Immune reactants in cryoproteins

Relationship to complement activation

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SUMMARY Cryoproteins were isolated from the serum of 5 patients with essential cryoglobulinaemia, 5 patients with rheumatoid arthritis, and 2 patients with Sjögren's syndrome. These cryoprecipitates contained IgG, IgM, and IgA as well as complement proteins C1q, C4, C3, and factor B. The cryoprecipitates were analysed further for content of antibody and antigen, and were tested for their ability to activate complement. In the cryoprecipitates of 2 patients with Sjögren's syndrome, nuclear antigen and antinuclear antibody characteristic of an immunological specificity found in Sjögren's syndrome were shown. The cryoprecipitates of 6 other patients contained rheumatoid factor and antibody to a lymphocyte nuclear antigen.

The solubilized cryoprecipitates were tested by *in vitro* assays for their ability to activate complement by the classical or alternative pathways. All 12 cryoprecipitates activated the classical pathway. 9 of the 12 cryoprecipitates also activated the alternative complement pathway under conditions which did not involve activation of C1 and C4. These studies show that a high percentage of cryoprecipitates consist at least in part of immune reactants. We discuss the relationship of these findings to pathogenetic mechanisms in disease.

Although proteins that precipitate from serum in the cold were first observed in multiple myeloma (Wintrobe and Buell, 1933), cryoproteins have since been detected in the sera of patients with many other diseases (Franklin, 1971; Brouet *et al.*, 1974). Cryoproteins of patients with autoimmune diseases such as systemic lupus erythematosus (SLE) have been shown to contain antigen-antibody complexes and to be rich in antibodies to nuclear ribonucleoprotein, single-stranded DNA (ss-DNA), and double stranded DNA (ds-DNA) (Winfield *et al.*, 1975). Cryoproteins have also been detected in patients with viral hepatitis and infectious mononucleosis (Kaplan, 1968; Kohler *et al.*, 1974). Hepatitis B antigen and antibody have been found in the proteins of some patients with extrahepatic manifestations of acute viral hepatitis such as arthritis and nephritis (Kohler *et al.*, 1974; Wands *et al.*, 1975). These data suggest that the phenomenon of serum cryoprecipitation may be related to the presence of immune complexes in the serum.

An association has been found between the presence of cryoproteins and decreased serum levels of complement (Hanauer and Christian, 1967; Stastny and Ziff, 1969). Several different studies suggest that complement activation occurs in patients with cryoproteinaemia. Complement proteins of the classical pathway, C1q and C4, have been detected in the cryoprecipitates of patients with SLE (Hanauer and Christian, 1967), and C4, C3, and C5 in the cryoprecipitates of patients with hepatitis (Wands *et al.*, 1975). Cryoprecipitates have also been shown to activate complement by *in vitro* assays (Wands *et al.*, 1975; Muller *et al.*, 1976). Stastny and Ziff (1969) reported that the isolated cryoprecipitates of a patient with SLE and of a patient with rheumatoid arthritis (RA) consumed complement proteins *in vitro*. Tesar *et al.* (1973) have described activation of the alternative complement pathway by mixed cryoglobulins from the synovial fluid and sera of patients with RA.

We studied the cryoprecipitates of patients with essential monoclonal and mixed cryoglobulinaemia, as well as cryoproteinaemia associated with RA and Sjögren's syndrome. We also studied the ability of these cryoprecipitates to activate complement by the classical or alternative complement

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pathways. We found that the cryoprecipitates of patients with Sjögren's syndrome contained nuclear antigen and antinuclear antibody characteristic of an immunological specificity found in Sjögren's syndrome (Alspaugh and Tan, 1975). A number of cryoprecipitates contained rheumatoid factor and an antibody to a lymphocyte antigen which has recently been described (Alspaugh *et al.*, 1976). All 12 cryoprecipitates activated the classical complement pathway in *in vitro* assays, and 9 also activated the alternative pathway independently.

Materials and methods

PATIENTS

We studied 5 patients with essential cryoglobulinaemia, 5 patients with cryoglobulinaemia associated with RA, and 2 patients with Sjögren's syndrome. 4 were male and 8 were female, ages ranging from 48 to 72 years. Clinical data are summarized in Table 1. 5 patients (Cases 3–7) had essential cryoglobulinaemia and purpura of the type described by Meltzer *et al.* (1966). 3 (Cases 3, 4, 5) had renal disease shown by microscopical haematuria and 1–2 g urinary protein/24 hours. Percutaneous renal biopsy of Case 3 showed proliferative glomerulonephritis.

Five patients with RA (Cases 8–12) met the criteria of the American Rheumatism Association for classical or definite RA (Ropes *et al.*, 1958). One had purpura, and 2 had peripheral neuropathy. The 2 patients with Sjögren's syndrome (Cases 1, 2) had keratoconjunctivitis sicca, xerostomia, positive Schirmer's test, and lip biopsies consistent with Sjögren's syndrome. One of these patients (Case 1) had purpura, arthralgia, and renal disease, microscopical haematuria, and 3–5 g urinary protein/24 hours. Percutaneous renal biopsy showed acute

proliferative glomerulonephritis. The second patient (Case 2) had a myocardiopathy with congestive heart failure. All 7 patients with purpura (5 with essential cryoglobulinaemia, 1 with RA, and 1 Sjögren's) had skin biopsies consistent with vasculitis showing infiltration of the dermal vessels with polymorphonuclear neutrophils.

COLLECTION OF SERA AND ISOLATION OF CRYOPRECIPITATE

Blood was withdrawn from patients into warmed sterile glass tubes and allowed to clot at 37°C in a water bath. Serum was separated from the clot by centrifugation at 1500 *g* for 10 minutes at 37°C. 3 ml serum were kept at 4°C for 72 hours. The precipitates which formed were isolated by centrifugation and washed three times with 3 ml cold 0.05 M phosphate buffer (pH 7.4), and were solubilized with constant stirring in 3 ml 0.01 M phosphate buffered 0.15 M sodium chloride (PBS) (pH 7.4) at 37°C for one hour. The isolated cryoprecipitates showed no reaction in double immunodiffusion against antihuman serum albumin, showing no detectable coprecipitation of serum proteins. To detect activation of the classical and alternative complement pathways, the cryoprecipitates were prepared from fresh serum or serum that had been stored at –70°C. Protein concentration in the cryoprecipitates was measured by the Lowry method using a standard protein curve from Cohn fraction II (Lowry *et al.*, 1951).

ANALYSIS OF CRYOPRECIPITATES

Immunoglobulins in the isolated cryoprecipitate were analysed by the Ouchterlony technique (0.6% agarose in PBS) with monospecific antisera to human IgG, IgM, IgA, κ and λ light chains (Meloy Laboratories, Springfield, Va.). Dr. Hans

Table 1 Clinical data and analysis of cryoprecipitates of patients

Case no.	Age	Sex	Cryoprecipitates						Clinical features
			Amount (mg/ml)	Immunoglobulin	C1q	C4	C3	Factor B	
1	62	F	0.80	IgG, IgM	+	+	+	+	Sjögren's syndrome, purpura, arthralgia, nephritis
2	64	F	0.50	IgG, IgM	–*	+	–	+	Sjögren's syndrome, myocardiopathy
3	53	M	0.80	IgG, IgM	+	+	–	+	Purpura, arthralgia, nephritis
4	54	F	1.00	IgG, IgM	+	+	+	+	Purpura, arthralgia, nephritis
5	54	F	2.10	IgG, IgM	+	+	+	+	Purpura, arthralgia, nephritis
6	48	M	3.60	IgG	+	+	+	+	Purpura, arthralgia
7	63	F	0.90	IgG	+	+	–	+	Purpura, arthralgia
8	68	F	0.20	IgG, IgM	+	+	–	+	RA, purpura
9	72	F	2.00	IgG, IgM	+	+	+	+	RA, peripheral neuropathy
10	72	F	0.30	IgG, IgA	–	–	–	–	RA
11	62	M	0.80	IgG, IgA	–	–	–	–	RA
12	72	M	0.85	IgG, IgA	–	–	–	–	RA, peripheral neuropathy

* <1 µg/ml.

Spiegelberg (Scripps Clinic and Research Foundation, La Jolla, Calif.) subtyped the monoclonal IgG cryoprecipitates of 2 patients (Cases 6, 7). C1q, C4, C3, C5, C6, C9, and factor B were detected in the cryoprecipitates by single radial immunodiffusion (Mancini *et al.*, 1965) and counterimmunoelectrophoresis (Arroyave and Tan, 1976). Monospecific antisera to complement proteins prepared in our laboratory were used in these assays. All antisera were prepared with highly purified complement proteins and showed single precipitin lines with the respective antigens in the Ouchterlony technique. No precipitin lines were detected against other serum proteins.

DETECTION OF SPECIFIC ANTIBODIES IN SERA AND CRYOPRECIPITATES

Sera and cryoprecipitates were tested for antinuclear antibodies of specificities present in Sjögren's syndrome, SS-A and SS-B (Alspaugh and Tan, 1975), by the Ouchterlony technique using standardized reference sera from patients with Sjögren's syndrome. Rheumatoid arthritis precipitin, antibody to human lymphocyte antigen, was demonstrated by the Ouchterlony technique using standardized reference sera from patients with RA (Alspaugh *et al.*, 1976). An extract of cellular antigens from a human lymphoid cell line (Wil₂) was used as a source of SS-A, SS-B, and RA precipitin antigens (Alspaugh and Tan, 1975). Antibodies to ss-DNA and ds-DNA were detected by methods described by Picazo and Tan (1975). Antibodies to Sm antigen and nuclear ribonucleoprotein, two acidic nuclear proteins, were identified by the Ouchterlony technique using reference prototype sera from SLE patients and patients with mixed connective tissue disease (Northway and Tan, 1972). Antibody titre was determined by the passive haemagglutination assay (Tan and Peebles, 1976). Antiglobulin antibodies were determined by latex agglutination (Hyland Div., Travenol Laboratories).

COMPLEMENT ASSAYS

Fresh sera from 3 patients (Cases 1, 5, 6) were immediately tested for total haemolytic complement (CH50) activity by the method of Mayer (1961). Serum levels of C1q, C4, factor B, and C3 were also measured by single radial immunodiffusion with monospecific antisera to these proteins (Mancini *et al.*, 1965). An aliquot of the fresh serum was frozen immediately at -70°C for 48 hours, and the CH50 and serum complement levels determined after thawing in a 37°C water bath. There was no significant difference in the complement levels of the fresh serum or frozen serum, showing that in sera

known to contain cryoglobulins the procedure of freezing and thawing serum once did not appear to alter the complement levels significantly.

ACTIVATION OF CLASSICAL COMPLEMENT PATHWAY BY ISOLATED CRYOPRECIPITATES

Activation was determined by the per cent of haemolytic C4 (C4H50) consumed when normal human serum was incubated with isolated cryoprotein (0.5 mg/ml PBS). 1 volume of cryoprotein was added to 2 volumes of fresh normal human serum and incubated at 37°C for 60 minutes. The effective molecule titration of C4 remaining was then measured by adding dilutions of this serum to EAC1 human cells (1.5×10^8) and fresh C4-deficient guinea pig serum (final dilution 1:120) to provide the terminal complement proteins (Arroyave *et al.*, 1977). The per cent of haemolytic C4 consumed was determined by comparing the cryoprecipitate-activated fresh normal serum with a control containing only PBS and fresh normal serum. Any cryoprecipitates which produced $>10\%$ consumption of C4 were considered positive for activation of the classical complement pathway. All assays for C4H50, which were performed in triplicate on the same day on three separate occasions, had a coefficient of variance of 2.5%, giving a high degree of reproducibility for this assay.

ACTIVATION OF ALTERNATIVE COMPLEMENT PATHWAY BY ISOLATED CRYOPRECIPITATES

Activation was determined by two different methods. The first assay determined the ability of the cryoprecipitate to produce lysis of glutathione-sensitized human erythrocytes (GSHE) under conditions in which the early steps of the classical pathway were inhibited by ethylene glycol tetra-acetic acid (EGTA). GSHE cells are sensitive to lysis by the C5b-9 complex in the presence of magnesium (Götze and Müller-Eberhard, 1972). Fresh normal human serum in EGTA was the source of C5b-9 complexes if this had been generated during alternative pathway activation. Briefly, GSHE (5×10^6 cells) were incubated with $50\ \mu\text{l}$ cryoprecipitate and $50\ \mu\text{l}$ fresh normal serum in the presence of EGTA (10 mmol/l) and MgCl_2 (0.5 mmol/l). These conditions block classical complement pathway activation but permit activation of the alternative complement pathway. After incubation in a water bath at 37°C for 60 minutes, 2 ml cold saline was added. After mixing and centrifugation, the amount of free haemoglobin was determined spectrophotometrically at 412 nm. Normal serum activated by inulin in the presence of EGTA and Mg^{++} was used as the standard for 100% lysis.

The second method was factor B conversion into split products. 5 μ l cryoprecipitate (0.5 mg/ml in PBS) and 20 μ l fresh normal human serum in the presence of EGTA (10 mmol/l) and $MgCl_2$ (0.5 mmol/l) were reacted at 37°C for 30 minutes. The mixture was then electrophoresed on a microscope slide coated with 1.5% agarose using veronal buffer pH 8.6 (ionic strength 0.05) and EDTA (0.02 mol/l). The slide was developed with monospecific antisera to factor B. Conversion of factor B indicating alternative complement pathway activation was detected by the appearance of split products with alpha and gamma mobility (Arroyave *et al.*, 1976).

SUCROSE DENSITY GRADIENT (SDG) ULTRACENTRIFUGATION STUDIES

The isolated cryoprecipitates were dissolved in 0.06 M glycine-HCl buffer (pH 3.2). After solubilization, a 0.25 ml sample (4 mg/ml) was layered on to a 4.5 ml linear 10–40% sucrose gradient made in the same buffer. The gradients were formed by using a Buchler automatic density gradient maker (Buchler Instruments Division, Nuclear Chicago, Fort Lee, N.J.). All samples were centrifuged in a SW 50.1 rotor at 50 000 rpm for 16 hours at 4°C in L2 ultracentrifuge (Spinco Div., Beckman Instruments, Palo Alto, Calif.). Samples were run in triplicate or quadruplicate. Marker proteins of 4.5S (human haemoglobin), 7S (human IgG), and 19S (human myeloma IgM) were centrifuged separately with each experiment. The SDG was separated into 0.35 ml fractions by collecting drop by drop from the bottom of the tube. Fractions were dialysed overnight against PBS to return them to neutral pH for immunofluorescence and other studies.

IMMUNOFLUORESCENCE STUDIES

The sera and SDG fractions were tested for the presence of antinuclear antibody (ANA) by the methods of Northway and Tan (1972). For immunofluorescent blocking experiments, the IgG fractions of a human reference serum containing an ANA specificity found in Sjögren's syndrome (SS-B) (Alsbaugh and Tan, 1975) was isolated by 0-(diethylamino-ethyl) cellulose (DEAE) chromatography (Fahey and Terry, 1967) and labelled with fluorescein isothiocyanate (FITC) (Fromhagen, 1965). This FITC-labelled IgG stained nuclei of tissue substrates in a speckled pattern to an end-point titre of 1:64.

The immunofluorescent blocking experiments to detect SS-B antibodies in the SDG fractions of the cryoprecipitates were performed as follows. The SDG fraction was incubated on mouse kidney

substrate for 30 minutes at room temperature. The slide was washed in cold PBS for 5 minutes. Then the FITC-labelled IgG at a dilution of 1:8 was reacted with the substrate for 30 minutes at room temperature. If the speckled nuclear immunofluorescent pattern was blocked by the SDG fraction, the fraction was presumed to contain SS-B antibodies. Controls for these studies included the demonstration that other fractions of the same SDG did not block nuclear staining.

If ANA of the SS-B specificity was detected in the 7S fractions of the SDG, we then determined whether the acid conditions used might have dissociated antigen-antibody complexes and homologous antigen might be detected in other fractions. The 7S fractions were preincubated with other SDG fractions at 37°C for 1 hour and at 4°C for 12 hours, then tested for ANA on tissue substrates. Rheumatoid factor was first removed from the SDG fractions with unsolubilized IgG (Avrameas and Ternynck, 1967) before incubation with the 7S fractions. If the ANA activity was removed by certain SDG fractions, the SS-B antigen was presumed to be present in these fractions. Enrichment of ANA in the cryoprecipitates was determined by the following formula:

$$\frac{\text{amount of IgG in serum}}{\text{amount of IgG in cryo}} \times \frac{\text{titre of ANA in cryo}}{\text{titre of ANA in serum}}$$

Results

ANALYSIS OF CRYOPROTEINS

The amount of protein in the cryoprecipitates ranged from 0.20 to 3.60 mg/ml. 7 patients had mixed IgG-IgM cryoprecipitates. In the cryoproteins of 2 patients (Cases 6, 7) the only immunoglobulin detectable was monoclonal IgG₃ κ (Case 7) or IgG₁ λ (Case 6). 3 patients (Cases 10, 11, 12) had IgG-IgA cryoprecipitates.

C1q, C4, C3, and factor B of the alternative pathway were detected in most of the isolated cryoprecipitates (Table 1). The C3 which was detected in the cryoprecipitates had the electrophoretic mobility of split products C3c and C3d by counterimmunoelectrophoresis (Arroyave and Tan, 1976). C3b was not detectable in the cryoprecipitates, perhaps because any C3b formed during activation of C3 was further degraded into C3c and C3d by the serum C3b inactivator enzyme, C3bI, though we have no direct evidence for this.

In 7 of 9 patients the terminal complement proteins C5, C6, and C9 were also detected in the cryoprecipitates together with the earlier complement components. No complement proteins were found

Table 2 Antibody studies of patients with cryoproteinaemia

Serum				Cryoprecipitates		
Case no.	ANA/pattern* titre	Antigammaglobulins†	RAP‡	ANA/pattern* titre	Antigammaglobulins†	RAP‡
1	128 speckled	320	+	32 speckled (7S)	19S	+
2	256 speckled	640	+	4 speckled (7S)	19S	—
3	Neg	160	+	—	19S	+
4	„	640	+	—	19S	+
5	„	640	+	—	19S	+
6	„	Neg	+	—	—	—
7	„	Neg	—	—	—	—
8	64 homogeneous	1280	+	—	19S	+
9	256 „	5120	+	—	19S	+
10	16 „	2560	+	—	—	—
11	16 „	640	—	—	—	—
12	64 „	640	+	—	—	—

*ANA of serum expressed as the reciprocal of antinuclear antibody titre. ANA of cryoprecipitates represents studies on sucrose density gradient fractions.

†Antigammaglobulins expressed as reciprocal of latex fixation titre in serum. Antigammaglobulins of cryoprecipitates expressed as the fraction of the SDG which was positive.

‡Rheumatoid arthritis precipitin was tested in immunodiffusion using serum and whole solubilized cryoprecipitates.

+ = positive; — = negative.

in the cryoprecipitates of 3 patients (Cases 10, 11, 12), but in all 3 the cryoprecipitates contained IgG and IgA but no IgM.

DETECTION OF SPECIFIC ANTIBODIES IN SERA AND CRYOPRECIPITATES

Antibody activities in sera and cryoprecipitates of the patients are summarized in Table 2. The sera of 10 patients had a significant titre of antigammaglobulin ($>1:40$). The cryoprecipitates of 7 of these patients had antigammaglobulin activity found only in the 19S fractions by SDG studies of the solubilized cryoprecipitates. No activity was found in the 7S fractions.

The sera and whole cryoprecipitates of each patient were tested for rheumatoid arthritis precipitin which was found in approximately 75% of seropositive RA patients (Alsbaugh *et al.*, 1976). These antibodies were tested by immunodiffusion and were detected in the sera of 11 patients and in the cryoprecipitates of 6 patients.

The relative difference in titre of RA precipitin between the serum and cryoprecipitates was found by determining the titre of the samples to an end-point against a standard Wil₂ antigen extract using the Ouchterlony technique. There was 32- to 80-fold enrichment of these antibodies in cryoprecipitates compared to sera.

Indirect immunofluorescence was used to detect ANA in the sera of the 12 patients. The sera of 7 patients were positive for ANA. The sera of both patients with Sjögren's syndrome produced a speckled nuclear pattern. In order to detect ANA in the cryoprecipitates, acid dissociated SDG proteins (see 'Methods') were tested for ANA by indirect immunofluorescence. Fig. 1 shows an SDG done on

Fig. 1 Sucrose density gradient (SDG) study of an acid-dissociated cryoprotein of Case 1. ● = the protein profile. Antinuclear antibody activity is represented as the reciprocal of the titre. This activity was in the 7S fraction of the SDG. The corresponding antigen was shown in the 19S and heavier SDG fractions.

the solubilized cryoprecipitates of Case 1. Protein peaks were present in the 7S and 19S regions of the gradient. The 7S fractions contained IgG, and the 19S fractions contained IgM. As Fig. 1 shows, ANA was detected only in the 7S region of the gradient in this patient. Using Wil₂ cells as a substrate, the speckled nuclear pattern produced by 7S fractions of Case 1's SDG can be seen in Fig. 2. ANA activity was also present in the dissolved cryoprecipitates of the other patient with Sjögren's syndrome, but was not detected in the cryoprecipitates of the other 10 patients.

Fig. 2 Speckled nuclear immunofluorescence produced by the 7S SDG fraction of Case 1. Wil₂ cells, a human B lymphoid cell line, was used as the substrate. $\times 785$.

We decided to analyse the ANA in the cryoprecipitates of the 2 patients with Sjögren's syndrome for the immunological specificities SS-A and SS-B, previously found in the sera of such patients (Alspaugh and Tan, 1975). However, the concentration of antibody in the SDG fractions was either insufficient or incapable of forming precipitin reaction with tissue extracts in immunodiffusion techniques, so an immunofluorescent blocking experiment was used (see 'Methods') (Table 3). The SDG fractions of the cryoprecipitates of both patients with Sjögren's syndrome were reacted first with the tissue substrate followed by the FITC-labelled reagent. Only the 7S fractions blocked the speckled nuclear pattern, indicating the presence of SS-B antibodies in these fractions. The antibodies were enriched 12- and 32-fold in the cryoprecipitates compared to sera.

Table 3 Immunofluorescent blocking experiments to detect SS-B antibodies in sucrose density gradient fractions of cryoprecipitates

Preincubation of tissue with:	Staining with FITC-SS-B antibody	Titre
Buffer	+	1:8
Case 1		
(Sjögren's syndrome) 7S*	—	0
(Sjögren's syndrome) 19S	+	1:8
Case 2		
(Sjögren's syndrome) 7S	—	0
(Sjögren's syndrome) 19S	+	1:8
Case 5		
(essential cryoglobulinaemia) 7S	+	1:8
(essential cryoglobulinaemia) 19S	+	1:8

*Sucrose density gradient fraction.
FITC = fluorescein isothiocyanate.

In order to determine if antigen was present in the cryoprecipitates of both Sjögren's patients, the 7S fractions were absorbed with other fractions of the gradient. The results (Fig. 1) for Case 1 show that only the 19S and heavier fractions were able to absorb the speckled nuclear immunofluorescent pattern of the 7S fraction, suggesting the presence of antigen in the heavier fractions of the gradient.

No antibodies to SS-A, another ANA specificity characteristic of Sjögren's syndrome (Alspaugh and Tan, 1975), were detected in the sera or cryoprecipitates. Antibodies to ss-DNA, ds-DNA, nuclear ribonucleoprotein, and Sm antigen were not detected in the solubilized cryoprecipitates or SDG fractions of any of these patients.

SERUM COMPLEMENT STUDIES

The serum levels of CH50 and C1q, C4, and factor B are given in Table 4. 7 patients had CH50 levels below normal, 6 patients had decreased levels of C1q and C4, and 2 had levels of factor B which were lower than normal.

The ability of the isolated cryoprecipitates to activate the classical complement pathway was determined by the per cent consumption of haemolytic C4 when the cryoprecipitates were incubated with fresh normal human serum. Activation of the alternative complement pathway by isolated cryoprecipitate was determined by lysis of GSHE cells and immunoelectrophoretic conversion of factor B when the cryoprecipitates were incubated with fresh normal human serum under conditions where C1 and C4 activation was inhibited (see 'Methods') (Table 5). The cryoprecipitates of all but one patient activated the classical complement pathway. Consumption of C4 ranged from 8 to 66%. 8 of the 12 cryoprecipitates produced more than 10% lysis of

Table 4 Serum complement levels of patients with cryoglobulinaemia

Case no.	CH50*	C1q (g/l)	C4 (g/l)	Factor B (g/l)
1	10†	0.059†	<0.01†	0.295
2	68	0.166	0.35	0.18
3	50†	0.07†	0.117†	0.117†
4	10†	0.019†	0.20†	0.175
5	22†	0.04†	0.08†	0.25
6	10†	0.09†	0.105†	0.20
7	70	0.156	0.8	0.265
8	11†	0.139†	0.15†	0.122†
9	56†	0.19	0.3	0.265
10	82	0.21	0.75	0.21
11	87	0.153	0.715	0.30
12	80	0.199	0.64	0.215
Normal values				
± 2 SD	67–107	0.14 $\pm$ 0.2	0.24 $\pm$ 0.8	0.15 $\pm$ 0.3

*Total haemolytic complement.

†Values below normal limits for this determination.

Table 5 Activation of the classical and alternative complement pathways by isolated cryoprecipitates*

Case no.	Classical pathway activation C4 (% consumption)†	Alternative pathway activation	
		GSHE (% lysis)‡	Factor B conversion§
1	31	34	+
2	8	33	+
3	46	30	+
4	47	33	+
5	42	21	+
6	37	45	+
7	15	18	+
8	32	27	+
9	66	48	+
10	12	3	—
11	10	<2	—
12	23	<2	—

*Cryoprotein adjusted to a concentration of 0.5mg/ml for testing.

†>5% consumption of C4 considered significant for activation of the classical pathway.

‡>5% lysis is considered significant for activation of the alternative pathway.

§Immunoelectrophoretic conversion of factor B to Bb and Ba.

GSHE = glutathione-sensitized human erythrocytes.

GHSE cells, which in our laboratory is significant for activation of the alternative complement pathway. The per cent lysis produced by the cryoprecipitates ranged from 18 to 48%. In addition, the same 9 isolated cryoprecipitates produced conversion of factor B into split products of alpha and gamma mobilities. Heating the cryoprecipitates to 56°C for 30 minutes before testing caused no change in the per cent lysis of GHSE or conversion of factor B.

Discussion

The presence of antigen-antibody complexes in the cryoprecipitates of patients with immunological diseases has been demonstrated (Winfield *et al.*, 1975; Wands *et al.*, 1975). Winfield *et al.* described an enrichment of antibodies to nuclear ribonucleoprotein, ss-DNA, and ds-DNA, as well as DNA antigen in the cryoprecipitates of SLE patients. Hepatitis B antigen and antibody have been detected in the cryoprecipitates of patients with extrahepatic manifestations of viral hepatitis, such as nephritis and arthritis (Kohler *et al.*, 1974; Wands *et al.*, 1975). In each of these studies the immune complexes shown in the cryoprecipitates appeared to be of specificities which were characteristic of the disease in which they were found.

Our patients had cryoglobulinaemia associated with the various clinical features of Sjögren's syndrome, rheumatoid arthritis, and idiopathic purpura. In the majority mixed cryoglobulins were shown, in agreement with previous reports that this type of cryoglobulin is more frequent in autoimmune disorders. In 7 of our 12 patients, autoantibodies could

be detected in the cryoproteins. 2 patients with Sjögren's syndrome had ANA of the SS-B specificity, a type which has been found in the sera of approximately one-half of patients with Sjögren's syndrome and to a lesser extent in other autoimmune diseases (Kurata and Tan, 1976). ANA activity could only be detected in cryoproteins after procedures which dissociated and separated antigen-antibody complexes. In addition, the homologous SS-B antigen was detected in the dissociated cryoproteins.

In 5 patients (3 with essential cryoproteinaemia, 2 with RA) conventional ANA was not detected in the cryoproteins although 2 had ANA in their sera. In 5 patients the cryoproteins were rich in rheumatoid arthritis precipitin. This had been described as precipitating antibody to a lymphocyte-associated antigen and is devoid of rheumatoid factor activity (Alspaugh and Tan, 1976). These 5 patients also had 19S rheumatoid factor in their cryoproteins.

The detection of a wide variety of immune reactants and complement in cryoproteins adds to the evidence that cryoprecipitation from serum may be a manifestation of pre-existing immune complexes in the circulation. In the 2 patients with Sjögren's syndrome the immune complexes may have consisted of SS-B antigen and antibody, since they were both present in the cryoproteins. In 5 patients the immune complexes may have consisted of RA precipitin and its homologous lymphocyte antigen. We were unable to show the presence of the latter antigen in the cryoproteins, perhaps because of lack of sensitivity of our assay system. Finally, other serum proteins such as rheumatoid factor and complement may be associated with cryoprecipitation, either by making a primary contribution to the property of cryoprecipitability or secondarily by their association with antigen-antibody complexes. It has been suggested that C1q plays a major role in cryoprecipitation, since IgG and C1q isolated from cryoglobulins precipitated in the cold when mixed together (Stastny and Ziff, 1969). No complement proteins were detected in the cryoprecipitates of 6 patients. These were the patients with no C1q in the cryoprecipitates. It is possible that complement proteins were present in the cryoprecipitates but at levels below the sensitivity of the detection system.

The pattern of interaction of cryoglobulins with complement resembles the pattern of complement activation of mixed cryoglobulin, described by others in that they activated preferentially the early complement components. The classical pathway was activated by the cryoprecipitates of all 12 patients, and it has been shown by other workers that cryoprecipitates, like aggregates of human immunoglobulins, will activate complement (Rother *et al.*

1972; Muller *et al.*, 1976). Of great interest was the fact that 9 of the 12 cryoprecipitates activated the alternative pathway. Since the initial reactions of classical pathway were blocked in our *in vitro* assays by the presence of EGTA, activation of the alternative pathway probably occurred independently. Similar activation of the alternative pathway was shown by Rother *et al.* (1972) who incubated different cryoglobulins in C4-deficient guinea pig sera and found a decrease in the C3-9 haemolytic activity. The loss of C3-9 under these conditions was considered evidence for direct activation of the alternative pathway by cryoglobulins. It is important to consider that the alternative pathway was activated via the C3b feedback cycle (Medicus *et al.*, 1976). However, the only antigenic C3 detected in our solubilized cryoprecipitates had the electrophoretic mobility of C3c/C3d. These proteins, unlike C3b, are *not* known to participate in the feedback cycle of complement activation via factor B.

The monoclonal IgG cryoprecipitates of 2 patients (Cases 6, 7) activated the alternative pathway, as did that of a patient with a similar type monoclonal cryoprecipitate reported by Muller *et al.* (1976). Thompson (1972) has shown that immunoglobulin fractions rich in IgG₃ isolated from patients with chronic hypocomplementaemic glomerulonephritis activated the alternative pathway. Others have also reported that certain specific subclasses of IgG myeloma may activate the alternative pathway (Spiegelberg and Götze, 1972; Frank *et al.*, 1976).

The demonstration of the immune complex nature of most mixed cryoglobulins suggests that such complexes may be responsible for acute vasculitis *in vivo*. Clinically there seemed to be some correlation between the presence of cutaneous vasculitis and cryoprecipitates which activated the complement system. 7 patients with skin biopsies compatible with vasculitis had cryoprecipitates which activated both the classical and alternative pathways. Of the 2 patients without cutaneous vasculitis who had such cryoprecipitates, one (Case 2) had myocardiopathy and one (Case 9) had peripheral neuropathy, conditions which might have been associated with localized vasculitis. All 9 patients had proteins of the classical and alternative complement pathway in their cryoprecipitates. When immunofluorescent studies were performed in 2 patients the fresh vasculitic skin lesions showed deposits of two alternative pathway proteins, properdin and factor B, in dermal vessels. In the skin lesion of Case 1 no C1q could be detected but this was present in the other patient, Case 5. Immunopathological studies of the skin were not available for the other patients.

The significance of cryoproteinaemia is not yet

fully understood, but as evidence accumulates that immune complexes of various kinds may be present in cryoprecipitates the presence of immune reactants should be sought in both tissues and sera of patients. Immune complex vasculitis may be found to be an important factor causing many different forms of tissue injury in these patients.

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References

- Alspaugh, M. A., and Tan, E. M. (1975). Antibodies to cellular antigens in Sjögren's syndrome. *Journal of Clinical Investigation*, **55**, 1067-1073.
- Alspaugh, M. A., and Tan, E. M. (1976). Serum antibody in rheumatoid arthritis reactive with a cell-associated antigen. *Arthritis and Rheumatism*, **19**, 711-719.
- Alspaugh, M. A., Talal, N., and Tan, E. M. (1976). Differentiation and characterization of autoantibodies and their antigens in Sjögren's syndrome. *Arthritis and Rheumatism*, **19**, 216-222.
- Arroyave, C. M., and Tan, E. M. (1976). Detection of complement activation by counterimmunoelectrophoresis. *Journal of Immunological Methods*, **13**, 101-112.
- Arroyave, C. M., Levy, R. M., and Johnson, J. S. (1976). Genetic deficiency of the fourth component (C4) in Wistar rats. *Immunology* (in press).
- Arroyave, C. M., Wilson, M. R., and Tan, E. M. (1977). Serum factors activating the alternative complement pathway in autoimmune disease. Description of two different factors from patients with systemic lupus erythematosus. *Journal of Immunology*, **116**, 821-826.
- Avrameas, S., and Ternynck, T. (1967). Biologically active water-insoluble protein polymers: their use in isolation of antigens and antibodies. *Journal of Biological Chemistry*, **242**, 1651-1660.
- Brouet, J. C., Clauvel, J. P., Dannon, F., Klein, M., and Seligmann, M. (1974). Biologic and clinical significance of cryoglobulins. A report of 86 cases. *American Journal of Medicine*, **57**, 775-788.
- Fahey, J. L., and Terry, E. W. (1967). Ion exchange chromatography and gel filtration. *Handbook of Experimental Immunology*, 1st ed., p. 19. Ed. by D. M. Weir. Davis, Philadelphia.
- Frank, M. M., Gaither, T., Adkinson, F., Terry, W. D., and May, J. E. (1976). Activation of the alternate complement pathway by human immunoglobulins. (Abst.) *Journal of Immunology*, **116**, 1733.
- Franklin, E. C. (1971). Cryoglobulinemia. *American Journal of Medical Science*, **262**, 51-60.
- Fromhagen, L. H. (1965). The solubility and other physicochemical properties of human-globulin labeled with fluorescein isothiocyanate. *Journal of Immunology*, **95**, 442-446.
- Götze, O., and Muller-Eberhard, H. J. (1972). Paroxysmal nocturnal hemoglobinuria. Hemolysis initiated by the C3 activator system. *New England Journal of Medicine*, **286**, 180-184.

- Hanauer, L. B., and Christian, C. L. (1967). Studies of cryoproteins in systemic lupus erythematosus. *Journal of Clinical Investigation*, **46**, 400-408.
- Kaplan, M. E. (1968). Cryoglobulinemia in infectious mononucleosis: quantitation and characterization of the cryoproteins. *Journal of Laboratory and Clinical Medicine*, **71**, 754-765.
- Kohler, P. F., Cronin, R. E., Hammond, W. S., Olin, D., and Carr, R. I. (1974). Chronic membranous glomerulonephritis caused by hepatitis B antigen-antibody immune complexes. *Annals of Internal Medicine*, **81**, 448-451.
- Kurata, N., and Tan, E. M. (1976). Identification of antibodies to nuclear acid antigens by counterimmunoelectrophoresis. *Arthritis and Rheumatism*, **19**, 574-580.
- Lowry, O. H., Rosebough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- Mancini, G., Carbonara, R. D., and Heremans, J. T. (1965). Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry*, **2**, 235-254.
- Mayer, M. M. (1961). Complement and complement fixation. *Experimental Immunochemistry*, 2nd ed., p. 133. Ed. by E. A. Kabat and M. M. Mayer. Thomas, Springfield, Illinois.
- Medicus, R. G., Schreiber, R. D., Götze, O., and Muller-Eberhard, H. J. (1976). A molecular concept of the properdin pathway. *Proceedings of the National Academy of Sciences of the USA*, **73**, 612-616.
- Meltzer, M., Franklin, E. C., Elias, K., McCluskey, R. T., and Cooper, N. (1966). Cryoglobulinemia - a clinical and laboratory study. II. Cryoglobulins with rheumatoid factor activity. *American Journal of Medicine*, **40**, 837-856.
- Muller, S., Rother, U., and Westerhausen, M. (1976). Complement activation by cryoglobulin. Evidence for a pathogenic role of an IgG (kappa) cryoglobulin in cutaneous vasculitis. *Clinical and Experimental Immunology*, **23**, 233-241.
- Northway, J. D., and Tan, E. M. (1972). Differentiation of antinuclear antibodies giving speckled staining patterns in immunofluorescence. *Clinical Immunology and Immunopathology*, **1**, 140-146.
- Picazo, J., and Tan, E. M. (1975). Specificities of antibodies to native DNA. *Scandinavian Journal of Rheumatology*, **11**, Suppl., 35-41.
- Ropes, M. W., Bennett, G. A., Cobb, S., Jacox, R., and Jessar, R. A. (1958). Revision of diagnostic criteria for rheumatoid arthritis. *Bulletin on Rheumatic Diseases*, **175**-176.
- Rother, U., Rother, K., Flad, H. D., and Miescher, P. A. (1972). Bithermic complement activation in cryoglobulinemic serum. *European Journal of Clinical Investigation*, **59**-65.
- Spiegelberg, H. L., and Götze, O. (1972). Conversion of C3 proactivator and activation of the alternate pathway of complement by different classes and subclasses of human immunoglobulins. (Abst.) *Federation Proceedings*, **31**, 2478.
- Stastny, P., and Ziff, M. (1969). Cold insoluble complexes and complement levels in systemic lupus erythematosus. *New England Journal of Medicine*, **280**, 1376-1381.
- Tan, E. M., and Peebles, C. (1976). Quantitation of antibodies to Sm antigen and nuclear ribonuclear protein by hemagglutination. *Manual of Clinical Immunology*, 1st ed., p. 666. Ed. by N. R. Rose and H. Friedman. American Society for Microbiology, Washington, D.C.
- Tesar, J., Kazmar, R., and George, S. (1973). Activation of the alternate pathway of complement by serum and synovial fluid cryoglobulin. (Abst.) *Arthritis and Rheumatism*, **16**, 575.
- Thompson, R. A. (1972). C3 inactivating factor in the serum of a patient with chronic hypocomplementemic proliferative glomerulonephritis. *Immunology*, **22**, 147-158.
- Wands, J. R., Mann, E., Alpert, E., and Isselbacher, K. (1975). The pathogenesis of arthritis associated with acute hepatitis-B surface antigen-positive hepatitis. *Journal of Clinical Investigation*, **55**, 930-936.
- Winfield, J. B., Koffler, D., and Kunkel, H. G. (1975). Specific concentration of polynucleotide immune complexes in the cryoprecipitates of patients with systemic lupus erythematosus. *Journal of Clinical Investigation*, **56**, 563-570.
- Wintrobe, M. M., and Buell, M. V. (1933). Hyperproteinemia associated with multiple myeloma, with report of a case in which an extraordinary hyper-proteinemia was associated with thrombosis of the retinal veins and symptoms suggesting Raynaud's disease. *Bulletin of the Johns Hopkins Hospital*, **52**, 156-165.