Review

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Analysis, detection and quantitation of mixed cryoglobulins in HCV infection: brief review and case examples

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Abstract: Considering the high incidence of cryoglobulins in hepatitis C virus (HCV) infection together with the high worldwide prevalence of HCV infection, identification of clinically apparent mixed cryoglobulinemia syndrome is increasingly important as most patients who are identified can now be successfully treated. Different approaches for the detection, analysis and reporting of cryoglobulins have been described and there is a wide variation in results reported, ranging from a qualitative "negative" or "positive", to a quantitative report including cryoglobulin type and the total protein. Protein and immunofixation (IFE) electrophoresis are generally used to identify and characterize cryoglobulins, as these methods quantify and phenotype. Here, we present a brief review of the literature and demonstrate a case oriented approach for identifying mixed cryoglobulinemia from the preanalytical phase, leading up to and including the analytical phase with characterization by IFE. Most patients with mixed cryoglobulinemia can now be treated with success. Nevertheless, the high cost may limit treatment of those with symptoms unless there is laboratory evidence for mixed cryoglubulinemia. Low levels of cryoglobulins can be associated with severe symptoms; as a result, accurate identification of cryoglobulins may be of increasing importance since clear identification may be a good reason to initiate treatment.

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Introduction

Nature of cryogobulins

Cryoglobulins are immunoglobulins or immunoglobulin complexes that precipitate at temperatures below 37 °C and usually dissolve upon warming to 37 °C. Cryoglobulins have long been classified as: monoclonal, type I; mixed, type II; and polyclonal, type III [1]. Monoclonal type I is usually associated with plasma cell dyscrasia or other hematological malignancies such as multiple myeloma or Waldenstrom macroglobulinemia, and accounts for about 10%–15% of identified cryoglobulins while type II and type III account for about 50%–60% and 25%–30%, respectively [2]. Mixed type II and polyclonal type III are generally associated with diseases where there is a chronic infection, where the endogenous antibody cannot clear the antigen. Cold sensitivity in monoclonal type I cryoglobulins seem to be dependent on the conformation of the monoclonal Ig while mixed type II and polyclonal type III are largely dependent on the formation of circulating immune complexes.

To more appropriately direct diagnostic, monitoring, or therapeutic interventions, in heptatis C virus (HCV) patients with mixed type II cryoglobulinemia adequate analysis, detection and quantitation of mixed cryoglobulins is important. Mixed type II is generally associated with diseases where there is a chronic infection such as HCV, chronic hepatitis B or human immunodeficiency virus infection [3], and in autoimmune diseases such as Sjogren's syndrome [4], although cases have been idiopathic or associated with lymphoma or leukemia [5]. In the 1990s, it became apparent that more than 90% of patients with mixed type II cryoglobulinemia were

infected with HCV [6–8]. Serum cryoglobulins in most of the patients exist in low concentrations (100–300 mg/L) among the high concentrations (60,000–80,000 mg/L) of normal serum proteins. It is difficult to isolate such small amounts of cryoglobulin without contamination from normal serum proteins. Analytical methods for cryoglobulins have not kept up with recent advances in technology. For example, the sample volumes required for cryoglobulin tests are large (5–10 mL of serum), the rate of analysis is low (3–7 days), and routine quantification techniques need refinement. Also, the wide variability in the prevalence of mixed cryoglobulins in HCV infection may reflect differences in analytical sensitivity and technique. For example, in a previous study done to examine the prevalence of serum cryoglobulin, a gel filtration method was shown to detect cryoglobulin with greater sensitivity and specificity than the conventional precipitation method [9].

Cryoprecipitate

Several components can be found in cryoglobulin precipitates in addition to immunoglobulins, including rheumatoid factor (RF), albumin, fibrinogen, fibronectin, viruses, and bacteria. Some of these are contaminants or co-precipitants [3] (e.g. albumin and the normal immunoglobulins). In mixed cryoglobulin (type II) many components can be detected including RF, which is usually composed of monoclonal IgM κ [3]. Because of the RF activity, they can cause immune-complex vasculitis in target organs such as skin, nerves, kidneys, liver, and joints [3].

Therapies

Immunosuppressive therapies and plasma exchange remain the first line therapy in cases of idiopathic associated cryoglobulinemia and may have value for reducing vasculitis in patients with mild symptoms [2]. Nevertheless, although the increased RF is an immune response, usually long term therapies with immunosuppressive agents are not preferred because of side effects and questionable long term effectiveness [10].

Rituximab, an immunosuppressive therapy that is effective in mixed cryoglobulinemia vasculitis may form a complex with RF – positive IgM-κ leading to accelerated cryoprecipitation and induced severe systemic reactions [3]. In vitro cryoprecipitation experiments after addition of rituximab to serum containing a HCV-positive type II mixed cryoglobulin induced the appearance of a visible cryoprecipitate within <30 min [3]. Therefore, patients on high doses of rituximab with mixed cryoglobulin and high levels of complement activation are prone to accelerated formation of immune complexes between rituximab and RF-positive mixed cryoglobulin in a rituximab dose- and mixed-cryoglobulin serum level-dependent manner.

The preferred therapies are to treat the cause. In monoclonal type I cryoglobulinemia therapies should be directed against a clonal plasma cell/B-lymphocyte disorder [2]. In HCV infection, a complete and lasting disappearance of cold sensitive symptoms was observed in 88% of patients with a complete viral response to therapy [10]. Prior therapies for HCV infection were poorly tolerated and effective in only a minority of patients, but new therapies with viral serine protease and nucleotide polymerase inhibitors are well tolerated and effective in up to 95% of patients [10–13]. Thus, the correct identification of clinically apparent cryoglobulinemia may have increased importance since most patients who are identified can now be successfully treated.

Pathogenesis

Due to decreased solubility cryoglobulins tend to precipitate in the small vessels (venules, capillaries, arterioles) of various tissues, causing a cryoglobulin vasculitis [3]. Histologically, a leukocytoclastic vasculitis secondary to vascular deposition of immune complexes is seen. This can lead to ischemia, necrosis and purpura. Especially, the kidney, skin, musculoskeletal system and peripheral nervous system may be involved [3, 14]. Not all patients with mixed cryoglobulins develop clinical symptoms of vasculitis, though type II cryoglobulinemia is more associated with vasculitis and HCV is highly associated with type II cryoglobulinemia.

Rheumatoid factor

The majority (95%) of cryoglobulins are immune complexes that contain RF. Such cryoglobulins are known as “mixed” cryoglobulins to differentiate them from the cryoglobulins with monoclonal bands [3]. The RF cross-reacts with other autoantigens and binds to microorganisms covered with specific IgG antibodies, leading to agglutination and complement activation [3]. It is postulated that HCV infects circulating B lymphocytes, stimulating them initially to synthesize polyclonal IgM RF. However, unknown factors induce a shift to abnormal proliferation of a single clone of B cells that produces monoclonal IgM-κ RF, leading to type II mixed cryoglobulinemia. IgM-κ RF

binds avidly to anti-HCV IgG to the IgG-HCV immune complex leading to the presence of cryoglobulinin the serum [3]. Elevated levels of RF seem to be associated with higher incidence of cryoglobulin.

It is interesting that in mixed type II, although antigen and antibody from the chronically infecting organism are found in the cryoprecipitate [7], the major species responsible for the precipitate are RFs [14, 15]. These seem to be formed in abundance in chronic infection, apparently in an attempt to enhance clearance of the offending antigen [3]. Thus, the formation of cryoglobulins is an immune response. Usually, the RF is monoclonal or polyclonal IgM, although other Ig may be found [16]. In about 10% of cases, polyacrylamide gel electrophoresis indicates IgM microheterogeneity causing the formation of oligoclonal IgM that seem to form a type II variant. It has been suggested that this represents and intermediate in a continuous B-cell expansion from polyclonal to oligoclonal and finally to monoclonal RF [2]. Moreover, monoclonal or oligoclonal expansions of lymphocyte infiltrates have been found in the bone marrow and liver of HCV infected patients. Thus, it appears that the formation of identifiable cryoglobulins represents a type of induced plasma cell dyscrasia [2].

Organs affected

About 50%–70% of symptomatic patients with cryoglobulinemia have liver involvement, arthralgia, and asthenia, and about 25% have renal involvement [3]. The incidence of nervous system involvement is 36%.

Cryoglobulins in viral infection

Though mixed cryoglobulins are associated with viral infections, including HIV, HBV, and HCV, the prevalence of cryoglobulin is 2–4 times higher in HCV than in HBV [3]. In acute viral hepatitis of various etiologies, cryoglobulins appear in the acute period of the disease triggered by the viruses [3]. The cryoprecipitate mostly is type II, containing monoclonal IgM (RF) and polyclonal IgG in addition to the viral proteins and viral RNA. Low levels of circulating mixed cryoglobulins can be detected in 40%–66% of HCV-infected individuals, mostly asymptomatic [3].

Pathogenesis of HCV-induced cryoglobulinemia

Between 42% and 98% of patients with mixed cryoglobulinemia are affected with HCV [17]. HCV causes cryoglobulinemia by chronic stimulation of immune responses and by causing dysfunction of the reticuloendothelial system, thereby leading to the production and persistence of antibodies that are capable of precipitating below core body temperatures [17]. There is a higher prevalence of IgG3 responses to HCV antigens in those patients who are HCV- and mixed cryoglobulin-positive than in patients who are HCV-positive and mixed cryoglobulin-negative. Immunoglobulin G3 fixes complement most efficiently among the subclasses, thereby leading to activation of the classical pathway [17].

Severity of symptoms in HCV-induced cryoglobulinemia

Normal persons have very low serum concentrations of cryoglobulin (0–60 mg/L). Because cryoglobulins are heterogenous compounds that vary in chemical composition, typing, thermal properties, and ability to stimulate complement, it should not be expected that their serum levels would correlate well with the severity of symptoms. When HCV patients with cryoglobulinemia are compared as a group to those without cryoglobulinemia, the severity of disease is higher in the cryoglobulin-positive group [18].

Studies have also shown a lack of correlation between serum cryoglobulin concentration, and severity of disease [19–21]. Many patients have cryoglobulinemia without symptoms even when serum cryoglobulin levels are high. This indicates that clinical symptoms and severity do not depend simply on the serum cryoglobulin concentration. Nevertheless, it is important to document cryoglobulins in symptomatic patients as this places patients into the highest risk category where treatment should not be delayed. Also, the cryoglobulin level may be useful for following the response to treatment [17].

Analytical aspects

Several factors should be considered before performing cryoglobulin analyses. There is no internationally accepted reference value, and this lack of standardization can lead to missed diagnosis of mixed type II cryoglobulinemia [22]. Various authors have reported 20–80 mg/L as an upper cutoff [3, 22, 23]. This wide variation in reference range for total protein content in cryoprecipitate limits the clinical use of reference values for total protein content [22]. The variation could be due to co-precipitation of other proteins. The lack of

reference values hampers interpretation by the clinicans as healthy individuals may have low amounts of detectable cryoglobulins [22].

Also, while precipitation of monoclonal type I and mixed type II cryoglobulins are usually apparent by 24 h, polyclonal type III cryoglobulins are often barely visible upon precipitation may require several days before a weak precipitate is visible [23] and they are difficult to type by immunofixation electrophoresis (IFE) [3].

Total protein content and cryocrit are measures of cryoglobulin concentration but these also measure other co-precipitating proteins and are not well standardized [2]. A more complete way to identify and characterize cryoglobulins remains protein (PE) and IFE [23, 24]. To ensure the sample has been adequately collected, the blood should be pre-warmed, allowed to clot at 37 °C and kept warm prior to electrophoresis [23]. In our experience, turbidity due to lipid particles, non-immunoglobulin cryoprecipitates or other debris at refrigerated temperatures cannot be easily differentiated from cryoglobulins.

Therefore, we recommend centrifuging all suspect samples at 4 °C and looking for a pellet. Lipoproteins will float or the serum will remain turbid. If a precipitate is found, it should be vigorously washed [23], re-dissolved in warm saline or buffer and assayed for protein prior to PE/IFE. IFE will allow appropriate characterization of the precipitate. Here, we describe our approach for measuring and characterizing cryoglobulins in a case orientated report.

Materials and methods

Sample preparation

Blood in a red top tube was transported to the laboratory in a thermos container. This blood was clotted in 40 °C water. The clot was taken from the bath and immediately separated in a standard centrifuge for only 5 min. The serum was immediately removed and placed in a refrigerator at about 4 °C. The sample was examined for turbidity or a precipitate up to 72 h. If the sample remains clear, it was reported as negative.

If a precipitate or turbidity was present, the volume of the serum was determined and then centrifuged in a refrigerated centrifuge (4 °C), and the supernatant was removed, with a Pasteur pipette, taking care not to disturb the pellet. If no pellet was observed, it was reported as negative.

The pellet was kept cold and washed three times with 3.0 mL of ice cold saline and vigorous vortex between washes. After each wash the sample was separated in the refrigerated centrifuge and fluid was removed with a pasteur pipette. The final pellet was dissolve in 0.5 mL of warm saline with vigorous shaking and kept in a 37 °C bath until application to PE/IFE.

Measurement of protein

The amount of protein in the cryoglobulin solution was measured by the cerebrospinal fluid protein assay in a Vitros [25] Chemistry Analyzer (Ortho Clinical Diagnostics, Rochester, NY, USA). A biuret assay is preferred for measuring protein in cryoprecipitates since it measures peptide bonds and thereby all types of proteins more equally. Generally dye binding methods are not preferred as they are more selective for some proteins.

The Vitros uses a dye-binding method where a cupric salt and a pyridyl-azo dye or a preformed cupric-pyridyl-azo dye complex is reacted in an aqueous medium having a pH in excess of 12. This reaction forms a color having an intensity which is inversely proportional to the amount of unreacted dye. The analytical method provided by this invention for determining protein is more sensitive than analytical methods based on the biuret reaction. The amount of protein was corrected for volume based on the volume of the original serum.

Although this method appears to be as efficacious, it is important to qualitatively estimate the amount of cryoglobulin from the density of stain seen on the PE/IFE and compare this with the cryoglobulin protein concentration since quantification using methods other than biuret may be suspect.

Electrophoresis

Electrophoresis was performed with no dilution. For PE standard high-resolution split-β gels (SPIFE, Helena Laboratories, Beaumont, TX, USA) were used and for IFE, Titan Gel Immunofix-plus equipment and antisera (also Helena) were used, all in accordance with the manufacturer's instructions.

Ideally washing the cryoprecipitate and checking the re-solubility at 37 °C is recommended to avoid contamination from precipitation of normal serum proteins [26, 27]. Due to increasing limitations in person power, we have replaced re-precipitation with vigorous vortex and washing. To help ensure that the washings are complete,

no albumin should be seen upon PE of the re-dissolved pellet. If no albumin was seen on electrophoresis, IFE using antibody against IgG, IgM, IgA, κ and λ was performed on the 0.5 mL cryoglobulin solution. If albumin was seen, additional washing was performed.

Estimation of protein concentration from the electrophoresis

We always check the measured protein in the washed precipitate against the apparent density of the immunoglobulins in the stained gel to see, if in our judgment, they correspond. We expect total protein less than about 2000 mg/L to be very light and we report these as low concentration. Those between about 2000 mg/L to about 4000 mg/L are expected to stain darker and are reported as moderate concentration, and those above about 4000 mg/L are expected to stain very dark and are reported as large. Because we are using a dye binding method, in our judgment, if the gel density staining does not correspond to the measured protein concentration in the precipitate, the concentration estimated from the gel staining is emphasized in the interpretive report.

Case reports with results and interpretations

Case 1

A 61-year-old man with HCV infection was admitted to hospital because of hematuria, proteinuria and increased serum creatinine. Liver biopsy showed mild activity and mild to moderate fibrosis. The patient had a trial of pegylated interferon and ribavirin but it had been discontinued due to side effects.

Table 1 shows pertinent laboratory results. The increased serum creatinine, BUN, potassium and urine protein are consistent with rapidly progressing glomerular nephritis. Negative tests for antinuclear antibodies, antiglomerular basement membrane antibodies, antistreptolysin O antibodies, and antinuclear cytoplasmic antibodies (ANCA) rule against lupus or similar systemic autoimmune disease, Goodpasture disease, antistreptococcal nephritis, and ANCA associated vasculitis such as granulomatus polyangitis (Wegner granulomatus), respectively [28]. Although the aspartate aminotransferase (AST) (EC 2.6.1.1) and the alanine transaminase (AST) (EC 2.6.1.1) ALT (EC 2.6.1.2) were normal, these can wax and wane in chronic hepatitis. The platelets were low and the patient was HCV positive with mild to moderate fibrosis. Cryoglobulins were positive with a protein concentration of about 3500 mg/L. In this case, the positive cryoglobulin test, along with the low complement, strongly suggests renal disease due to cryoglobulin deposition. A kidney biopsy was put off until it was determine how the patient would respond to treatment.

Table 1: Laboratory results.

Test	Result	Reference range
Chemistry		
Blood urea nitrogen (BUN)	220	50–200 mg/L
Creatinine	25	5–14 mg/L
Albumin	2.7	35–48 g/L
AST	39	0–50 IU/L
ALT	36	0–45 IU/L
Potassium	5.3	3.5–5.1 mmol/L
Hematology		
White blood cells	5.5	$4.8–10\times10^9$/L
Hemoglobin	107	140–180 g/L
Platelets	148	$130–430\times10^9$/L
Urinalysis		
Protein	0.5	<0.15 g/24 h
Serology		
Complement C3	62.5	808–2010 mg/L
Complement C4	125	160–470 mg/L
Antinuclear antibodies (ANA)	<40	<40 dilution
Antibasement membrane antibodies (AGBMA)	Negative	Negative
Anitstreptolysin O (ASO)	<200	<200 IU
Antinuclear cytoplasmic antibodies (ANCA)	Negative	Negative
Cryoglobulin	3500	<60 mg/L

The results of PE/IFE are shown in Figure 1A. No albumin is seen in the PE suggesting that the precipitate was sufficiently washed. The densitometer profile of the PE shows an apparent protein spike and a polyclonal distribution. This is confirmed by the IFE that shows monoclonal IgM-κ staining and polyclonal IgG, κ and λ staining, indicating a mixed type II cryoglobulin. The final report indicated a mixed type II, moderate to large concentration cryoglobulin, consistent with HCV infection.

Case 2

Figure 1B shows an PE/IFE from a patient with IgG-κ multiple myeloma. This cryoprecipitate was voluminous and the cryoglobulin protein was 10,000 mg/L. The PE showed a peculiar pattern. The standard washing procedure failed to

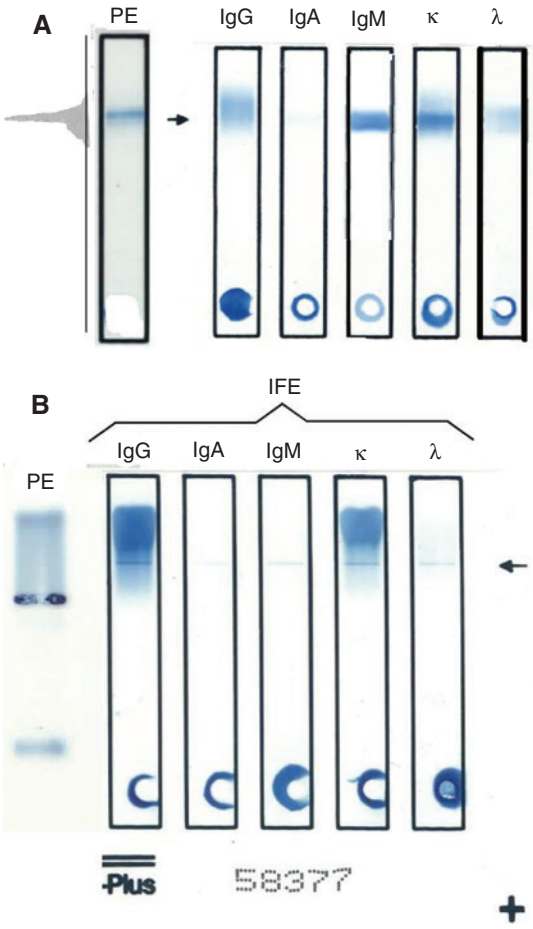

Figure 1: Protein (PE) and immunofixation (IFE) electrophoresis. The type of immunoglobulin fixed is indicated. The direction of migration is down towards the positive electrode (+). Arrow indicates the origin. Circles at the bottom of the gel indicated control wells. (A) Case 1; (B) Case 2.

remove all the albumin and an albumin band is seen near the anodal end. Nevertheless IFE shows the cryoglobulin is IgG-κ with almost no staining for λ, IgA or IgM. There is a tiny band at the origin in all lanes where the sample was placed indicating some of the precipitate was trapped and did not migrate in the gel. Based on the patient's clinical history and the IFE patterns, it was concluded that the cryoglobulin was monoclonal type I, IgG-κ and no additional washings were conducted. The final report indicated a very large concentration monoclonal, type I cryoglobulin, consistent with monoclonal gammopathy.

This is an interesting case, which requires a bit of interpretation. The PE shows a peculiar pattern that does not appear to be a simple monoclonal protein but appears as if it is mixed with a slightly dense band toward the cathode, a denser band in the front of the γ region and what appears to be polyclonal between them. Nevertheless, IFE shows the major density is towards the cathode. Also, there is a tiny bit of λ staining at the origin. But in this case, the exceptional size of the precipitate suggests, it would be difficult to wash away all of the absorbed protein. The PE shows a small albumin band indicating less than a complete wash. It was concluded that the small amount of λ did not represent polyclonal free soluble λ but a small amount of λ absorbed to the monoclonal protein. Moreover, the monoclonal was IgG and not IgM that is commonly found in type II mixed cryoglobulinemia, finally, this patient had an IgG-κ myeloma. One can only conclude this is a monoclonal IgG-κ type I cryoglobulin.

Discussion

As there is now effective treatments for HCV hepatitis and because most cryoglobulins are associated with this disease, it is likely that analysis of these species will become increasingly important. The type of cryoglobuin may not change the therapeutic approach in patients with HCV induced cryoglobulinemia but the cost of these treatments currently approaches $100,000, so that treatments may be limited for patients with minimal liver disease [29]. Although it is now recommended to treat all patients with HCV infection, due to the high cost of the drugs [30], patients at highest priority should be treated first. These include those with advanced fibrosis, compensated cirrhosis, liver transplant recipients, and those with severe extrahepatic complications, such as cryoglobulinemia http://hcvguidelines.org. Thus, clear identification of a mixed type II cryoglobulin in a symptomatic patient may be a good reason to begin treatment.

IFE interpretations are often clear but may be more complex, as shown in Figure 1B, where a peculiar PE pattern was interpreted as a monoclonal type I based on IFE and history. It is important to remember that cryoglobuliemia can be mild, requiring no intervention and the severity of cold sensitive symptoms do not necessarily correlate with the amount or type of cryoglobulin [3, 15]. Besides, testing for cryoglobulins is complicated by the lack of reference range, standards, and stringency in maintaining testing temperature conditions [16], and that contamination by other proteins can cause false positive results, which can be a problem when low concentrations of cryoglobulins are present [22].

Moreover when low concentration cryoglobulins are present in mixed cryoglobulinemia, the diffuse polyclonal pattern may be sufficiently indistinct so that only the predominant monoclonal IgM may be seen and the pattern may be misinterpreted as a type I. As a result of the methodological and clinical complexities, as illustrated

in case 2, it is essential to consider the patient's clinical history as well as the methodological findings when finalizing these interpretations. Thus, a patient with a hematological malignancy may also have a monoclonal protein with RF activity that may appear as a mixed cryoglobulinemia. In such a case chronic viral infection should be ruled out. Besides, it is not impossible that that a patient with HCV infection could also have plasma cell dyscrasia. Thus, identification of a monoclonal type I in a HCV patient should lead to a workup that would rule out likely plasma cell dyscrasia.

IFE or an equivalent technique is essential in making these interpretations. The hospital laboratory scientist usually has the clinical information available and is in a unique position to synthesize the methodological and clinical information to relay the interpretation to the clinician.

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