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CRYOGLOBULINS IN ACUTE AND CHRONIC LIVER DISEASES

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SUMMARY

Cryoglobulins were detected in the sera of thirteen patients with acute viral hepatitis and of twelve with chronic hepatic diseases (active chronic hepatitis, primary biliary cirrhosis and cryptogenic cirrhosis). Their nature and antibody activity was studied. In both groups, most of them consisted of mixed cryo-immunoglobulins (IgM, IgG and/or IgA), but some were single-class immunoglobulins with one or both types of light chains. Unusual components were also found.

α_1-fetoprotein was present in four cryoprecipitates: in two as the single constituent and in two associated to immunoglobulins; hepatitis-associated antigen co-existed in one of the latter. Some cryoglobulins showed antibody activity against human IgG, smooth muscle and mitochondrial antigens. In one case, the IgM-kappa of the cryoprecipitate had antibody activity against α_1-fetoprotein; this antigen was also present in the cryoprecipitate, suggesting immune-complex formation.

Autoantibodies were also looked for in the sera of the twenty-five patients; apart from the most common ones, antibodies to α_1-fetoprotein were found in two patients.

INTRODUCTION

The presence of cryoglobulins in patients with lymphoproliferative disorders (Grey *et al.*, 1968), connective tissue diseases (Christian, Hatfield & Chase, 1963; Arana *et al.*, 1971), chronic bacterial infections (Bonomo & Damacco, 1971), cutaneous vasculitis (Cream, 1972) and other diseases (Ritzman & Lewin, 1961) is well documented.

Physicochemical and immunological studies have shown that they most frequently consist of mixed immunoglobulins (two or three of the major classes), but also one class of immunoglobulin and even monoclonal cryoglobulins may occur.

While surveying the presence of cold precipitates in different sera, a group of patients

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with acute viral hepatitis and chronic hepatic disorders associated with autoimmune phenomena (Doniach & Walker, 1969; Sherlock, 1970), turned out to have cold precipitates. The presence and characterization of serum cryoglobulins in hepatic diseases had not yet been formally investigated.

The purpose of this report is to describe the constituents and biological activities of these precipitates.

MATERIALS AND METHODS

Out of 4,571 sera tested for cryoglobulins, 689 positive results were obtained, 121 belonging to patients with hepatic disorders. Twenty-five among them were selected for this study due to accuracy in diagnosis and follow-up possibilities.

Group I consisted of thirteen patients with acure viral hepatitis (AVH) and Group II of twelve patients with chronic hepatic diseases, including seven cases of active chronic hepatitis (ACH), three of primary biliary cirrhosis (PBC) and two of cryptogenic cirrhosis (CC).

Diagnosis was based on accepted clinical, biochemical, and—for patients in Group II—histological data. Follow-up of patients in Group I confirmed the diagnosis.

Thirty millilitres of blood were allowed to clot at 37°C. An aliquot was kept at 4°C for at least 10 days as a screening for cold precipitation, and the bulk of the serum was stored at −20°C until tested, using sodium azide as a preservative.

Cryoglobulin concentration in sera ranged from 0·2 to 1 mg/ml. Cryoglobulins were purified from 5 to 8 ml of serum by centrifugation at 4°C and 10,000 ***g***, and approximately ten successive cold washes with phosphate-buffered saline (PBS) (pH 7·2) at 8000 ***g*** until the supernatant was free of proteins as confirmed by a Beckman DU-2 spectrophotometer at 280 nm.

The cryoprecipitates were studied at concentrations ranging from 1 to 7·7 mg/ml; antibody activities were initially looked for at a concentration of 1 mg protein/ml in every case. Protein contents were measured by the microbiuret technique after heating the purified precipitates for 30 min at 40°C.

Double diffusion in agarose plates was used for the detection of immunoglobulins, fibrinogen and C3, employing 0·85% agarose in 50 mM veronal buffer (pH 8·2).

Antisera

Antisera to IgG and IgA were prepared by immunizing rabbits with pooled normal IgG or IgA myeloma proteins, and rendered monospecific for γ and α chains by further absorptions.

Rabbit and goat antisera to human IgM, IgD, IgE, kappa and lambda chains and C3 were obtained commercially (Hyland and Hoechst Laboratories). They were also absorbed until shown to be monospecific.

Antiserum to fibrinogen was raised in rabbits by immunization with whole human plasma followed by absorption with normal human serum (NHS).

Antisera to human liver antigens were obtained by immunizing rabbits with normal human liver homogenates. After adequate absorptions with normal human plasma, the antisera revealed one alpha and one beta precipitin line when tested by immunoelectrophoresis with the original liver homogenates; no reactions were observed with normal serum or plasma components.

Antibody tests

Antibodies to homologous and heterologous γ-globulins were looked for means of the latex fixation test (FII) (Singer & Plotz, 1956), and the sheep cell agglutination test (SCAT) (Roitt & Doniach, 1969c).

LE cell tests were done according to Zimmer (1954). Antibodies to native calf thymus deoxyribonucleic acid (DNA) (Worthington Biochemical Corporation), single-stranded, heat-denatured DNA (SSDNA) and soluble thymus antigens (SSTE: saline-soluble calf thymus extract) (Tan & Kunkel, 1966) were investigated by complement fixation and precipitin tests (Cannat, Arana & Seligmann, 1967).

Fluorescent antibodies reacting with nuclei (ANA), cytoplasm of gastric parietal cells (GPCA), smooth muscle (SMA), non-organ-specific cytoplasm (NSCytA) and mitochondria (AMA) were studied by means of an indirect immunofluorescence technique (Hammard, Cannat & Seligmann, 1964; Roitt & Doniach, 1969b) on unfixed cryostat sections of mouse liver, kidney and stomach. Anti-human γ-globulin (IgG, IgM and IgA) was prepared in a goat and labelled with fluorescein isothiocyanate (12·5 μg/mg protein) as described by Nairn (1968). The protein:fluorescein ratio was 1:5. The antiserum was used at a 1:8 dilution in PBS (pH 7·2); standard sera were used to check activity titres.

The presence of hepatitis-associated antigen (HAA) and antibody (HAAb) and of α_1 fetoprotein (α_1-FP) and anti-α_1 fetoprotein (anti-α_1-FP) was investigated by double diffusion and immuno-osmoelectrophoresis (Florin-Christensen & Arana, 1973) in 0·85% agarose–veronal 50 mM (pH 8·2). The presence of α_1-FP was also sought by means of single radial immunodiffusion (Mancini, Carbonara & Heremans, 1965). Reference sera for HAA and HAAb were at first provided by Austragen Laboratories and later obtained from poly-transfused haemophylic patients from the Academia Nacional de Medicina, Buenos Aires. Reference sera for α_1-FP and its antibody were obtained from Hoechst Laboratories.

RESULTS

Group I

Composition of the cryoprecipitates. In all cases the cryoglobulins precipitated at 4°C, were partially soluble at 37°C and completely soluble at 40°C. Incubation at the latter temperature did not affect subsequent reprecipitation when cooled to 4°C.

They were mostly mixed cryoimmunoglobulins; three consisted of all three major classes and six contained IgM and IgG. Four were single-class cryoglobulins; one was a polyclonal IgG and one a polyclonal IgM. The other two were an IgG and an IgM reacting only with anti-λ and anti-κ sera, respectively.

α_1-FP coexisted with an IgM–IgG and with the IgM–κ precipitates. The former also contained HAA. No C3 was found in any of the cryoprecipitates.

Antibody activities in cryoprecipitates. Two cryoglobulins were positive in the latex fixation test from 1 mg/ml down to 100 and 50 μg/ml respectively. Another had fluorescent antibodies to smooth muscle down to a concentration of 100 μg/ml and one showed anti-α_1-FP activity.

Serum studies. None of the patients showed LE cell formation. However, one had ANA up to a serum dilution of 1:50, and another had antibodies to DNA and SSDNA demonstrated by precipitin tests. Follow-up of this patient for over 3 years showed no manifestations suggestive of systemic lupus erythematosus.

Three had FII titres of 640–5120. Nine had SMA, three had GPCA and one showed NSCytA in fluorescence tests. Seven sera were positive for HAA. Two had α_1-FP and co-existing anti-α_1-FP activity.

The correlation between serum and cryoglobulin antibody activities is shown in Table 1.

Group II

Composition of the cryoprecipitates. Solubility of the precipitates was poor at 37°C and increased only moderately at 40°C. This treatment did not prevent subsequent precipitation at 4°C.

TABLE 1. Group I: acute viral hepatitis

Patient number	Cryoglobulin		Serum antibodies (titres in parentheses)
	Nature	Antibody activity	
1	IgM, IgG, IgA	—	SMA, GPCA
2	IgM, IgG, IgA	—	SMA
3*	IgM, IgG, IgA	RF	SMA, GPCA, RF (5120)
4*	IgM, IgG, α_1-FP, HAA	—	anti-α_1-FP
5	IgM, IgG	—	SMA, RF
6	IgM, IgG	—	ANA, SMA, NSCytA
7*	IgM, IgG	—	—
8*	IgM, IgG	—	SMA, GPCA
9	IgM, IgG	RF	SMA, RF (Negative)
10*	IgM	—	ANA (anti-DNA SSDNA)
11*	IgG	SMA	SMA (50)
12	IgG (λ)	—	—
13*	IgM (κ)–α_1-FP	Anti-α_1-FP	SMA, RF, anti-α_1-FP

* HAA-positive sera. Explanation of abbreviations in text.

Two consisted of IgG, IgM and IgA; two contained IgG and IgM and one IgM and IgA. One was a polyclonal IgG and another proved to be a monoclonal IgG of the kappa type. Two precipitates had α_1-FP free of any other serum component.

The remaining three cryoglobulins could not be classified by accepted methods, since they did not react with antisera to NHS, fibrinogen, C3, human γ-globulins or monospecific α, γ, μ, κ or λ chains. Neither did they react with antisera to α_1-FP, HAA, IgA-secretory piece or human liver homogenates. They were also tested in low-level immunoplates for IgM, IgG, IgA and the foetal antigen with negative results. Their protein concentrations after purification were 1·5, 2·6 and 7·7 mg/ml. The antiserum raised in a rabbit with these pooled cryoprecipitates showed only IgM and IgG precipitin lines when tested by immuno-electrophoresis against NHS, plasma, concentrated human saliva, human cord serum and liver homogenates. No precipitin lines were obtained when the rabbit antiserum was tested by double diffusion in agarose against each of the three cryoprecipitates. No C3 or HAA were found in the cryoprecipitates of this group.

Antibody activities in cryoprecipitates. Three cryoglobulins were positive in the FII test

down to concentrations of 30, 60 and 100 μg/ml. Three had SMA down to 50, 50 and 500 μg/ml. One had AMA at a dilution of 100 μg/ml. The 'unidentified' precipitates showed no antibody activity.

None of these cryoglobulins had anti-α_1-FP activity.

Serum studies. LE cell tests were negative in all patients. Fluorescent antibodies to nuclei, however, were present in four sera, in titres ranging from 10 to 100. One of them had anti-DNA and SSDNA antibodies and another anti-SSTE activity.

Anti-human γ-globulins were present in six sera, their FII titres ranging from 160 to 1280.

SMA were found in four sera; fluorescent antibodies to mitochondriae were disclosed

TABLE 2. Group II: chronic hepatic disorders

Patient number	Diagnosis	Cryoglobulin		Serum antibodies (titres in parentheses)
		Nature	Antibody activity	
1	ACH	IgM, IgG, IgA	SMA, RF	ANA, RF (640), SMA (100)
2	CC	IgM, IgG, IgA	—	ANA, NSCytA
3	PBC	IgM, IgG	AMA, RF	AMA (1000), RF (640)
4	PBC	IgM, IgG	—	AMA
5	PBC	IgM, IgA	SMA	SMA (10)
6	ACH	IgG	RF	RF (320), NSCytA, anti-SSTE
7	ACH	IgG (κ)	SMA	SMA (100), RF
8	ACH	α_1-FP	—	ANA, NSCytA
9	CC	α_1-FP	—	RF, NSCytA
10	ACH	*	—	—
11	ACH	*	—	ANA (anti-DNA SSDNA)
12	ACH	*	—	SMA, RF

* See text.

ACH: active chronic hepatitis. PBC = primary biliary cirrhosis. CC = cryptogenic cirrhosis. AMA = antimitochondrial antibodies. SSTE = saline soluble thymus extract.

Explanation of other abbreviations see text.

in two cases, both from PBC patients. Specificity was confirmed by absorption with rat liver and kidney mitochondrial-rich fractions, prepared according to Roitt & Doniach (1969a). Four sera had NSCytA.

No HAA or HAAb were found in these sera, and neither could anti-α_1-FP activity be demonstrated (Table 2).

DISCUSSION

This study demonstrates the presence of abnormal amounts of cryoglobulins in the sera of patients with acute viral hepatitis, active chronic hepatitis, primary biliary cirrhosis and cryptogenic cirrhosis.

In both groups these precipitates most often consisted of mixed cryoglobulins, especially IgM and IgG, although IgA was also frequently detected. In this respect they do not differ from cryoprecipitates described in other diseases (Meltzer & Franklin, 1966). It is of interest

that none of these mixed cryoimmunoglobulins contained complement. The presence of α_1x-lipoprotein was not investigated, and the possibility of its contribution to cryoprecipitation in some cases remains open.

Some of the precipitates contained only one class of immunoglobulin, and three proved to be monoclonal. Apart from cases of Waldenström's macroglobulinemia and multiple myeloma, single monoclonal cryoglobulins are rare.

Peculiar components were observed in these precipitates. In one case HAA was found in association with a mixed cryoglobulin. α_1-FP was detected twice coexisting with immunoglobulins, and twice again as the only constituent of the precipitate. This antigen occurs in patients with hepatocellular carcinoma and other malignancies, especially in those arising from embryonic tissues (Tatarinov, 1964; Adinolfi & Adinolfi, 1971). More sensitive techniques have shown it to be a normal serum constituent (Purves & Geddes, 1972) and disclosed increasing concentrations in pregnant women's sera (Seppälä & Ruoslahti, 1972).

In this report, the findings of α_1-FP 's extended to cryoglobulins, and its ability to precipitate in the cold has been demonstrated.

The three cryoprecipitates in Group II which could not be identified by conventional methods were shown to contain at least IgM and IgG with no detectable antibody activity. The difficulty in reacting by gel diffusion could be due to an impaired migration due to molecular aggregation.

Antibody activities found in the cryoglobulins were directed against human γ-globulin, nuclear, smooth muscle and mitochondrial antigens and α_1-FP. It seems interesting that a monoclonal cryoglobulin (IgG-kappa) had anti-smooth muscle activity. Monoclonal antibodies to smooth muscle were previously reported in two myeloma sera by Wager *et al.* (1971). Naturally occurring antibodies against the foetal antigen have been reported in a previous paper (Florin-Christensen & Arana, 1973). These specificities were also present in the sera after removal of the cryprecipitate without significant reduction of titre, possibly due to the cryo-antibodies being complexed to antigens.

Immune-complex formation has been proposed as a possible mechanism for cryoprecipitation (Lospalluto *et al.*, 1962). This could explain mixed cryoglobulins with anti-IgG activity, complexes involving immunoglobulins and identifiable antigens, such as HAA or α_1-FP and possibly other self antigens (Bluestone *et al.*, 1970).

In other cases immune-complex formation with unidentified antigens, or molecular instability (Saha *et al.*, 1968, 1969) might be responsible for cryoprecipitation.

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