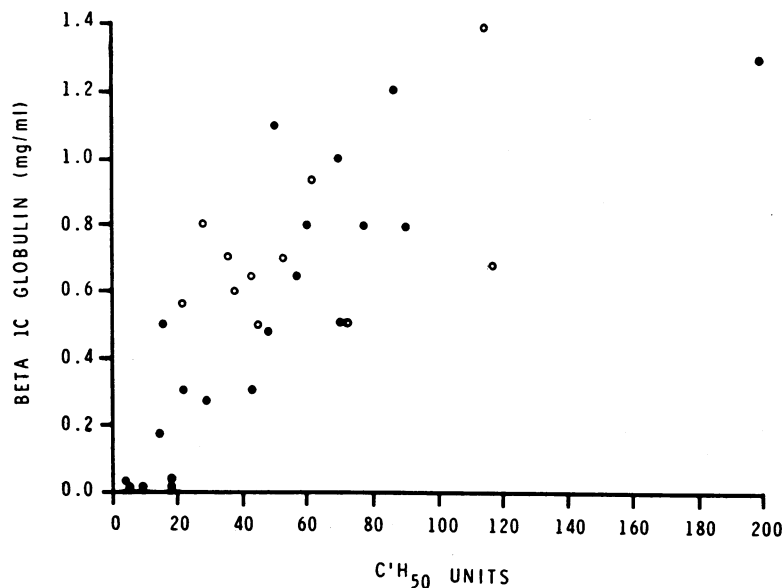


FIGURE 3 Relationship between the  $\beta$ 1C-globulin concentration and the total hemolytic complement activity of the same synovial fluid specimens. The open circles designate fluids which showed a conversion product of C'3. Each circle is the determination from a single patient.

TABLE III  
Relation of a Conversion Product of C'3 in Synovial Fluid to the Presence of Serum Rheumatoid Factor

Source of synovial fluid	Rheumatoid factor	Conversion product of C'3		Fourfold contingency	
		Present	Absent	$\times 2$	<i>P</i>
All joint diseases (47)	Positive	12	4	24.9	<0.001
	Negative	8	23		
Only inflammatory joint diseases (41)	Positive	12	4	5.5	<0.025
	Negative	8	17		

Number in parenthesis indicates number of joint fluids (also patients) studied.

normal human serum (average of seven experiments was  $0.12 \pm 0.02$  mg).

### DISCUSSION

The mechanisms postulated to explain the persistent depression of serum levels of C'3 in diseases like glomeru-

lonephritis, hypocomplementemic nephritis, and systemic lupus erythematosus include: (a) fixation to tissues, (b) decrease production, and (c) increased destruction. By immunofluorescent techniques, many workers have demonstrated C'3 (or C'3i) in the renal lesions of these diseases (19, 20). In vitro studies of the reaction mechanism of C'3 in immune hemolysis have shown that only 5–10% of the available C'3 attaches to red blood cells or antigen-antibody complexes. The remainder (90–95%) of whole serum C'3 is converted to C'3i (21). It appears likely that during in vivo complement activation C'3 is converted to C'3i in the plasma or extravascular spaces, and only a small proportion of the C'3 is fixed to tissues. Therefore, probably only a fraction of

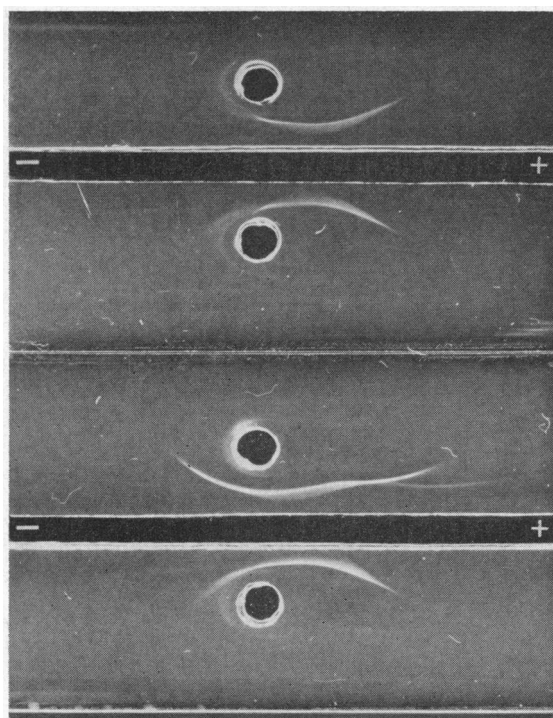


FIGURE 4 Immunoelectrophoretic patterns produced by the addition of reduced, aggregated  $\gamma$ -globulin to fresh human serum. The troughs contain an antiserum to human C'3. The well above each trough contains fresh human serum incubated with the reduced, aggregated  $\gamma$ -globulin, and the well below the same serum incubated with saline. Incubation of reduced, aggregated  $\gamma$ -globulin with fresh normal human serum (top pattern) shows no conversion of C'3. Incubation of the same concentration of reduced, aggregated  $\gamma$ -globulin with a serum containing rheumatoid factor (titer, 1:1024) results in a more anodal migration of the main arc and an additional faster migrating precipitin band (bottom pattern). No change occurs in the control serum.

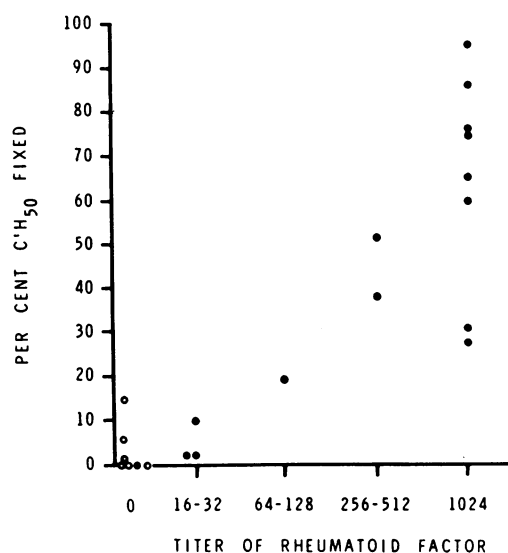


FIGURE 5 The per cent of total hemolytic complement fixed by the addition of reduced, aggregated  $\gamma$ -globulin to fresh human sera containing varying titers of rheumatoid factor. Equal quantities (0.5 ml) of fresh human serum and reduced, aggregated  $\gamma$ -globulin (10 mg/ml) were incubated together at 37°C for 60 min and the residual complement determined. The open circles designate normal sera; the closed circles are sera from patients with rheumatoid arthritis. Each point is a single determination on a different subject.

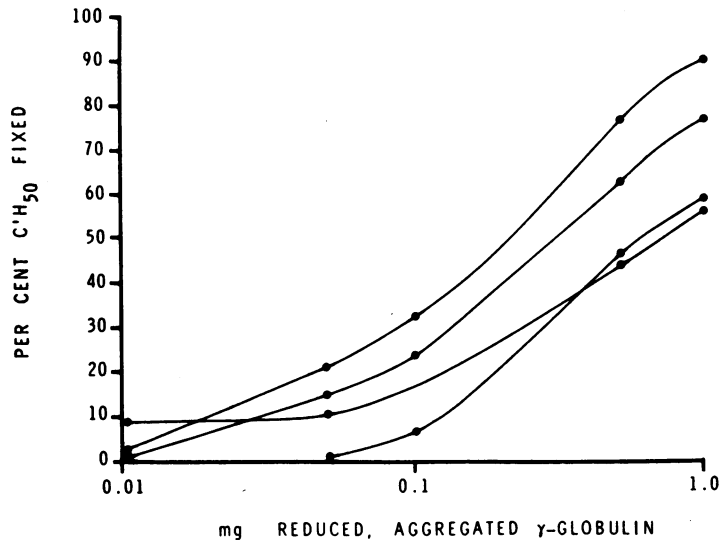


FIGURE 6 The relationship of the per cent complement fixation in four fresh human sera, containing rheumatoid factor to the amount of reduced, aggregated  $\gamma$ -globulin. Each point represents the per cent of the total hemolytic complement activity fixed in 0.5 ml of fresh human serum after incubation for 60 min at 37°C with 0.5 ml of varying concentrations of reduced, aggregated  $\gamma$ -globulin. Each line designates an individual serum containing a high titer of rheumatoid factor (bentonite flocculation test of 1:1024 or greater).

the decrease in concentration of serum C'3 is explained by attachment to tissues. In patients with low C'3, studied by Alper and Rosen, both decreased production and (or) increased destruction could be demonstrated in some patients with acute glomerulonephritis and systemic lupus erythematosus (22).

The situation in an extravascular space, like the articular cavity, is of interest since many of the variables present in renal diseases are not applicable. Hemolytic complement activity is depressed for months and even years in the joint effusions of patients with rheumatoid arthritis (4, 23). The low levels can not be explained entirely by complement binding in the synovial membrane, since immunofluorescent studies have only shown C'3 as small localized deposits, and multiple tissue sections are often required to demonstrate its presence (24, 25). Decreased production is not at fault, since the complement in inflammatory effusions appears to be derived from the serum, and serum hemolytic complement activity is normal or increased in rheumatoid arthritis (4, 23).

The finding of some conversion of C'3 to C'3i and the presence of another breakdown product of C'3 in the fluids from most patients with rheumatoid arthritis is suggestive of in vivo complement activation. Our ability to demonstrate only small amounts of C'3i, in spite of the presence of a fast migrating breakdown product of C'3, is remarkably similar to the observations of West

et al. in patients with hypocomplementemic nephritis (3). The ability to detect material with an electrophoretic mobility of a  $\beta$ 1A-globulin (C'3i) in plasma from patients with low concentrations of hemolytic complement has been variable. Morse, Müller-Eberhard, and Kunkel demonstrated a conversion product in one of nine patients with systemic lupus erythematosus (SLE) (26), and Lachmann identified a  $\beta$ 1A-globulin by immunoelectrophoresis in fresh plasma from two subjects with that disorder (27). Soothill found an altered form of  $\beta$ 1C-globulin in the fresh plasma of 15 of 16 patients with acute glomerulonephritis (with or without low levels of complement) and some patients with nephrotic syndrome due to proliferative glomerulonephritis and membranous glomerulonephritis (28). Some plasma specimens showed only material with the appearance of  $\beta$ 1A-globulin, but others had faster migrating proteins with a reaction of partial identity to  $\beta$ 1C-globulin. Alper and Rosen found no conversion products in the fresh plasma from their subjects with SLE or progressive glomerulonephritis. They found some C'3i in a few patients with acute glomerulonephritis, but only in plasma samples obtained within the first 48 hr after the onset of symptoms (22).

The reason for the lack of agreement in these various studies is not easy to ascertain. Differences in patient material and in antisera are probably significant variables. Of greater importance, however, is the demonstra-



tion that the fractional catabolic rate of C'3i is 5-10 times faster than the parent molecule (22). The conversion product is rapidly cleared from the plasma, has only a minimal urinary excretion, and is presumed to be destroyed in extravascular areas. Thus, very significant amounts of C'3 must have to be converted to C'3i to detect it in plasma. There is no information on the metabolic fate of other fast migrating fractions, but the urinary excretion of  $\alpha_2d$  is not significant (3). The demonstration in synovial fluid of a fast moving breakdown product of C'3, and only small amounts of C'3i could be explained by a differential catabolic rate for the two in this extravascular space. It is also possible that C'3i is more susceptible to the proteolytic enzymes which reside in an inflamed joint.

Treatment of C'3 with plasmin or trypsin results in the elaboration of split products with fast anodal electrophoretic migration (29, 30). Thus, it could be argued that the observed fast migrating fraction is simply a degraded protein produced by proteolytic enzymes which have been activated during joint inflammation. The fact that the fast migrating fraction was found mainly in rheumatoid effusions, but not in fluids from other articular diseases with comparable degrees of inflammation, makes this a less likely explanation.

An antigen-antibody reaction in the articular cavity has been proposed as one explanation for the decreased hemolytic complement activity in rheumatoid synovial effusions (4). Even admitting that the demonstration of a breakdown product of C'3 in some synovial fluids is evidence for in vivo activation of complement, it does not identify the initial immunologic reaction. The correlation noted of the fast moving fragment and rheumatoid factor activity suggests one possible candidate. Rheumatoid factor is present in the serum and synovial fluid of the majority of patients with rheumatoid arthritis (31). Hedberg reports an excellent correlation between the titer of rheumatoid factor and the depression of synovial fluid hemolytic activity (23). Aggregated  $\gamma$ -globulin has been identified in some rheumatoid effusions (5). It would therefore be attractive to suggest that the interaction of aggregated  $\gamma$ -globulin (or antigen-antibody precipitates) with rheumatoid factor is the complement-fixing reaction which is responsible for the in vivo complement activation in the rheumatoid joint.

A number of studies have analyzed the influence of rheumatoid factor on complement fixation. In all of them, isolated rheumatoid factor, rheumatoid euglobulin preparations, or heat-inactivated serum containing rheumatoid factor has been shown to compete with complement for binding sites on  $\gamma$ -globulin (32-36). Wiedermann, Miescher, and Frankin demonstrated that when 7S human antibodies or heat-aggregated  $\gamma$ -globulin were reduced with 2-mercaptoethanol they lost their ability

to fix complement while retaining the capacity to react with rheumatoid factor. They concluded from this that the sites on  $\gamma$ -globulin which fix complement and react with rheumatoid factor are separate, but possibly close together. They suggested that rheumatoid factor could interfere with complement fixation by steric hindrance or by producing conformational changes in the  $\gamma$ -globulin molecule (12). From all of these studies it has been inferred that the reaction between rheumatoid factor and aggregated  $\gamma$ -globulin does not fix complement. This hypothesis could not be tested directly, however, because aggregated  $\gamma$ -globulin was too anticomplementary by itself (11). Such an analysis is possible, however, if  $\gamma$ -globulin is reduced with 2-mercaptoethanol before aggregation; for reduced, aggregated  $\gamma$ -globulin retains the ability to react with rheumatoid factor but can no longer fix complement (13). Therefore, any complement fixation observed must result from the interaction of the reduced, aggregated  $\gamma$ -globulin and rheumatoid factor. Utilizing this system it has been demonstrated that preformed complexes of rheumatoid factor and reduced, aggregated  $\gamma$ -globulin can fix human complement at 37°C. No fixation of either human or guinea pig complement was observed when aggregated, reduced  $\gamma$ -globulin was added to heat-inactivated sera with various titers of rheumatoid factor (13). This finding conflicts with the results in the present study, which showed significant complement fixation when reduced, aggregated  $\gamma$ -globulin was added to fresh serum containing rheumatoid factor. In this system, the test serum serves both as the source of complement and of rheumatoid factor.

Several reasons can be proposed to explain the discrepancy, but none are entirely satisfactory. Preformed complexes of rheumatoid factor and aggregated, reduced  $\gamma$ -globulin fix human complement but not guinea pig complement (13). It may be that this reaction is most efficient with autologous complement. There is some evidence showing that human 19S antibody fixes human complement better than guinea pig complement, but there is no information comparing autologous to homologous complement fixation (37). The changes produced when human serum is heated to destroy complement activity may be important.  $\gamma$ -globulin is susceptible to aggregation at temperatures above 37°C (38). Epstein and Ross demonstrated that when undiluted human serum containing rheumatoid factor was heated at 56°C for 30 min, there was a significant decrease in its ability to form a precipitate on the addition of pooled human FII (39). These data were interpreted as showing that a portion of the rheumatoid factor activity was heat labile. An alternative explanation might be that some  $\gamma$ -globulin in the serum was aggregated and combined with rheumatoid factor before the addition of FII. Thus, in the process of deplementing rheumatoid serum

by heating at 56°C,  $\gamma$ -globulin may be aggregated and reacts with, or covers up, the complement fixing sites on the rheumatoid factor molecule. The subsequent introduction of reduced, aggregated  $\gamma$ -globulin would then not demonstrate complement fixation.

Since reduced  $\gamma$ -globulin does not occur naturally, one can question the relevance of these in vitro observations to the situation in patients with rheumatoid arthritis. Nevertheless, rheumatoid factor does have the potential of fixing human complement. It remains to be seen whether conditions in the rheumatoid joint are appropriate to utilize this ability. Only then will it be possible to state that the presence of a breakdown product of C'3 in the synovial effusions of most patients with seropositive rheumatoid arthritis, and the ability of rheumatoid factor to fix complement, are related phenomena.

### ACKNOWLEDGMENTS

The author wishes to acknowledge the expert technical assistance of Mrs. Jennifer Robinson, Mrs. Annelore Serenyi, and Miss Maria Martinez. Each contributed in significant fashion to some aspect of the study. Secretarial aid was provided by Miss Patricia McKaig.

Support for these studies was provided by the U. S. Public Health Service (AM 5042 and 5140), and some patients utilized the facilities of the Georgetown University Clinical Study Unit (FR-60).

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