CRYOGLOBULINS AND PYROGLOBULINS: AN OVERVIEW

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In 1933 WINTROBE and BUELL[88] described a case of multiple myeloma in a 56-year-old woman whose serum formed a precipitate when cooled, that redissolved when the serum was warmed to 37 °C. The patient had hepatosplenomegaly, Raynaud's phenomenon, purpura, as well as venous thrombosis of the retina. In 1941 PETERS and HORTON[66] reported on a patient with 'allergic purpura' that appeared after exposure to cold. The patient, a 53-year-old man living in Minnesota, wore heavy clothes even on mild days, since exposure to cold induced purpura, urticaria and arthralgia. Desensitization with histamine did not help, but his symptoms decreased when he moved to the much warmer climate of Arizona. Three years later he was seen at the University of Minnesota by LERNER and WATSON[45], who detected in his serum a protein that precipitated in the cold, characterized it as a gammaglobulin and named it *cryoglobulin*.

DEFINITION AND CLASSIFICATION

Cryoglobulins are presently defined as a group of serum proteins with temperature-dependent solubility. While most proteins are highly soluble and remain in solution in the cold up to concentrations above 100 mg/ml, cryoglobulins precipitate or form a gel below 37 °C, even at concentrations of 0.1 mg/dl[14,21,55]. The temperature at which they precipitate is a function of their concentration, the higher the concentration the higher the temperature of precipitation. At low concentrations they precipitate only around 4 °C and completely redissolve when the serum is reheated to 37 °C. The pH of the solution is also important for cryoprecipitation[56]. Usually cryoprecipitation does not occur at pH below 5.0 or above 8.0.

Key-words: Cryoglobulins; Fibronectin; Immune complexes; Immunoglobulins; Pyroglobulins; Rheumatoid factor.

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It should be emphasized that small amounts of cryoglobulin may occur in human serum normally, in concentrations of about 30 μg/ml[17]. Therefore, cryoglobulins should be considered to be abnormal increases in proteins normally present in serum in relatively small amounts.

The etiological heterogeneity of the cryoglobulins is suggested by the fact that cryoproteins have been described in patients with many different diseases[33] (tab. 1). Cryoglobulinemia associated with one of these diseases is called *secondary cryoglobulinemia*, whereas patients for whom no associated pathology can be found are said to have *essential* or *idiopathic cryoglobulinemia*. This last form is known as the LoSpalluto-Meltzer syndrome and is characterized by a triad of symptoms, namely purpura, arthralgia and weakness. Renal failure often follows these.

For several years it has been thought that most, if not all, cryoglobulins are immune complexes[21] made up of antigen (Ag) + antibody (Ab), sometimes also combined with complement. This hypothesis was confirmed by studying the immunochemical properties of several cryoproteins, by showing their frequent occurrence in diseases associated with immune complexes and by detecting the deposition of immune complexes in different organs and tissues in patients with mixed cryoglobulinemia[21].

Table 2 shows the immunochemical composition of many cryoglobulins that have been studied. Such structural heterogeneity is the basis for the classification proposed by BROUET et al.[14] and accepted by most investigators. In this classification three types of cryoglobulin are reported:

1. Cryoglobulins of the *first type* are made up of a single monoclonal Ig, may be found almost exclusively in patients with multiple myeloma or Waldenström's macroglobulinemia, and account for lower than 20% of cryoglobulinemias. Most often the monoclonal Ig is IgM, less often IgG usually belonging to γ_2 or γ_3 heavy chains, less frequently γ_1. Only one case of IgG4 cryoglobulinemia has been reported[64]. In rare cases the cryoglobulins of the first type are made up of IgA[68], perhaps with the properties of pyroglobulins as well[85], or of light chains that can reach high serum levels once renal insufficiency has developed[2,14,47]. Occasionally single IgG cryoglobulins have been shown to consist of IgG-IgG immune complexes[21].

2. Cryoglobulins of the *second type* are mixed, usually IgM-IgG, and one of the two fractions is monoclonal (tab. 2). These have an incidence of approximately 40%. As a rule, neither of the two components is able to precipitate in the cold individually, but it is the IgM fraction that behaves as an incomplete cryoglobulin, in that when this is reacted with an IgG from another subject, cryoprecipitation does occur. Vice versa, if the IgG component of the cryoglobulin is reacted with homologous IgM, cryoprecipitation is no longer detectable[49].

The most frequent form in this group is the so-called 'essential mixed cryoglobulinemia' (EMC), characterized by the occurrence in the serum of IgM-IgG complexes. While the IgG is always polyclonal, the IgM is monoclonal, usually possesses kappa light chains and is endowed with rheumatoid factor activity. In addition to classical rheumatoid factor activity, anti-idiotypic activity has been demonstrated in a significant number of the IgM fractions from these mixed cryoglobulins[31]. These cryoglobulins are often associated with vasculitis, skin reactions and glomerulonephritis[55]. Metabolic studies of the constituent fractions from patients with cryoglobulinemia have shown no significant differ-

1) *lymphoproliferative diseases*
- multiple myeloma [74]
- Waldenström's macroglobulinemia [12]
- chronic lymphatic leukemia and lymphoma [14]
- angioimmunoblastic lymphadenopathy [32]

2) *autoimmune and immune complex diseases*
- systemic lupus erythematosus [16]
- rheumatoid arthritis [86]
- Sjögren's syndrome [91]
- Felty's syndrome [86]
- scleroderma [39]
- polyarteritis nodosa (HBsAg-positive and -negative) [73]
- Behçet's syndrome [71]
- Schönlein-Henoch purpura [30]
- autoimmune vasculitis [17]
- glomerulonephritis [53]
- autoimmune thyroiditis [56]
- polymyositis [27]
- sarcoidosis [79]
- celiac disease [23]
- pemphigus vulgaris [59]

3) *infectious diseases*

A) *viral*
- acute hepatitis [75]
- chronic hepatitis B virus infection [54]
- infectious mononucleosis [82]
- cytomegalovirus infections [82]

B) *bacterial*
- lepromatous leprosy [11]
- subacute bacterial endocarditis [38]
- acute poststreptococcal glomerulonephritis [53]
- syphilis [43]
- venereal lymphogranuloma [44]

C) *parasitic*
- echinococcosis [10]
- malaria [1]
- toxoplasmosis [81]
- leishmaniasis [62]

D) *fungal*
- coccidioidomycosis [29]

4) *liver diseases*
- biliary cirrhosis [83]
- Morgagni-Laennec's cirrhosis [19]
- alcoholic cirrhosis [34]

Tab. 1 - A tentative list of diseases which may be associated with cryoglobulinemia.

IgG	single-type cryoglobulins
IgM	
IgA	
light chains	
IgM + IgG	mixed-type cryoglobulins
IgG + IgA	
IgG + IgG	
IgG + C1q	
IgG + IgA + IgM	
IgG + IgM + complement	
IgG + IgM + α_2-macroglobulin	
IgM + IgG + DNA*	

fibronectin appears to be consistently present in both the mixed and single forms

* Nucleic acids may be found in other forms of mixed cryoglobulins.

Tab. 2 · Immunochemical composition of the cryoglobulins.

ences between the metabolism of IgG purified from the complex and normal IgG[72].

Other mixed cryoglobulins of the second type are made up of IgA-IgG, with IgA usually being the monoclonal component[61], or else IgG-IgG in which one IgG is monoclonal with rheumatoid factor activity and the other is polyclonal[14].

3. Cryoglobulins of the *third type* include the mixed polyclonal cryoglobulins which appear to be fairly common, with an incidence higher than 40%. In this group, in addition to some of the 'essential' cases apparently unassociated with concomitant diseases, the forms that accompany infectious and/or inflammatory diseases are usually found.

Under the nosological point of view, single-type monoclonal cryoglobulinemia (type I) and mixed cryoglobulinemia with one monoclonal component (type II) should be considered lymphoproliferative diseases. Indeed, in the first group clinical diagnoses have mostly been either Waldenström's macroglobulinemia or multiple myeloma. The large majority of patients with type II cryoglobulins either have EMC or Waldenström's macroglobulinemia, in which the monoclonal component (IgM) has rheumatoid factor (anti-immunoglobulin) activity, thus accounting for the presence of circulating IgM-IgG immune complexes. Even cases with IgA-IgG and IgG-IgG cryoglobulins should possibly be considered lymphoproliferative diseases, with monoclonal IgA or IgG components able to react with polyclonal IgG.

The nosological classification of mixed polyclonal cryoglobulinemias (type III) is somewhat more complex. While some forms (those secondary to acute or chronic infectious diseases) might be considered to be specific antibody responses (often transitory and not necessarily abnormal) to some antigenic stimuli such as streptococcal antigens[36] or hepatitis B virus (HBV)[46], type III EMC should be considered a lymphoproliferative disease because of the peculiar clinical picture, with its characteristic clinical development into progressive damage of various organs.

PATHOGENESIS

The mechanisms by which cryoglobulins originate are still largely unknown. Some investigators[6,21] have suggested that, in response to persistent antigenic stimulation by antigens of various types, the immune system produces a secondary antibody response, most often IgG that, after reaction with the antigen, becomes autoantigenic itself. The next response to this is the production of IgM, which causes formation of immune complexes (primary antigen-IgG-IgM) that precipitate in the cold for not yet understood reasons. BALLARD et al.[4] studied a cryoprecipitable monoclonal IgM with high affinity for the antigen, obtained by stimulating the splenocytes of already immunized mice with fluorescein isothiocyanate, and concluded that cryoglobulins are the expression of a hyperimmune response. In fact, the process of maturation of the immune response in terms of the affinity of the binding to Ag might cause somatic mutations of the Ig-secreting cells, causing them to produce an Ig with elevated affinity and with cryoglobulin properties.

Some patients with systemic lupus erythematosus were found to have mixed IgG-IgM cryoglobulins containing DNA, which could not be studied further because it was present in only very small amounts[6]. In more than a few patients with mixed cryoglobulinemia it has been possible to identify denatured DNA in the cryoprecipitated immune complexes, intimately associated with the complex itself[8]. In fact, this DNA can be attacked by DNAse only at pH 3, whereas the enzyme is not active at neutral pH. This is unlike the situation for the DNA bound to normal immunoglobulins, which is accessible to DNAse at all pH values[5]. Possibly the DNA so closely associated with the IgG-IgM complex and inaccessible to DNAse at pH 7 becomes accessible at strongly acid pH because of the dissociation of the IgG-IgM components known to occur at acid pH. The DNA might come either from increased destruction of host cells or from viral particles in the form of single-stranded DNA and might, in at least some cases, be the antigen that initiates the immunological reaction responsible for cryoprecipitation[6,21].

In 1977 LEVO et al.[46] reported a significantly high correlation between EMC and chronic infection with hepatitis B virus. HBsAg or anti-HBs were found in 52% of the sera from patients with EMC, while positive responses for at least one of the markers for HBV infection were found in 74%. In addition, electron microscopy revealed that some cryoprecipitates contained spherical or tubular particles of 20 and 27 nm referable to HBV and also contained Dane particles. The association of EMC with exposure to HBV was thought to be so strong that some investigators proposed calling it mixed cryoglobulinemia secondary to hepatitis B virus instead of EMC[48]. This opens up a possibility of explaining another frequently found association, that of cryoglobulinemia and chronic liver disease. In these cases, HBV might on one hand be the etiological agent for the chronic liver disease, and on the other it might be the antigen responsible for immunologic stimulation that brings about formation of the IgM-IgG-HBV immune complexes which may have cryoprecipitating properties.

Additional evidence in favor of the etiological responsibility of HBV for EMC was obtained by DRÜEKE et al.[24], who studied patients on hemodialysis. Three of 266 carriers of HBsAg developed EMC, with HBsAg and anti-HBs in

the cryoprecipitate, followed by necrotizing vasculitis, while none of the patients without HBsAg developed either cryoglobulinemia or vasculitis.

More recently, a few studies[67,75] have somewhat reshaped the importance of the association of EMC and HBV, suggesting that there is not such a large presence of at least one of the markers of HBV in the cryoprecipitates[28]. In addition, even in those cases that do have HBV markers, it was possible that the chronic HBV infection became established in subjects who, by already having cryoglobulinemia, should possibly be considered more prone to infection by HBV in view of their immunological abnormalities. What is more, the cryoprecipitating property should not be considered specific for the IgM-IgG-HBV immune complexes, since cryoglobulins are found with even greater frequency than HBV in subjects with many infectious and parasitic diseases.

Mixed cryoglobulinemia was produced in the rabbit by repeated inoculations with streptococcal vaccine or with live streptococci B[36]. In this infectious disease, in which the pathogenic agent disappears once it has been cured, cryoglobulinemia is transient and cannot be demonstrated within a few weeks or months after recovery. Therefore, formation of cryoglobulins during antibody response to whatever antigen should be considered neither exceptional (since it occurs in different diseases) nor abnormal. What is abnormal in the cryoglobulinemic syndrome, therefore, is the accumulation of cryoglobulins in the blood, which does not probably result from hyperproduction only, but also from reduced catabolism by the macrophages.

MECHANISMS OF THE CRYOPRECIPITATION

Very little is known about the molecular basis for the different solubility in the cold of the cryoglobulins as compared with other immunoglobulins. The most likely explanation is that cryoprecipitation is a simple solubility phenomenon based on an unfavourable interaction between cryoglobulins and the solvent at low temperatures[58]. The solubility of proteins depends on various factors, namely concentration, temperature, pH, ionic strength of the solution, as well as surface charge (which depends on the amino acid residues and the carbohydrate content).

A series of data from MELTZER and FRANKLIN[55] indicates that 90% of the total quantity of monoclonal IgG or IgM cryoglobulins in a serum is precipitated at 0 °C in 0.15 M NaCl solution when the cryoglobulin concentration is 0.5 to 4 mg/ml and the pH is between 4.5 and 8.5.

Several investigators have reported changes in the primary structure of the heavy and light chains of the cryoglobulin that they consider to be responsible, at least in part, for the different solubility of the cryoglobulins. CUMMINGS[18] found low percentages of tyrosine and tryptophan in a monoclonal IgG cryoglobulin and UKI et al.[80] and MIDDAUGH et al.[58] found a deficiency of tyrosine in the heavy chains of two different monoclonal IgM cryoglobulins. UKI et al.[80] also detected in many cryoglobulins low basic amino acid (lysine and arginine) and aromatic amino acid (phenylalanine and tyrosine) contents associated with increased glutamic acid. On the other hand, WANG et al.[84] reported that the light chains of IgG and IgM cryoglobulins had low serine content.

Under normal conditions, the polar amino acid residues (aspartic acid, glutamic acid, arginine, histidine, lysine, serine, tyrosine, threonine) are on the surface

of the molecule and provide most of the charge and solubility of the molecule itself, whereas the non-polar residues are located in the hydrophobic inside of the molecule. Decreases in the polar residues will, therefore, decrease the negative charge and the solubility of the protein, but within certain limits this can be overcome by the formation of dimers or trimers, which increases the negative charge on the surface in proportion to the mass, so that the same number of negative charges is distributed over a smaller total surface as compared with that of the individual monomers[3]. This preserves the solubility of the aggregates of cryo- or pyroglobulins under suitable thermal conditions. However, decreasing the temperature causes further changes in the steric conformation of the entire molecule, thus exposing non-polar residues with further loss of solubility and the formation of cryoprecipitate. Reheating to 37 °C reestablishes the initial steric conformation of the molecule[79].

The *pH* of the solution is also important for the solubility of proteins, affecting the secondary and tertiary structures of the molecule. At pH below 5 or above 8, for example, the loss of negative surface charges becomes less critical, so that cooling does not cause precipitation[56]. There are, however, exceptions such as one particular monoclonal IgM described by MIDDAUGH et al.[58] that precipitated at 0 °C even in solutions at pH 10.

In addition, *steric changes in the molecule* that follow its binding to antigen are very important for function, since they expose the binding site to complement and can also decrease the surface charge and thus the solubility at low temperatures.

Even though the *carbohydrate content* of many cryoimmunoglobulins is within normal limits, some investigators have shown it to be reduced or absent in a few instances. SAHA et al.[69] described an IgG cryoimmunoglobulin with a low glycosamine content. ZINNEMAN et al.[92] found no sialic acid in the IgG component of a mixed IgG-IgM cryoglobulin and made an artificial mixed cryoglobulin by combining an anti-IgG IgM with a normal IgG that had been desialylated by incubation with neuraminidase.

Even uncharged carbohydrates (fucose, hexose, hexosamine) contribute indirectly to the solubility of the molecule[3]. When these carbohydrates are removed with glycosidase, a decreased solubility occurs up to 37 °C. One patient with Sjögren's syndrome had a monoclonal IgG cryoglobulin with low fucose and hexose content that after precipitation had to be reheated to 52 °C before it dissolved[91]. Attempts to localize the segments of the molecule that were responsible for cryoprecipitation by enzymatic fragmentation of the cryoglobulin gave unclear results, but the various fragments obtained [Fab, $F(ab')_2$, Fc, monomeric subunits of IgM] no longer cryoprecipitated[58].

Fibronectin has recently been shown to play an important role in cryoprecipitation. It is a protein with molecular weight 450,000 daltons, consistently appears to be a component of mixed cryoglobulins (plate 1E), especially those from patients with autoimmune diseases, and seems possibly responsible for the formation of the cryoprecipitate[7,19,89]. Fibronectin has also been found in the precipitates of single-type monoclonal cryoglobulins[20], in which it accounts for 10-14% of the total protein[77]. Its role in the formation of the cryoprecipitate is emphasized by the fact that, when fibronectin is removed from the plasma chromatographically, there is a notable decrease in the ability of the cryoglobulins to precipitate in the cold. The capacity to cryoprecipitate is regained by

the addition of fibronectin. However, the possible interactions of fibronectin and cryoglobulin during cold precipitation are still poorly understood. The consistent presence of fibronectin in the cryoprecipitate might be due either to contamination or to the binding of fibronectin to some component (not necessarily an immunoglobulin) of the cryoglobulin. This latter appears to some extent supported by the following observations: 1) fibronectin has been found in association with all the polyclonal cryoglobulins studied; 2) repeated solubilization and reprecipitation of cryoglobulins do not result in any loss of fibronectin, as it does of other contaminating plasma components such as albumin; 3) the electrophoretic mobility of fibronectin is significantly changed when the cryoprecipitate is immunoelectrophoresed directly without having been solubilized[89]. Since the only cryoglobulin components that are essential for formation of cryoprecipitate are the immunoglobulins, it would seem that they are indeed the effective substrate for fibronectin attachment. Finally, the change in electrophoretic mobility of the Ig and of fibronectin might be due to their interactions.

It is possible that some Ag-Ab complexes that are able to bind fibronectin are soluble at physiological temperatures but, when they are combined with fibronectin at low temperature, they form a precipitate[89]. Another possibility is that fibronectin can be bound to the collagen-like portions of C1q that are present in many cryoglobulins, especially those in patients with connective tissue diseases[87]. Finally, it seems also possible that a number of large normal serum proteins, including fibronectin, may be endowed with cryoprecipitability. In the course of inflammatory situations such proteins would indeed behave as acute-phase reactants and increase to a threshold beyond which intermolecular attractive forces become prevalent. The molecular aggregation between these non-Ig proteins and immune complexes would be enhanced at low temperatures, thus resulting in the cryoprecipitation of otherwise cold-soluble immune complexes[35].

STRUCTURAL STUDIES

Several investigators have studied the ultrastructure of a number of cryoprecipitates. Single monoclonal IgG cryoglobulins precipitate in cylindrical tubules, the transverse sections of which have a ring appearance, sometimes with electron-dense bridges between them, while longitudinal sections look like the rungs of a ladder. The walls of the tubules probably consist of protein subunits made up of the Fab components, while the Fc components project outside the cylinder[76]. The precipitate consisting of mixed IgG-IgM cryoglobulins looks under the electron microscope like a structure with a central tubule surrounded by material in the form of the spokes of a wheel. The subunits that form the walls of the central tubule look in longitudinal section like the rungs of a ladder[76]. The material arrayed around the tubule might be IgM bound to the Fc component of the IgG that probably makes up the wall of the tubule.

LABORATORY DATA

Blood samples drawn from patients with cryoglobulinemia into a syringe are warmed to 37 °C and set to coagulate in a 37 °C water bath or oven. The serum (which may be turbid) is stored in a refrigerator at +2 to 4 °C for a

few days. The cryoglobulin precipitates in the bottom of the tube (plate 1B) and can be easily washed with cold buffered saline solution to remove the other serum components, including normal Ig. Cryoglobulins are then ready for immunological typing by immunoelectrophoresis. If the whole serum is centrifuged at 1,400 rpm and at 4 °C in a Wintrobe tube, one can obtain the percent of cryoprecipitate in total serum, which is called the *cryocrit*. After electrophoresis there is often a homogeneous band, usually in γ position, and occasionally in β position. The immunoelectrophoretic pattern is often characteristic (plate 1C). There may be either polyclonal hypergammaglobulinemia or hypogammaglobulinemia. The erythrocyte sedimentation rate is usually high, especially for patients with hypergammaglobulinemia. Analytical ultracentrifugation of the isolated cryoglobulins often shows typical patterns (plate 1D).

Some cryoglobulins, especially the IgM monoclonal cryoglobulins with rheumatoid factor activity that are seen in Waldenström's macroglobulinemia and the monoclonal components of mixed cryoglobulins, have anti-immunoglobulin activity. In addition, mixed cryoglobulins may have anti-complementary activity[92]. There have also been descriptions of decreased serum bactericidal activity and inhibition of chemotaxis, probably due to coating of the leucocytes with cryoglobulin[92]. In general, there are low serum complement levels, especially of C4 which may reach values lower than 5 mg/dl[32]. Cell-mediated immunity and suppressor cell activity have also been found to be impaired[5,57].

For largely unknown reasons 70% of the patients are anemic, their hematocrits being less than 35%. There may also be a spurious leucocytosis because the electronic counters include aggregates of cryoglobulin in the counts[25]. In patients with mixed cryoglobulinemia, bone marrow examination reveals focal lymphocyte aggregation or a slight increase in plasma cells[32], while the bone marrow appearance of patients with monoclonal cryoglobulinemia is typical of those with multiple myeloma or Waldenström's macroglobulinemia.

CLINICAL MANIFESTATIONS

Our experience is based on the study of over 180 patients with cryoglobulinemia (tab. 3), one third of whom have been followed up for several years. Clinical diagnoses include lymphoproliferative diseases, connective tissue diseases and other autoimmune disorders, as well as chronic infectious diseases. Among patients belonging to the infectious disease category, a special mention deserve 3 patients with HTLVIII/LAV-related positive serology associated with significant amounts of circulating type III cryoglobulin, suggesting that patients with acquired immunodeficiency syndrome (AIDS), AIDS-related complex and lymphadenopathy syndrome should be carefully screened for the occurrence of cryoglobulinemia. However, no associated disease could be clearly detected in 68 out of 181 patients despite thorough clinical evaluation and prolonged follow-up. Thus, this group of patients classified as having 'essential' mixed cryoglobulinemia (EMC) is the largest one in this series (tab. 3), as well as in most large series published so far[14,32,33,56].

Not all subjects with cryoglobulinemia have specific signs and symptoms[60], although 80-85% of the patients with monoclonal cryoglobulinemia have clini-

diagnoses	cryoglobulin			
	type I (single type)	type II (mixed, with a monoclonal component)	type III (mixed, polyclonal)	total
multiple myeloma	15	–	–	15
Waldenström's macroglobulinemia	11	9	–	20
chronic lymphatic leukemia and malignant lymphoma	5	2	–	7
bacterial endocarditis	–	1	1	2
lepromatous leprosy	–	1	6	7
liver cirrhosis and chronic active hepatitis	–	9	24	33
systemic lupus erythematosus	–	5	8	13
rheumatoid arthritis	–	3	4	7
progressive systemic sclerosis	–	1	2	3
Sjögren's syndrome	–	2	2	4
periarteritis nodosa	–	–	1	1
idiopathic thrombocytopenic purpura	–	1	1	2
LAS (HTLVIII/LAV-positive lymphadenopathy syndrome)	–	–	3	3
'essential' mixed cryoglobulinemia	–	37	31	68
total	31	71	83	185 *
(%)	(17.1)	(39.2)	(45.8)	

* A few patients had more than one diagnosis.

Tab. 3 - Clinical diagnoses in 181* patients with cryoglobulinemia. Cumulative experience collected in the Institutes of *Patologia Medica* and 2nd *Clinica Medica*, University of Bari, up to June, 1986.

cal manifestations after exposure to cold or at other times[33]. In the patient population of GOREVIC et al.[32] more than two-thirds of the patients were *females*, with mean age of 50.7 years, range 21 to 72 years, and without any significant racial disproportions. In addition, cryoglobulinemia has been repeatedly reported to affect several members of the same family[22,63]. The most frequent signs and symptoms of mixed cryoglobulinemia are listed in tabs 4A and 4B.

Skin reactions are seen very frequently (plate 1A). Many patients are seen first by dermatologists, to whom they go because of purpura, leg ulcers, Raynaud's phenomenon, edema and urticaria. The *purpura* usually does not cause itching, appears intermittently on exposed parts of the body, especially the legs, fingers, ears, nose[13]. It occurs most frequently in the winter, lasts for 3-10 days for each poussée (sometimes the poussées follow each other with striking frequency), and eventually leaves an area that is hardened and diffusely hyperpigmented. Biopsies of the cutaneous purpuric lesions show vasculitis, with endothelial swelling, extravasation of blood, perivascular infiltration with mononuclear and polymorphonuclear cells and damage to the subcutaneous interstitium. Immunofluorescence studies show IgM, IgG and/or C4 in the vessel walls of the lesioned skin.

Chronic ulcers of the legs are especially frequent in the supramalleolar regions, are always associated with purpura and are probably due to vasculitis.

signs and symptoms	approximate percentages
purpura	90
hepatomegaly	70
splenomegaly	50
arthralgia	60
weakness	60
arterial hypertension	35
Raynaud's phenomenon	40
leg ulcers	30
cold urticaria	*
papules, pustules	*
gangrene of the fingers/toes	*
edema of the legs, anasarca	*
symmetrical/asymmetrical polyneuropathy	*
mental confusion, coma	*
cerebrovascular accidents	*
intestinal vasculitis with abdominal pain	*
Sjögren's syndrome	*
hyperviscosity syndrome	*
congestive heart failure	*
pericarditis, cardiac infarction	*
pleural effusion, pulmonary fibrosis	*
hemoptysis	*

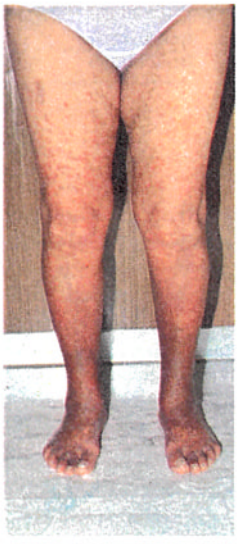
* Usually lower than 30%.

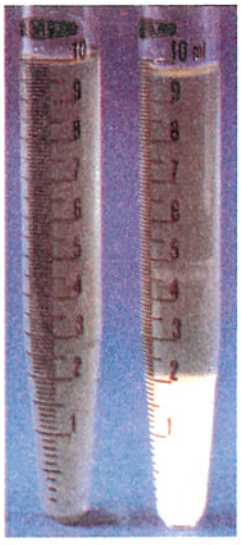
Tab. 4A - Clinical signs and symptoms frequently observed in patients with mixed cryoglobulinemia.

finding	approximate percentages
mild to heavy proteinuria	50
renal failure	40
glomerulonephritis (renal biopsy)	35
abnormal liver function tests	30
active chronic hepatitis (liver biopsy)	30
fever of unknown origin	20
recurrent bacterial infections	20
thyroiditis	*
renal tubular acidosis	*
papillary necrosis	*
demyelinization	*
occlusion of the central retinal artery	*
uveitis	*
otitis media	*

* Usually lower than 20%.

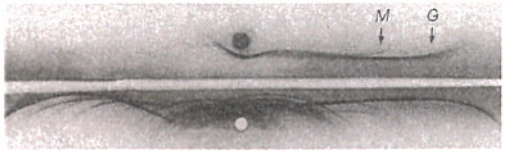
Tab. 4B - Other findings in patients with mixed cryoglobulinemia.



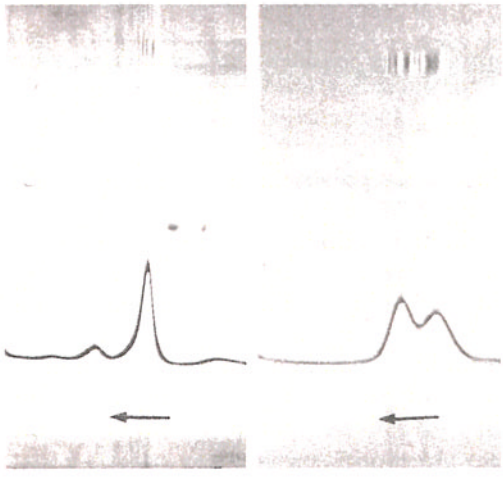


A) Chronic purpuric eruptions affecting legs and thighs of a patient with a 7-years' history of essential mixed cryoglobulinemia (EMC). Note the diffusely hyperpigmented dyschromias on the legs.

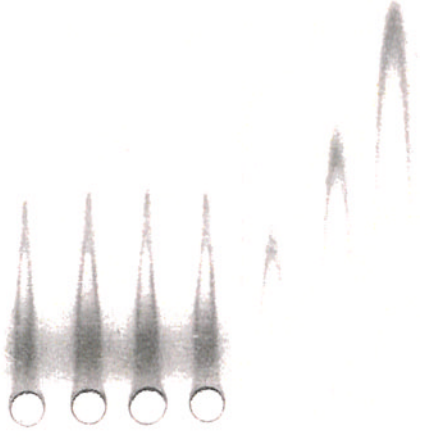
B) As compared with a pooled normal human serum (left), the serum in the right tube shows a whitish cryoprecipitate which accounts for approximately 20% of the total proteins. Tubes were kept for 72h at 4 °C and then centrifuged at 1,400 rpm for 10 min.



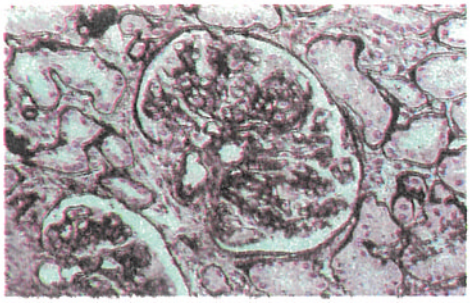
C) Immunoelectrophoresis of the purified cryoglobulin (upper pattern), isolated from the serum shown in the right tube of B) and, by comparison, of a pooled normal human serum (lower pattern). The trough was filled with a horse anti-human serum. The two lines recognizable in the cryoglobulin pattern can be identified as IgG and IgM by specific antisera and are fused for most of their length suggesting the immune complex nature of the cryoprecipitate. Owing to its poor solubility, a certain amount of protein remains in the upper well without migrating into the agar gel.



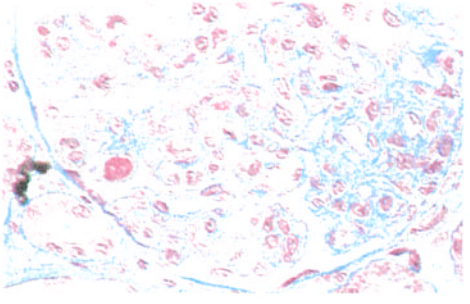
D) Analytical ultracentrifugation (speed: 59,780 rpm; temperature: 20 °C) of the isolated cryoglobulins from two patients affected with type I and type III cryoglobulinemia, respectively. Arrows indicate the direction of sedimentation. At pH 7.0 a single 19S peak is visible in the ultracentrifugal pattern on the left, along with much smaller amounts of proteins sedimenting faster and slower than the prominent 19S peak. The pattern on the right shows two main components, incompletely dissociated at this pH, with sedimentation coefficients of approximately 7S and 19S.



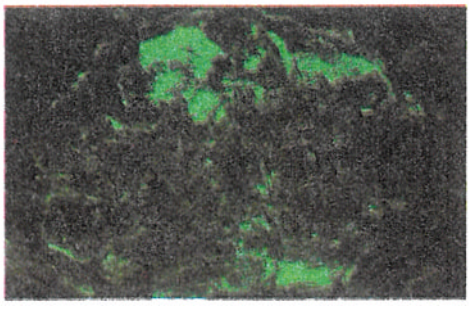
E) 'Rocket' (Laurell's) electroimmunoassay in 1% agarose gel containing a rabbit antiserum to human fibronectin (FN). Four different purified cryoglobulins (3 mg/ml) are placed in wells 1-4, whereas wells 5-7 are filled with standard FN solutions (500, 750 and 1,000 µg/ml, respectively). In spite of poor solubility and incomplete migration, a rocket is clearly visible for each cryoglobulin sample, indicating that appreciable amounts of FN are present in all the cryoproteins tested.



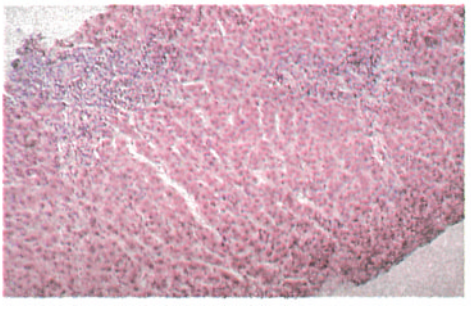
F) Renal biopsy (silver methenamine stain, x 40) from a patient with EMC. A representative glomerulus showing mesangiocapillary glomerulopathy with proliferation of mesangial cells and accentuated lobulation.



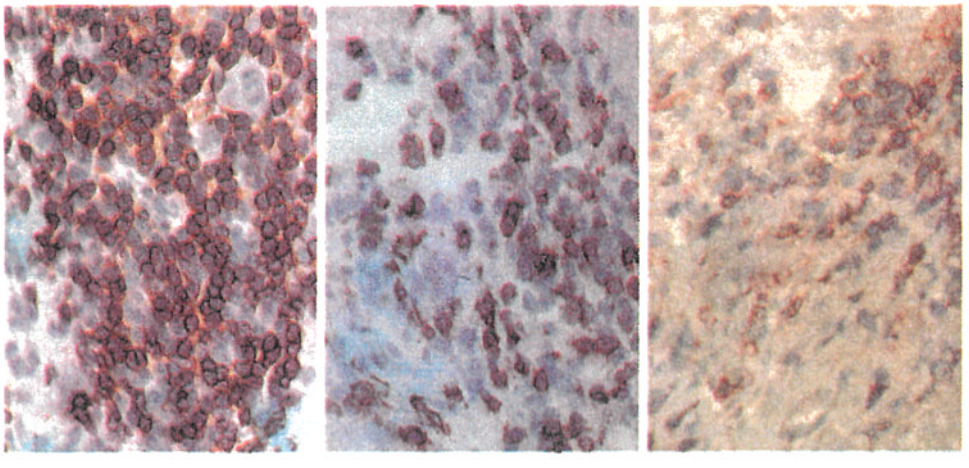
G) Renal biopsy from the same patient (acid fuchsin orange G stain, x 160). An occlusive thrombus can be seen in a capillary lumen.



H) Immunofluorescence on a renal biopsy specimen (fluorescein isothiocyanate conjugated anti-human IgM stain, x 250) showing subendothelial and intraluminal glomerular deposits of IgM. Focal 'hyaline thrombi' appear intensely fluorescent. Similar patterns were obtained in sections stained for IgG, C3 and kappa light chains.



I) Liver biopsy from a patient with EMC (hematoxylin and eosin stain, x 71). Dense infiltration of portal tracts with chronic inflammatory cells, somewhat extending into the surrounding periportal parenchyma, resulting in piecemeal necrosis.



L-N) Immunohistochemical staining (avidin-biotin-peroxidase, x 160) of T3+ lymphocytes (L) and of the T4+ helper-inducer (M) and the T8+ suppressor-cytotoxic (N) subpopulations. Heavy lymphocyte infiltration in the portal and periportal areas: phenotypic characterization shows T8+ cells to be largely prevalent.

When they appear in the absence of severe stasis dermatitis, one should suspect cryoglobulinemia. *Erythema bullosum* has been reported by KRIVO and MILLER[42].

Sometimes the first manifestation of the disease is *Raynaud's phenomenon*, which is present at the time of diagnosis in one-fourth of the cases[32]. It affects the distal parts of the limbs, the ears, the nose. There may or may not be gangrene. It occurs most frequently in patients with types I and II cryoglobulinemia.

More than 70% of the patients have *arthralgia*, intermittent, symmetrical and non-migrating[32]. It especially affects the joints of the hands and the knees (45%), but can also affect the hips and the elbows (25%).

Although skin manifestations and arthralgia are the most frequent in these patients, many other signs and symptoms develop during the course of the disease because of damage to other organs and systems[90]. The various organs may be affected at the same time as the skin or even earlier. *Renal damage* is characterized by diastolic hypertension; edema; proteinuria sometimes quite severe (up to 17 g/24h), usually associated with hematuria; and impairment of renal function that may even be as bad as renal failure. Sometimes it is the symptoms of renal damage that bring the patient to the attention of the physician, but usually they follow the appearance of the purpura after a mean interval of 4 years[32]. The histology of the kidney often shows necrosis of the tunica media of the renal arterioles and glomerular hypercellularity with polymorphonuclear lymphocyte infiltration (plate 1F, G). With immunofluorescence one often sees granular deposits of IgG, IgM (plate 1N) and complement along the glomerular basal membrane and the blood vessel walls. The tubules are dilated and separated by an edematous or slightly inflamed interstitium. The histological diagnosis is most often *proliferative* or *membranoproliferative glomerulonephritis*, which may be observed in more than one third of the patients and shows a progression to renal insufficiency in about half of them[41]. Biopsies taken from patients without renal impairment show no abnormalities, suggesting that one cannot use this to predict whether or not an asymptomatic patient will develop renal damage[32].

The organs in the abdomen are often involved. Physical examination shows *hepatomegaly* to occur in 70% of the patients and *splenomegaly* in about 50%, accompanied by high serum alkaline phosphatase levels. There are very few, if any, subjective symptoms due to enlargement of these organs[32]. As already emphasized, HBV-associated serology is not uncommon in patients with EMC and approximately 70% of these patients have evidence of hepatic dysfunction, although commonly at the subclinical level. Liver biopsy often shows a picture compatible with chronic active hepatitis (plate 1I), namely infiltration of mononuclear cells in the widened portal tract, also involving the limiting plate. An increased number of total T3+ lymphocytes can be detected by immunohistochemical methods, such cells being mainly accounted for by T8+ suppressor/cytotoxic lymphocytes (plate 1L-N).

A delayed manifestation of the disease may be *abdominal pain*, sometimes severe, in 20% of the patients, considered to be a manifestation of intestinal vasculitis.

The increased viscosity of the plasma may be the cause of the *hyperviscosity syndrome*, which is seen as retinal 'box-car' vessels, amaurosis, sensory and motor peripheral neuropathy[26], central nervous system compromise with transi-

tory hemiplegia and dysarthria, mental confusion to coma, pulmonary stasis with cyanosis, oligo-anuria. There is often congestive cardiac failure, probably due for the most part to the expansion of plasma volume because of the cryoglobulinemia.

Although pulmonary symptoms are usually absent or moderate in clinical terms, tests indicative of small airways disease may be markedly altered and roentgenographic signs of interstitial lung involvement are present in a significant number of patients[9].

Finally, there may be *complicating infections* (pulmonary tuberculosis, *herpes zoster*, bacterial pneumonia, etc.).

PYROGLOBULINS

Pyroglobulins are immunoglobulins that form a gel when the serum is heated to 56-60 °C for 30 min[50]. This precipitate is irreversible and does not respond to changes in temperature, pH or concentration, unlike the precipitates or gels formed by cryoglobulins or Bence Jones protein.

The phenomenon was discovered by chance toward the end of the last century during a complement fixation test for the diagnosis of syphilis, when the serum was inactivated by heating at 56 °C. It was interpreted at that time as being due to the presence of Bence Jones protein in the serum. Only in 1953 did MARTIN and MATHIESON[50] recognize that these proteins were not the same as the Bence Jones protein because their precipitate was irreversible. They named them pyroglobulins. Their existence was confirmed by HUISMAN et al.[37] in 1956, who analyzed their amino acid composition.

In most cases pyroglobulins are associated with multiple myeloma and macroglobulinemia or with lymphoproliferative diseases[40]. There have also been a few cases with metastatic carcinoma or systemic lupus erythematosus[51].

Pyroglobulins were found in 8 (2%) of 260 patients with monoclonal gammapathies studied by INVERNIZZI et al.[40], more frequently in patients with Waldenström's macroglobulinemia, especially if they also had cryoglobulinemia, than in patients with myeloma.

That M-components behaved as pyroglobulins was shown electrophoretically by the loss of the M-component in the serum after precipitation of the pyroglobulin. To date only single monoclonal pyroglobulins have been found. Many pyroglobulins are monoclonal IgG components; others are monoclonal IgM and are found in patients with Waldenströni's macroglobulinemia or lymphoma. MCCANN et al.[52] found IgM macroglobulinemia in a 65-year-old patient with erythrocytosis and hyperviscosity. In the literature very few reports of monoclonal IgA pyroglobulinemia can be found, usually in patients with multiple myeloma[40,78].

One possible explanation for the strict association of pyroglobulins with paraproteins is that pyroglobulins are normally present in the serum in very low amounts and increase to high concentrations when the clone that controls their synthesis is involved in an immunoproliferative process. An alternative to this is that pyroglobulins are abnormal immunoglobulins and are not to be found in normal serum.

Pyroglobulins do not by themselves cause clinical symptoms since they do not form the pyroprecipitate until the temperature is 55-56 °C, which is never

reached in a living human body. However, it is possible that applying heat to the skin of an individual with pyroglobulinemia could cause precipitation and obstruction of the cutaneous and subcutaneous vessels, with consequent necrosis of the skin. When they are present in high concentrations, they may cause hyperviscosity or coagulation disorders, such as increased thrombin time due to interference with fibrin polymerization, and abnormalities of platelet aggregation in response to ristocetin[52].

Investigations on the formation mechanisms of the pyrogel have not provided clear results. Some investigators have found a loss of thermoprecipitability of IgM pyroglobulins when they are splitted into 7S monomers[65]. Others have obtained pyroprecipitation with the monomers alone, but not with separated H or L chains[15,52].

Studies of the carbohydrate content, a decrease in which certainly affects the cryoglobulin solubility, have not shown quantitative or qualitative variations in the pyroglobulins[52]. However, amino acid sequence analysis of these peculiar proteins has shown the L chain to be normal, but the heavy chain to contain fewer cysteine and more glycine and leucine than normal[52]. This causes an imbalance between the hydrophobic non-polar residues and hydrophilic polar residues on the surface of the molecule, in favor of the non-polar, and this could be the reason for abnormal secondary and tertiary structures that would make the molecule soluble at normal temperature only by formation of aggregates. When the serum is warmed to 56 °C, there would be a further change in the steric conformation of the heavy chains, with exposure of other non-polar residues inside the hydrophobic portion, thus causing formation of an insoluble gel. Isolated H chains do not form pyrogels. Only when they are bound to L chains that normally change in solubility at 56 °C do they form strong and irreversible hydrophobic bonds.

Under the electron microscope the pyroprecipitate looks like a network of immunoglobulins strongly bonded to each other, therefore quite different from the appearance of amorphous coagulates of normal proteins precipitated at 80 °C. The hydrophobic nature of the bonding can be shown by adding an excess of sodium dodecylsulfate (SDS) to the pyrogel, in a molecular ratio of 30 to 1. The hydrophobic component of SDS interacts preferentially with the hydrophobic bonds, breaking them, while the sulfate radical confers a negative charge on the surface of the Ig. This completely solubilizes the pyrogel as Ig-SDS complexes.

The abnormal amino acid content of the heavy chain is not the only reason for the formation of the pyrogel. A conformational change, with exposure of non-polar residues, can have the same result. In addition, even an abnormality in the light chain can possibly result in pyroprecipitation.

Pyroprecipitation is not inhibited by changing the pH from 3 to 9, although it appears to be maximal at pH 7.3, nor is it affected by changing the ionic strength of the solution. In some cases, not even reduction and alkylation of the molecule with 2-mercaptoethanol followed by addition of iodoacetamide was able to prevent the pyroprecipitation[40].

SUMMARY

Cryoglobulins are serum proteins with heterogeneous etiopathogenetic and immunochemical properties. What they have in common is temperature-dependent insolubility, in that at tem-

peratures below 37 °C (often around 4 °C) they precipitate, and then redissolve at 37 °C. When the etiopathogenesis of the cryoglobulinemia is unknown, which is true for many patients, the condition is called *idiopathic* or *essential cryoglobulinemia*, whereas it is termed secondary whenever it appears to be associated with one of several diseases. Cryoglobulinemia has indeed been found in patients with lymphoproliferative and autoimmune disorders, liver diseases, infectious (viral, bacterial, fungal and parasitic) diseases, and so on. Cryoglobulins are usually classified according to their immunochemical properties as single-type monoclonal, mixtures of a monoclonal Ig with non-immunoglobulin material (DNA, lipoprotein, complement), mixed with one monoclonal Ig or mixed polyclonal, in which constitutive Ig fractions are polyclonal. As compared with normal Ig, cryoimmunoglobulins have sometimes been found to exhibit a peculiar amino acid structure of their heavy chains, less often of their light chains as well, and to have a lower carbohydrate content. Such structural abnormalities may contribute to their loss of solubility at low temperatures, possibly associated to the steric changes induced by the low temperature, causing the precipitate to form. The most common clinical features of cryoglobulins are correlated with vasculitis in the various organs and sometimes with increased viscosity of the plasma. Signs and symptoms include purpura, ulcers of the extremities, arthralgia, proteinuria, hepatic damage, abdominal pain, congestive heart failure, mental confusion, oligo-anuria, hemorrhagic diathesis, and coma. Pyroglobulins are also serum proteins with temperature-dependent insolubility. However, although they precipitate out of serum heated at 56 °C for half an hour, they do not resolubilize when the serum is returned to 37 °C. Pyroglobulins have been mainly found in patients with lymphoproliferative diseases (especially Waldenström's macroglobulinemia, with or without cryoglobulinemia), systemic lupus erythematosus, and neoplasia. So far, only single monoclonal IgG, IgM or IgA pyroglobulins have been described. Since they precipitate only at 56 °C, pyroglobulins do not cause clinical symptoms and they are usually discovered by chance.

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