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Research Article

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Sorbitol density gradient studies on whole synovial fluid before and after removal of this activity has shown that the complement-fixing activity migrates in the 19S and heavier regions and that portion is removed with cryoprecipitation. Cryoproteins found in nonrheumatoid synovial fluid are generally devoid of complement-fixing activity and predominantly contain fibrinogen. DNA and IgG are also present, with IgG occurring significantly less frequently than in rheumatoid cryoproteins. These findings are discussed in relationship to recent studies demonstrating the presence of complement-fixing antibody to denatured DNA in rheumatoid cryoproteins.

The Occurrence of Cryoproteins in Synovial Fluid; the Association of a Complement-Fixing Activity in Rheumatoid Synovial Fluid with Cold-Precipitable Protein

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Abstract A significant portion of a complement-fixing activity found in the synovial fluid of patients with rheumatoid arthritis, and previously implicated as a possible cause of the low synovial fluid complement levels in these patients behaves as a high solubility cryoprotein. Analysis of rheumatoid synovial fluid cryoproteins has revealed mixed immunoglobulins, bound complement components, fibrinogen, DNA, and rheumatoid factor.

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INTRODUCTION

The occurrence of cryoproteins in the sera of patients with diverse disease states is widely recognized, and has been the subject of numerous detailed studies (1–4) and reviews (5, 6). Analysis of serum cryoproteins generally has revealed a single protein moiety possessing the property of cold insolubility; or a mixture of two or more immunoglobulins, one possessing rheumatoid factor like activity. However, the occurrence of cryoproteins in synovial fluid has not been reported previously.

During studies on a complement-fixing activity in rheumatoid synovial fluid (7), it was noted that the greater portion of this activity as detected in whole synovial fluid was cold precipitable (8). These complement-fixing synovial fluid cryoproteins appear in some ways similar to the mixed cryoproteins occurring in the serum of patients with SLE, most recently studied by Christian, Hatfield, and Chase (9) and Hanauer and Christian (10) in that they are composed of multiple immunoglobulin types, bound complement components, and are associated with depressed supernatant fluid complement levels.

Cryoproteoins have been found in nonrheumatoid synovial fluids also, but with rare exception have been devoid of complement-fixing activity, and unassociated with depressed synovial fluid complement levels. In this paper, studies of the composition, sedimentation characteristics, and complement-fixing activity of synovial fluid cryoproteins are presented.

METHODS

Selection of patients. Patients were selected on the basis of availability of sufficient synovial fluid for arthrocentesis. Patients with rheumatoid arthritis satisfied the American Rheumatism Association's criteria for classical or definite rheumatoid arthritis (11), with the exception of one patient, W. H., who had juvenile rheumatoid arthritis. Nonrheumatoid patients satisfied generally accepted diagnostic criteria.

Collection and storage of specimens. Under aseptic conditions and local lidocaine anesthesia, synovial fluid was aspirated from the knee or shoulder, in the absence of anticoagulant, and placed in clean glass or plastic containers. A small aliquot was processed immediately for whole complement determination, while the remaining fluid was placed at 37°C for 2 hr. The fluid was then centrifuged at 15,000 *g*, 37°C, for 15 min, and the resultant supernatant assayed immediately for cryoproteins, or aliquots placed in vials,

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and shell frozen in a mixture of dry ice and 2-methoxyethanol, and stored at −70°C for studies at a later date. This procedure failed to generate additional complement-fixing activity, and usually fluids were free of precipitate on rapid thawing at 37°C.

Whole complement titration.[1] Complement determinations were performed on synovial fluid using a 7.5 ml reaction volume and under the conditions described in (12). Veronal-buffered saline, pH 7.4, was prepared as described. Gelatin, $CaCl_2$, and $MgSO_4$ were added to final concentrations of 0.1%, 0.15 mmole/liter, and 1 mmole/liter, respectively (Gel VB). The titer expressed in terms of CH_{50} units/ml is defined as the reciprocal of that dilution of synovial fluid, 1 ml of which will produce 50% hemolysis of the standardized red cell suspension.

Titration of C1. The first component of human complement (C1) was titrated using a modification (13) of the technique of Becker (14), employing guinea pig complement components.

Complement-fixing activity. Assays for complement-fixing activity were performed on whole synovial fluid, unwashed cryoprecipitate, and residual supernate as follows. 1 ml of synovial fluid was placed in a $\frac{1}{2} \times 2$ inch centrifuge tube, and kept at 0°C for 18 hr. The cryoprecipitate which formed was then resuspended, and a 0.1 ml aliquot removed for assay of whole fluid complement-fixing activity, and placed in a tube containing 0.4 ml of Gel VB. The 0.9 ml residual was then centrifuged at 0°–4°C, 15,000 *g*, for 15 min. The supernatant fluid was then removed, the tube wall wiped free of residual supernate, and the precipitate resuspended without washing in 0.9 ml of Gel VB. Precipitate resuspension was aided by a small motor with an eccentric rubber tip attached to its shaft. Aliquots of supernate and precipitate, 0.1 ml, were added to separate tubes containing 0.4 ml of Gel VB. 2 ml of an appropriate dilution of human complement containing a total of 7–8 CH_{50} units were added to all three tubes, and appropriate control tubes. The tubes were incubated for 1 hr at 37°C, following which 10 ml of Gel VB was added, and the residual complement titrated in the 7.5 ml reaction volume described above. Residual complement was calculated, and the results subtracted from the complement control, yielding the amount fixed by the test material. Results are expressed in terms of per cent complement fixed by 0.1 ml of test material, unless otherwise indicated.

Washing of synovial fluid cryoprecipitates. In initial assays not presented, washing of synovial fluid cryoprecipitates with isotonic buffer pH 7.4 resulted in loss of large and variable amounts of complement-fixing activity up to 63% of that originally present in the cryoprecipitate. In some fluids with small amounts of complement-fixing activity all of the activity was lost with washing. It was found that this loss could be decreased by washing in a low ionic strength buffer. Accordingly, a washing procedure consisting of two washes of cold (0°–4°C) 0.02 M, pH 7.4 Veronal buffer followed by final resuspension in 0.02 M, pH 7.4 Veronal-buffered saline, was employed. The wash volume was one-half the initial sample volume; and the tube wall was wiped dry between washes. At final resuspension, the cryoprecipitate was concentrated four times with respect to initial sample volume. This allowed the increased sensitivity necessary for some of the chemical determinations. The precipitates and supernates were then stored in aliquots at −70°C for chemical and immunochemical determinations at a later date. When it was noted that small amounts of complement-fixing activity were generated during the washing procedure in fluids devoid of this activity in the first resuspension, it was decided to perform this assay on unwashed precipitates as indicated above. Cryoprecipitate data presented are with respect to the amount present in whole synovial fluid. Chemical and immunochemical determinations on washed cryoprecipitates do not reflect the exact amount of material initially present, as variable amounts of aggregation and solubilization have been observed during the washing process.

Chemical determinations. Micromethod adaptations of standard procedures were performed using a Coleman Junior II spectrophotometer,[2] equipped with an Ultramicro adapter[3] (modified) and cell assembly,[4] and micropipettes[5] prepared and calibrated in our laboratory to an accuracy of within 1% of their stated value. Protein was determined by a modification of the Folin-Ciocalteau procedure (15). Whole human serum subjected to Kjeldahl nitrogen analysis served as a protein standard, using a factor of 6.25 to obtain the protein equivalent. DNA was determined by a modification of the diphenylamine reaction (16).

Immunochemical determinations. Antisera to human immunoglobulins initially were obtained commercially,[6] and later were prepared in rabbits (footpad immunization), using complete Freund's adjuvant and purified immunoglobulins prepared by the method of Fahey, McCoy, and Goulian for IgG (17), a modification of the method of Vaerman, Heremans, and Vaerman for IgM (18), and an unpublished method for IgA, from a serum pool of over 100 normal donors. All antisera were absorbed according to standard procedures. Antisera to β1C/β1A (C3) and β1E (C4) were kindly supplied by Dr. Hans Müller-Eberhard. Antiserum to fibrinogen and α_2-macroglobulin were obtained commercially.[7] All antisera were monospecific for their respective antigens. Antisera to washed unfractionated rheumatoid cryoprecipitates were prepared as above using 0.1 mg protein per rabbit per injection. Antisera to whole human serum and synovial fluid were prepared using complete Freund's adjuvant followed by intravenous immunization (19).

Immunoelectrophoresis was performed by the method of Scheidegger (20), using Ionagar in Veronal buffer, ionic strength 0.035, pH 8.2. Gel diffusion by the method of Ouchterlony was performed on 1×3 inch microscope slides using 0.5% agarose in Veronal-buffered saline, pH 7.4 containing 0.1% sodium azide. Molten agarose was filtered before use and 2.0 ml applied per slide. Wells 1.5 mm in diameter, holding 2.5 μl of antigen and separated from adjacent wells by 3 mm were cut in a linear fashion. Antiserum troughs 1 mm in width were cut parallel to the antigen wells, separated by a diffusion distance of 3 mm. Generally three rows of 15 antigen wells separated by two antiserum troughs were used per slide. Reactants were allowed to diffuse at room temperature, read at 24 and 48 hr, washed,

[1] The complement terminology used herein conforms with that published in the *Bull. World Health Organ.* 1968, **39:** 935.

[2] Model 6/20, Coleman Instrument Co., Maywood, Ill.

[3] No. 6-802, Coleman Instrument Co.

[4] No. 6-803, Coleman Instrument Co.

[5] Spinco Division, Beckman Instrument Corp., Palo Alto, Calif.

[6] Hyland Laboratories, Los Angeles, Calif.

[7] Behringwerke (Hoechst Pharmaceuticals) Cincinnati, Ohio.

TABLE I
Analysis of Rheumatoid

Patient	Date	Whole fluid: Complement titer (CH_{50} units)	Whole fluid: Complement fixing activity‡	Cryoprecipitate*: Protein	Cryoprecipitate*: Complement fixing activity‡	Immunoglobulins: IgG	IgM	IgA
				mg/ml				
1. R. C.	102566	<2	35%	0.066	46%	+	0	0
2. G. M.	102566	7.4	0	0.095	9%	+	+	0
	113066	10	0	0.075	2%	+	0	0
3. J. W.	R 102666§	3.3	12%	0.125	6%	+	0	0
	R 020167	5.0	7%	0.210	5%	+	+	0
	L 113066	5.8	14%	0.153	10%	+	0	0
4. L. M.	R 102666	7.9	0	0.068	0	0	0	0
	L 020167	9.2	0	0.205	0	+	+	0
5. M. R.	R 110966	4.2	0	0.230	0	+	0	0
	L 110966	2.6	0	0.340	1%	+	0	0
6. I. W.	111466	4.0	13%	0.125	19%	+	0	0
7. W. H.‖	111966	6.4	7%	0.410	10%	+	+	0
8. H. B.	112266	10.1	0	0.140	3%	0	0	0
9. G. S.	113066	9.0	10%	0.048	8%	0	0	0
10. L. B.	112366	2.1	46%	1.288	33%	+	+	+
11. W. B.	R 120566	3.1	35%	0.330	24%	+	+	+
	R 010467	3.4	36%	0.285	23%	+	+	+
	R 010967	2.8	35%	0.118	22%	+	+	0
	L 020167	1.6	26%	0.365	26%	+	+	+
	L 031367	4.1	33%	0.416	25%	+	+	0
	L 033167	<1.6	41%	0.610	35%	+	+	+
	L 041367	4.3	15%	0.110	11%	+	+	0
12. J. T.	R 030967		55%	0.305	30%	+	0	0
	L 030967	3.0	46%	0.328	24%	+	0	0
13. M. F.	011167	9.0	4%	0.110	6%	+	+	0
14. M. S.	032067	3.4	38%	0.400	29%	+	0	0
15. G. W.	041067	2.5	0	0.235	3%	+	0	0
16. A. D.	050367	3.0	69%	1.370	65%	+	+	+
17. E. P.	030967	17.2	0	0.140	0	+	0	0

* Assays for complement-fixing activity were performed on resuspended unwashed cryoprecipitates. All other chemical and immunochemical determinations sere performed on washed cryoprecipitates.
‡ Per cent of 7–8 CH_{50} units fixed by 0.1 ml.
§ Prefix R and L indicate right and left knee joints respectively.
‖ Juvenile rheumatoid arthritis.

dried, and stained. For the increased sensitivity required for the detection of trace amounts of immunoglobulin in the heaviest sedimenting portion of the density gradient studies, 6 mm diameter antigen wells (holding 40 μl) arranged in a circular fashion and separated from a 4 mm diameter center antiserum well (holding 15 μl) by a diffusion distance of 3.5 mm were used.

DNA was determined qualitatively by the method of Tan and Kunkel (21). Calf thymus DNA, used for identity reactions in gel diffusion studies, was obtained commercially.[8] The SLE serum (J. H.) used as a source of precipitating antibody to DNA in these studies had 0.089 mg abN/ml, by quantitative precipitin analysis with native DNA.

Rheumatoid factor was determined by the method of Singer and Plotz (22). A micromethod employing one-tenth the volume and 5×50 mm glass tubes was used for the density gradient studies.

Density gradient studies. All sucrose preparations examined, including a purified density gradient grade, contained nondialyzable dextran contaminants, confirming previous observations (23). These contaminants reacted with all normal human and guinea pig sera tested, activating the complement sequence and invalidating complement-fixation studies. After testing several sugars, sorbitol was found to be most satisfactory as a sucrose subtitute with respect to noninterference with the complement sequence, although it must be removed by dialysis before protein and DNA determinations. Synovial fluid density gradient analysis was performed using a 10–80% (wt/vol) gradient, in isotonic Veronal-buffered saline pH 7.4. Gradient solutions 10, 45,

[8] Mann Laboratories, New York. Cat. No. 873, Lot S-4494.

Cryoprecipitate*						Supernate				
DNA	Rheumatoid factor	Fibrinogen	β1C	β1E	α_2-Macroglobulin	Protein	Complement-fixing activity‡	DNA	Rheumatoid factor	Serum, rheumatoid factor
mg/ml						*mg/ml*		*mg/ml*		
0.002	<20	0	0	0	0	50.9	20%	0.020	320	2560
0.002	20	0	0	+	0	43.9	0	0.008	640	640
0.002	20	0	0	0	0	49.9	0	0.009	640	
0.002	20	+	0	+	+	50.0	6%	0.009	1280	1280
0.002	40	+	0	+	0	42.2	0	0.009		
0	<20	0	0	0	+	42.2	4%	0.009		
0.002	<20	0	0	0	0	49.0	0	0.009	320	640
0	<20	+	0	0	0	51.0	0	0.008	<20	
0.010	<20	+	0	0	0	34.7	0	0.012	<20	<20
0.012	<20	+	0	0	0	31.0	0	0.014	<20	
0.003	<20	0	0	0	0	35.7	1%	0.015	640	1280
0.026	<20	+	0	+	+	57.0	0	0.054	<20	<20
0.002	<20	0	0	0	0	39.0	0	0.009	<20	<20
0	20	0	0	0	0	48.4	0	0.018	1280	640
0.076	160	+	0	+	+	47.7	21%	0.075	2560	—
0.006	40	+	0	+	+	37.8	11%	0.021	2560	10,240
0.002	40	+	0	+	+	43.5	8%	0.018	5120	
0	40	+	0	+	+	41.8	9%	0.017	2560	
0.017	160	+	0	+	+	45.9	0	0.110	2560	
0.007	80	+	0	+	+	38.8	6%	0.025	2560	
0.079	80	+	0	+	+	42.2	1%	0.058	2560	
0	20	0	0	0	0	37.2	0	0.013	5120	
0.002	20	0	0	0	0	33.5	20%	0.009	2560	10,240
0.009	20	0	0	0	0	30.4	20%	0.043	2560	
0	40	0	0	+	+	41.8	0	0.018	1280	2560
0.004	<20	0	0	0	0	41.2	7%	0.051	2560	2560
0.003	<20	+	0	0	0	47.1	4%	0.019	640	1280
0.110	40	+	0	+	+	50.9	21%	0.126	320	1280
0	<20	0	0	0	+	36.0	0	0.016	<20	<20

and 80% were prepared in volumetric flasks using dry sorbitol and stock Veronal-saline buffer concentrate to assure uniform ionic strength throughout the gradient. Into a ½ × 2 inch centrifuge tube 1.4 ml of each gradient solution was layered, and the gradient "cut" twice with a platinum wire. The gradient was allowed to stand 6 hr at 30°C following which 0.5 ml of sample was layered on top. Centrifugation was performed in a Spinco SW 39 head, 35,000 rpm, at 30°C for 4 hr. The tube bottoms were then punctured with a 27 gauge needle and 0.5 ml fractions collected. Rrefractometry revealed a linear gradient in fractions 2 through 7. Samples were dialyzed against Veronal-saline buffer (0.005 M Veronal) pH 7.4 for 8 hr at 30°C, following which they were assayed immediately for complement-fixing activity.

RESULTS

Incidence and quantitation. The occurrence of cryoproteins in rheumatoid and nonrheumatoid synovial fluids is shown in Tables I and II respectively. Cryoproteins were found in all of 29 rheumatoid synovial fluids with a mean protein value of 300 μg/ml; and in 10/12 nonrheumatoid synovial fluids with a mean protein value of 187 μg/ml. Using a t test for nonpaired experiments, the differences in protein content between rheumatoid and nonrheumatoid cryoproteins is not statistically significant with a P value <0.10 and >0.05. However, these cryoproteins are quite dissimilar in their properties and protein composition. Cryoproteins were associated

TABLE II
Analysis of Nonrheumatoid

Patient	Diagnosis	Date	Whole fluid: Complement titer (CH_{50} units)	Whole fluid: Complement-fixing activity‡	Cryoprecipitate*: Protein§	Cryoprecipitate*: Complement-fixing activity‡
					mg/ml	
1. E. F.	Pseudogout	102466	6.5	0	0.040	0
		102666	7.4	0	0.055	0
2. R. J.	Gout	041467	9.0	0	0.260	0
3. R. C.	Osteoarthritis	122866	15.8	0	0.225	0
4. G. W.	Undiagnosed monoarthritis	032267	20.4	0	0	0
5. C. M.	Pyogenic Pneumococcal	033167	6.8	0	0.302	3%
6. J. N.	Pyogenic Streptococcal	041167	14.3	0	0	0
7. O. R.	Undiganosed Polyarthritis	041967	18.9	0	0.040	0
8. W. R.	Acute Polyarthritis	041567	19.2	0	0.045	0
9. E. L.	Pseudogout	041769	3	0	0.125	0
10. W. S.	Spondylitis (with knee involvement)	031567	17.8	0	0.085	0
		033067	21.7	0	0.697	0

* Assays for complement-fixing activity were performed on resuspended unwashed cryoprecipitates. All other chemical and immunochrmical determinations were performed on washed cryoprecipitates.
‡ Per cent of 7–8 CH_{50} units fixed by 0.1 ml.
§ 0.040 mg/ml was the lower limit of sensitivity for the amount of material used for protein determination on these samples.

with complement-fixing activity detectable in whole synovial fluids in 20/29 rheumatoid fluids, while examination of the isolated cryoproteins revealed complement-fixing activity in 25/29. Cryoprecipitation of complement-fixing activity occurs rapidly at 0°C (Fig. 1) with the greater portion of this activity, in some fluids all of this activity, being recovered in the cryoprecipitate (Fig. 2). In striking contrast, all nonrheumatoid cryoproteins were unassociated with whole fluid complement-fixing activity. Only 1/10 isolated nonrheumatoid cryoproteins possessed complement-fixing activity, this in a fluid from a patient recovering from pneumococcal pyarthrosis.

Immunoglobulins. In Ouchterlony analysis using specific antisera, immunoglobulins were detected in 26/29 rheumatoid cryoprecipitates (Table I). 12 were composed of IgG alone, 8 IgG and IgM, while 6 contained IgG, IgM, and IgA. Immunoglobulins were undetectable in three at the concentrations checked. In many fluids, the use of specific immunoglobulin antisera in Ouchterlony analysis revealed the presence of immunoglobulins undetectable by potent rabbit anti–whole human serum in immunoelectrophoretic analysis. Immunoglobulin was detected in 4/10 nonrheumatoid cryoprecipitates (Table II). In every instance it was IgG alone. IgG when present in the nonrheumatoid cryoprecipitates generally gave a weak precipitin band. Using the chi square test, the difference with respect to frequency of occurrence of IgG in rheumatoid versus nonrheumatoid cryoprecipitates is significant, with a P value < 0.01.

Identification of complement components by hemolytic assay. Following washing, the first component of complement (C1) was detected in five of six nonrheumatoid and all of twelve rheumatoid cryoprecipitates tested. In an attempt to relate the cryoprecipitate-associated complement-fixing activity to the consumption of C1, the per cent of total synovial fluid C1 which was cryoprecipitate bound is plotted (ordinate) as a function of whole complement-fixing activity by that cryoprecipitate (abscissa) (Fig. 3). In general, the greater the complement-fixing activity, the greater was the per cent of total synovial fluid C1 which was cryoprotein bound. In nonrheumatoid fluids, usually less than 1% of total C1 was cryoprotein bound.

Identification of complement components by gel diffusion. Cryoprotein-bound β1E determinants were present by Ouchterlony analysis in 13/29 rheumatoid cryoprecipitates, and absent in all 10 nonrheumatoid cryoprecipitates. β1C/β1A determinants were absent in both the rheumatoid and nonrheumatoid cryoprecipitates (Tables I and II). However, rabbits immunized with two rheumatoid cryoprecipitates showed precipitating antibody to β1C/β1A, demonstrating the presence of trace amounts of this component.

Identification of fibrinogen and other proteins. Fibrinogen was present in 15/29 rheumatoid, and 8/10 non-

Cryoprecipitate*									Supernate			
Immunoglobulins												
IgG	IgM	IgA	DNA	Rheumatoid factor	Fibrinogen	β1C	β1E	α_2-Macroglobulin	Protein	Complement-fixing activity‡	DNA	Rheumatoid factor
			mg/ml						*mg/ml*		*mg/ml*	
+	0	0	0.003	<20	+	0	0	0	39.8	0	0.015	<20
0	0	0	0	<20	+	0	0	0	32.2	0	0.015	<20
+	0	0	0.022	<20	+	0	0	0	52.1	0	0.119	<20
0	0	0	0	<20	+	0	0	0	27.3	0	0.010	<20
0	0	0	0	<20	+	0	0	0	39.0	0	0.017	<20
+	0	0	0.004	<20	+	0	0	+	43.4	0	0.228	<20
0	0	0	0	<20	0	0	0	0	33.5	0	0.025	<20
0	0	0	0	<20	0	0	0	0	52.1	0	0.048	<20
0	0	0	0	<20	+	0	0	0	52.1	0	0.024	<20
0	0	0	0.004	<20	+	0	0	0	36.5	0	0.016	<20
0	0	0	0.005	<20	0	0	0	0	60.2	0	0.043	<20
+	0	0	0.045	<20	+	0	0	+	36.0	0	0.073	<20

rheumatoid cryoprecipitates by Ouchterlony analysis. In 4/10 nonrheumatoid cryoprecipitates, fibrinogen was the only protein detectable immunochemically. In immunoelectrophoresis a cathodically faster moving, apparent fibrinogen fragment, showing identity with fibrinogen was observed in both rheumatoid and nonrheumatoid cryoprecipitates. This faster moving fragment is similar to that noted in whole synovial fluid by Schur and Sandson (24). By Ouchterlony analysis α_2-macroglobulin was present in 13/29 rheumatoid and 2/10 nonrheumatoid cryoprecipitates. At least two additional as yet unidentified proteins were present in the rheumatoid cryoprecipitates.

Identification of DNA. DNA was present chemically in 22/29, and immunochemically in 24/29 rheumatoid cryoprecipitates (Table I). Patients W. B. 010967 and M. F. showed trace amounts of DNA immunochemically which was not detected chemically. A typical immunochemical determination is seen in Fig. 4. In the nonrheumatoid cryoprecipitates, there was absolute agreement between chemical and immunochemical determinations, DNA being detected in 6/10 cryoprecipitates (Table II). In spite of a low ionic strength wash, considerable amounts of DNA were lost with washing, up to 65% of that initially present. Accordingly cryoprecipitate DNA values represent only a portion of that initially present.

DNA was present by chemical determination in all of 29 rheumatoid and 12 nonrheumatoid synovial fluid supernates (Tables I and II). However, in 1 of the 29 rheumatoid (W. B. 041367), and 2 of the 12 nonrheumatoid supernates (R. C. and G. W.) DNA was absent immunochemically. The finding of diphenylamine-reactive material in synovial fluid in the absence of DNA detectable by precipitin analysis, is in agreement with the findings of Tan, Schur, Carr, and Kunkel for serum (16). However, the values of 13, 10, and 17 μg/ml for the three fluids are in excess of the serum values of 1–3 μg/ml which those authors reported. In view of the range of variation, and since some synovial fluid supernates possessing as little as 8 or 9 μg/ml diphenylamine-reactive material gave positive precipitin reactions with anti-DNA, supernatant values have been left uncorrected.

Rheumatoid factor. Rheumatoid factor was present in the serum of 12/17 patients with synovial fluid cryoproteins, and was detectable in 17/29 washed rheumatoid cryoproteins. Nonrheumatoid patients lacked rheumatoid factor in their serum and were devoid of rheumatoid factor in their synovial fluid cryoprecipitates.

Density gradient studies. Density gradient ultracentrifugation of a rheumatoid and inflammatory nonrheumatoid (pseudogout) synovial fluid are shown in Fig. 5. In this assay, rheumatoid factor was determined qualitatively by precipitation of heat-aggregated gamma globulin in agarose gel, and 0.2 ml fractions were analyzed

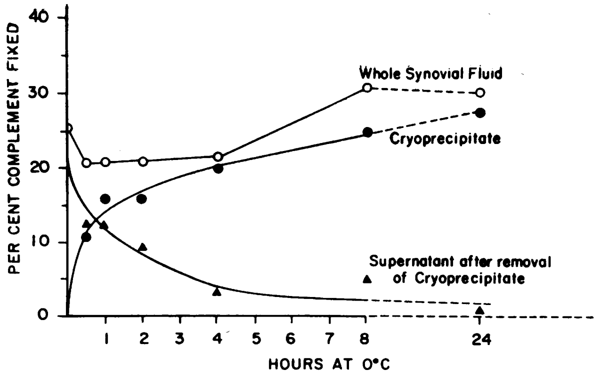

FIGURE 1 The kinetics of cryoprecipitation at 0°C, of rheumatoid synovial fluid complement-fixing activity (subject W. B.), relative to complement-fixing activities of whole fluid, cryoprecipitate, and supernate after removal of cryoprecipitate. At 0 time six tubes, each containing 1.0 ml of synovial fluid, were placed at 0°C, and synovial fluid in a seventh tube was analyzed for complement-fixing activity. At the indicated time, tubes were removed from the refrigerated bath, and whole fluid, supernate, and precipitate analyzed for complement-fixing activity.

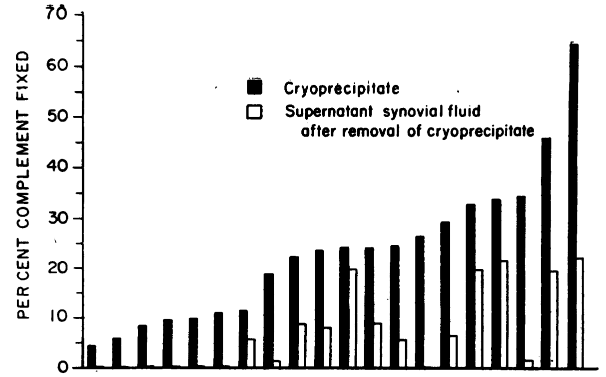

FIGURE 2 The distribution of complement-fixing activity in 20 rheumatoid synovial fluids after 18 hr at 0°C. Ordinate is per cent of 7–8 CH_{50} units fixed by 0.1 ml of cryoprecipitate or supernate.

for complement-fixing activity. Ouchterlony analysis employing small antigen wells failed to detect immunoglobulins in fractions 1–3. Complement-fixing activity sediments in the 19S and heavier regions.

Density gradient ultracentrifugation of a rheumatoid synovial fluid before and after cryoprecipitate removal is seen in Fig. 6. Samples were heated at 56°C for 30 min before application to the gradient to prevent the appearance of an artifactual heat-labile anticomplementary activity in the 19S region, possibly related to activated C1. Heating failed to generate significant anticomplementary activity in normal serum and nonrheumatoid synovial fluid. The position of peak IgG and IgM activity in normal serum in a third tube centrifuged simultaneously is indicated. In the synovial fluid, rheumatoid factor was detected throughout the gradient, with trace activity being detected in the lowermost fractions, and peak activity in fraction 5. Rheumatoid factor was detected in normal serum in the position of fraction 5, at a titer of 1/8 the lowest dilution tested. No correlation of complement-fixing activity and rheumatoid factor is observed. It is apparent that cryoprecipitation removes a portion of the heavy sedimenting complement-fixing activity. However, as indicated by protein determination, lighter than 19S material is removed as well. Application of the method of Martin and Ames (25) to this as-

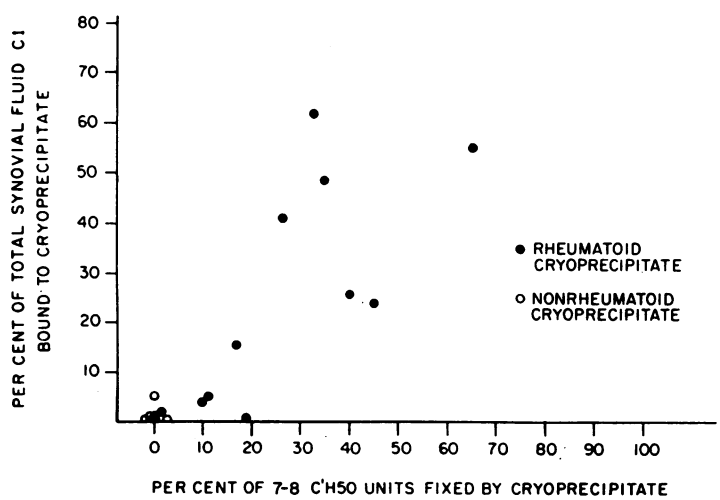

FIGURE 3 The per cent of total synovial fluid C1 which is cryoprecipitate bound (ordinate) in 12 rheumatoid (●) and 6 nonrheumatoid (○) cryoprecipitates, as a function of complement-fixing activity by that precipitate (abscissa).

say, yields an S value of approximately 43S for the heaviest sedimenting fraction.

A difference in terms of complement-fixing activity is observed if one compares whole fluid complement-fixing activity with the same fluid subjected to density gradient analysis (Fig. 6). Examination of noncentrifuged synovial fluid, and extrapolating to a 0.5 ml sample, revealed that a total of 7.3 CH_{50} units were fixed by the sample before cryoprecipitate removal, and 1.4 CH_{50} units after cryoprecipitate removal, reflecting removal of 81% of the complement-fixing activity with the cryoprecipitate. Following density gradient centrifugation of the cryoprecipitate-containing fluid, the sum of the amount of complement fixed by all nine fractions was 9.5 CH_{50} units, an increase of 2.2 U over the noncentrifuged sample. In contrast, in the fluid free of cryoprecipitate, the sum of the amount of complement fixed by all nine fractions was 6.7 CH_{50} units, an increase of 5.3 CH_{50} units over the noncentrifuged sample. This increase in complement-fixing activity following cryoprecipitate removal and ultracentrifugation suggests that the reactants in the complement-fixing process are in a dynamic rather than static state and that in native whole synovial fluid

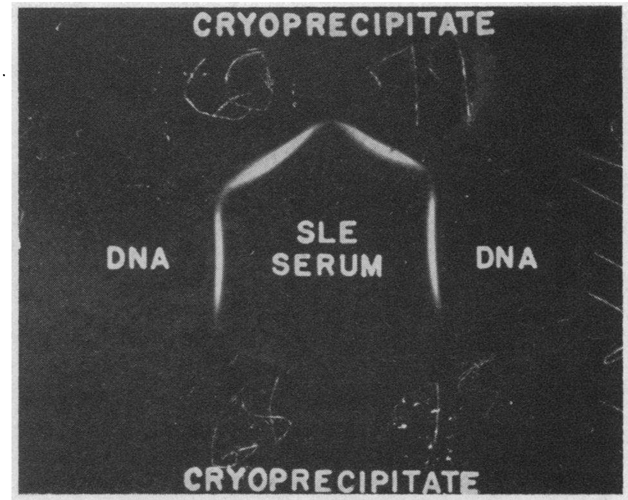

FIGURE 4 The presence of DNA in rheumatoid synovial fluid cryoprecipitates. Center well contains SLE serum with precipitating antibody to DNA. Upper and lower wells contain typical rheumatoid synovial fluid cryoprecipitates. Identity reactions seen best with the upper cryoprecipitates, are observed with native calf thymus DNA (1 mg/ml) in the side wells.

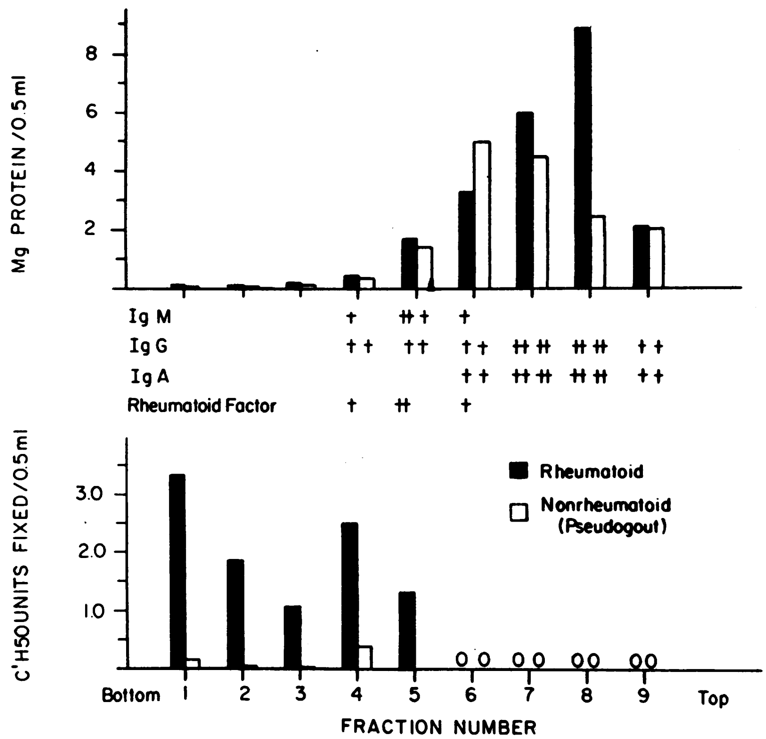

FIGURE 5 Density gradient ultracentrifugation of a rheumatoid (black bar) (W. B.), and inflammatory nonrheumatoid (white bar) (E. F.) synovial fluid. Following ultracentrifugation of 0.5 ml samples, 4 hr at 30°C, in 10–80% sorbitol, 0.5 ml fractions were collected and analyzed for complement-fixing activity (lower graph); immunoglobulins and rheumatoid factor (middle graph); and protein (upper graph).

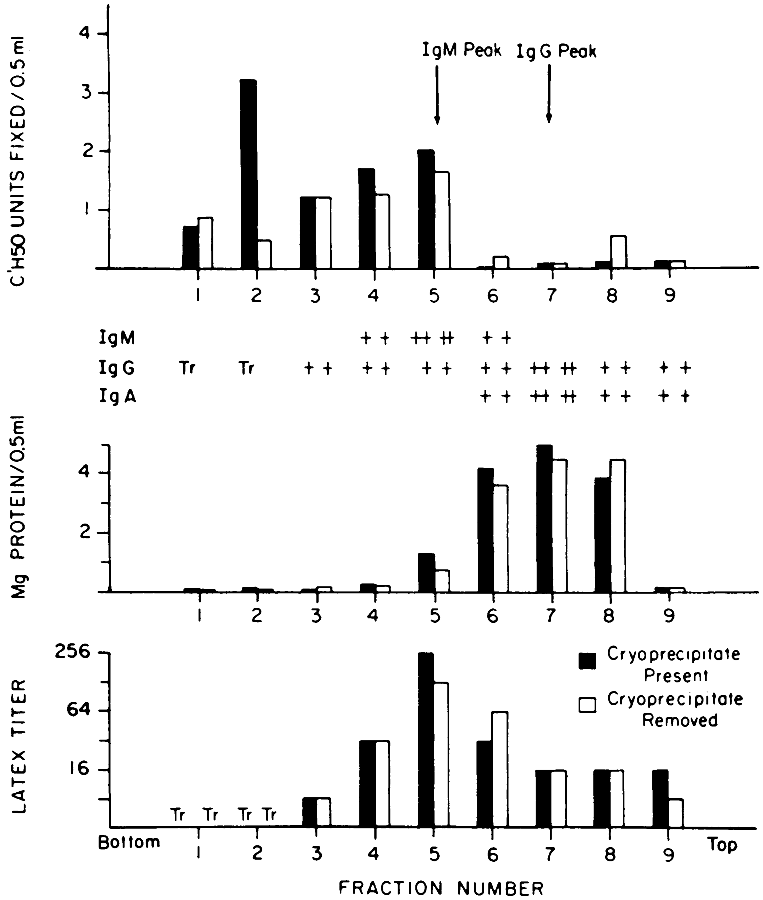

FIGURE 6 Density gradient ultracentrifugation of a rheumatoid synovial fluid (W. B) with cryoprecipitable protein present (black bar), and removed (white bar); under conditions as presented in Fig. 5. Trace rheumatoid factor activity was detected at a concentration of 1/8 in fractions 1 and 2. The position of peak IgM and IgG concentration in normal serum centrifuged simultaneously in a third tube is indicated by arrows in the upper graph.

the degree of interaction of these reactants may not be optimal for maximum complement fixation. Further, this increase may merely by due to the effect of reaction product removal, reflecting the law of mass action; although other possibilities, such as removal from presently undefined inhibitors, or noncomplement-fixing blocking antibody, must be considered. It should be noted (Fig. 6) that although IgG could be detected throughout the gradient with peak activity in fraction 7, it was the only immunoglobulin detected in the bottom fractions 1, 2, and 3.

DISCUSSION

Cryoprecipitation of small amounts of protein from synovial fluid has been demonstrated in these studies to occur in the majority of inflammatory fluids examined. The cryoproteins in rheumatoid synovial fluid resemble the cryoglobulins detected in serum of patients with disorders associated with circulatory immune complexes such as SLE. They contain in many instances mixed immunoglobulins, rheumatoid factor, DNA, and bound complement components, in addition to fibrinogen, α_2-macroglobulin, and other unidentified proteins. In contrast, cryoproteins from nonrheumatoid synovial fluid consist largely of fibrinogen, small amounts of IgG, and DNA.

The most striking difference in the cryoproteins in rheumatoid and nonrheumatoid synovial fluids relates to their capacity for complement fixation. Rheumatoid cryoprecipitates characteristically demonstrate complement fixation whereas nonrheumatoid cryoproteins do not. In fact, a significant portion of the complement-fixing activity in rheumatoid synovial fluid is demonstrated here to be cold precipitable. Since complement fixation has been shown in earlier studies to occur immediately after aspiration of rheumatoid synovial (7), cryoprecipitation per se must be considered a secondary phenomenon, unnecessary for complement fixation and reflecting the formation of larger, cold-insoluble aggregates from those already present in synovial fluid. The kinetic assay of cryoprecipitation (Fig. 1) further demonstrates this point. Density gradient studies (Fig. 6) confirm the presence of a heavy sedimenting fraction in rheumatoid synovial fluid which contains the complement-fixing activity.

It has been possible to utilize cryoprecipitation to separate the complement-fixing complex from the masking effect of complement components in whole synovial fluid, allowing the detection of small amounts of activity undetectable in whole synovial fluid. This was noted in one nonrheumatoid and five rheumatoid synovial fluids in the present study. Subsequently, using larger amounts of cryoprotein, it has been possible to demonstrate complement-fixing activity in cryoproteins from 90% of rheumatoid synovial fluids.

As demonstrated in Fig. 2, the greater portion of the complement-fixing activity detected in whole fluid is cold precipitable. However, the density gradient studies (Fig. 6) indicate that significant additional complement-fixing activity can be detected following synovial fluid fractionation. Although the manifold factors possibly controlling this increase have already been mentioned, the prime reason for its increase would appear to be the continued further aggregation of IgG either by itself, or in combination with specific antigen.[9] We have observed similar increases in complement-fixing activity accompanying manipulation of properly collected "anticomplementary" sera from patients with SLE and other disease states. It should be emphasized that care has been taken to exclude both a nonspecific, heavy-sedimenting, heat-labile anticomplementary activity present in normal serum and some synovial fluids which becomes manifest on ultracentrifugation, and the use of sucrose which contains nondialyzable dextran contaminants capable of activating the complement sequence.

Within the limits of methods used to detect immunoglobulins, IgG was the sole immunoglobulin in almost 50% of the rheumatoid cryoproteins. This suggests that a mixed immunoglobulin system is unnecessary for complement fixation and cryoprecipitation.

It is as yet unclear whether the C1 and C4 detected in the rheumatoid cryoproteins were bound to the complement-fixing complex in their soluble form at the time of aspiration, or whether they were fixed during the processing. Christian, Hatfield, and Chase have shown that C1q is required for precipitation of mixed cryoproteins in SLE serum (9). However, the bound C1 which we have detected using cell intermediates reflects a different portion of the C1 complex, mitigating valid comparison at the present time.

[9] Manuscript in preparation.

The association of fibrin or its breakdown products with rheumatoid synovial inflammation has been previously noted (26). A report that treatment of normal plasma with thrombin generates cryofibrinogen is most pertinent (27), since altered fibrinogen may be implicated as a single component of both the rheumatoid and nonrheumatoid cryoprecipitates with cold-insoluble properties. The finding that 40% of the nonrheumatoid cryoprecipitates devoid of complement-fixing activity contained fibrinogen alone, suggests that a portion of the fibrinogen in both the rheumatoid and nonrheumatoid cryoprecipitates is present independent of the other components, and unrelated to complement-fixing activity. The role of α_2-macroglobulin in synovial fluid cryoproteins, previously noted by Hanauer and Christian in SLE serum cryoproteins (10), remains unknown.

On the basis of experimental studies which have demonstrated the capacity of antigen antibody complexes and gamma globulin aggregates to produce tissue injury and inflammation (28, 29), and the liberation of biologically active fragments from C3 and C5 (30–32), one may presume that the complement-fixing complex described in these studies plays a role in producing some of the inflammatory manifestations of rheumatoid disease. Just how large a role is uncertain since the genesis of this complex in vivo is not clear. Whether these complexes are concerned with the basic pathogenesis of rheumatoid synovitis or represent in some way a product of the inflammatory process is a question of major importance. The striking difference with respect to composition and activity of the cryoproteins in rheumatoid and nonrheumatoid synovial effusions provides some evidence against a nonspecific response to inflammation, but more direct evidence is required.

If one assumes that the C1 and C4 present in the complex were bound in vivo, the finding of relatively little bound C3 suggests that following fixation of C2 and C3, the complex undergoes rapid phagocytosis, in a manner analogous to that described by Gigli and Nelson for the antibody-coated sheep erythrocyte (33). Accordingly one would expect to find more complex-bound C3 intracytoplasmically in the leukocyte than on complex free in the synovial fluid. It is apparent that a portion of the contents of intracytoplasmic inclusions in rheumatoid synovial fluid leukocytes noted by Hollander, McCarty, Astorga, and Castro-Murillo (34) and Vaughan, Barnett, Sobel, and Jacox (35) may represent the postphagocytic phase of the complex described herein. However, since the exact fate of this large complex in vivo is unknown and since leukocyte inclusions undoubtedly contain other materials, attempts at drawing a strict analogy between rheumatoid and nonrheumatoid leukocyte inclusions and cryoprecipitable proteins should be avoided.

The presence of DNA in all synovial fluids examined, and its detection in both rheumatoid and nonrheumatoid cryoprecipitates suggests that it may be a nonspecific component, there because of entrapment or nonspecific interaction with a protein moiety. The precipitation by DNA of both gamma globulin at acid pH (36), and C1q at alkaline pH (37), illustrate the propensity of DNA for ionic and apparent nonspecific interaction. Alternately, a portion of the DNA in the cryoprecipitate may be specifically combined with immunoglobulin as an antigen-antibody complex. This would be in agreement with observations of the occurrence of antinuclear factors in rheumatoid synovial fluid (38), and would support the hypothesis of Zvaifler that altered nuclear material could represent a potential antigen in rheumatoid synovial inflammation (39). It should again be emphasized that considerable amounts of both DNA and complement-fixing activity are lost with washing, and that the chemical and immunochemical determinations presented on the washed cryoprecipitates represent an exceedingly rough approximation of the materials initially present. In an attempt to clarify the role of DNA in these complexes, studies of inhibition of rheumatoid synovial fluid complement fixation have implicated a site or sites on denatured DNA as an antigenic determinant in these complexes (40). Subsequently, using a direct approach, it has been possible to dissociate partially the cryoprotein complex in a 15% sodium chloride, sorbitol gradient. Both IgG, and IgG- and IgM-containing fractions which contain variable amounts of DNA, have demonstrated the capacity for increased complement fixation upon addition of denatured calf thymus DNA. No significant complement fixation has been obtained to date with native DNA.[9] The role of rheumatoid factor in modifying complement fixation by this complex, the detection of antibody to denatured DNA in nonrheumatoid cryoprecipitates, and the role of other potential antigens must be clarified by additional studies.

The relationship between the complement-fixing cryoproteins described in these studies and synovial fluid IgG globulin aggregates described by Hannestad (41) and studied in further detail by Winchester, Agnello, and Kunkel (42), and soluble antigen-antibody complexes described by Baumal and Broder (43) is not entirely clear at the present time. It would appear that synovial fluid cryoproteins and the associated complement-fixing activity of rheumatoid cryoproteins per se, are distinct from the aforementioned, since these authors stored their fluid at 4°C, and Baumal and Broder removed cryoprecipitable material before assay (44). Nevertheless, there is a strong indication that we are dealing, at least in part, with the same activity, since a portion of the complement-fixing activity is associated with IgG sedimenting heavier than 19S, and cryoprecipitation effects only partial removal. However, the detection methods differ with regard to specificity, those of Hannestad (41), and Baumal and Broder (43) detect aggregates or soluble antigen-antibody complexes respectively, composed of IgG only; while our assay system utilizing complement fixation as described detects IgM and certain IgG antigen-antibody complexes or aggregrates, and possibly antigen-antibody-complement intermediates. This, plus the finding of DNA and specific antibody in association with the cryoprecipitable portion of this activity, suggests that any conclusive statement with regard to identity of these synovial fluid factors be deferred pending additional studies.

Note added in proof. After submission of this manuscript, a report on the occurrence of cryoproteins in synovial fluid was published (Barnett, E. V., R. Bluestone, A. Cracchiolo, L. S. Goldberg, G. L. Kantor, and R. M. McIntosh. 1970. Cryoglobulinemia and disease. *Ann. Intern. Med.* **73**: 95.).

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