Review:
Cryoglobulins: An Important but Neglected Clinical Test

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Abstract. Cryoglobulin (CR) denotes a serum immunoglobulin that precipitates at temperatures below 37°C and dissolves on re-warming. CRs are heterogeneous in chemical composition and behave differently in vivo and in vitro. The majority are mixed antigen-antibody complexes that occur with high incidence in autoimmune and infectious disorders. Their measurement is important in the management of patients with vasculitis. CRs elicit variable symptoms in patients, mostly purpura, weakness, and arthralgias, and they require various methods of treatment. Sometimes CRs are not associated with any symptoms; but they can be associated with very severe conditions such as nephropathy and neuropathy. Treatment depends on the symptoms and causes, and on the phenotyping of the CR. Considering the high incidence of CR in diseases such as hepatitis C virus (HCV) infection, together with the high worldwide prevalence of this disease, it is clear that testing for CR is underutilized in clinical practice. CR testing has been neglected in routine clinical laboratories and by clinicians due to several factors, such as the lengthy time for serum CR analysis and failure to appreciate that low levels of CR can be associated with severe symptoms. In a series of 194 serum samples that gave positive tests for CR at our institution, the majority contained low CR concentrations (65% of the samples were type II with a mean of 372 mg/L and 39% of type III with a mean of 216 mg/L; reference range 0-60 mg/L). Case studies are presented to illustrate the importance of such low levels of CR. There is a need for more rapid and more reliable methods for quantification and phenotyping of low concentrations of serum CR. Based on our experience in the routine analysis, quantification, and phenotyping of serum CR, some practical solutions to these problems are presented.

Keywords: cryoglobulins, immunoglobulins, immune complexes, rheumatoid factor, hepatitis C, vasculitis, multiple myeloma, Waldenstrom's macroglobulinemia, Sjogren's syndrome, SLE, purpura

Introduction

Cryoglobulin (CR) analysis is an important test in the care of patients with vasculitis. However, in routine clinical laboratories and clinical practice this test has not received adequate attention. This is due to several problems: 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 CR without contamination from normal serum proteins [1]. CRs, like other immunoglobulins, are variable in amino acid composition and are heterogeneous [1]; they behave differently in vivo and in vitro. Analytical methods for CR have not kept up with recent advances in technology. For example, the sample volumes required for CR tests are large (5-10 ml of serum), the analysis is slow (3-7 days), and routine quantification techniques need refinement [1]. Standards and controls are lacking. In addition, clinical laboratory personnel often lack experience in interpreting the electrophoretic patterns and the quantitative results of serum CR tests; they have scant appreciation of the importance of low CR levels.
A wide gap exists between the knowledge of CR in research and in routine analysis. At the present time, on the NIH Pub-Med website under the word “cryoglobulins” there are close to 3000 references. This vast bibliography reflects the great interest and complexity of this subject in basic and clinical research. On the other hand, most clinical laboratories only offer a simple qualitative CR analysis (visual detection). As discussed later, positive CR tests have been reported in many autoimmune disorders and infections, but requests for CR testing are far less frequent than would be expected. The aim of this review is to provide an overview of cryoglobulinemia and to focus attention on two areas: first, the importance of CR testing in patient care, especially for the low levels; and second, to suggest ways to improve the analytical techniques. The goal is to stimulate CR testing in routine patient care.

**Fundamental Considerations**

**Definition.** Cryoglobulins (CRs) are special serum immunoglobulins that precipitate at temperatures <37°C, but mostly at 0 - 4°C, and that dissolve when re-warmed to 37°C [2,3]. The majority (95%) of CRs are immune complexes (IC) that contain rheumatoid factor (RF). Such CRs are known as “mixed” cryoglobulins to differentiate them from the CRs with monoclonal bands that do not contain RF or antigen-antibody complexes. The antigens present in the mixed complexes may include RNA and proteins (eg, hepatitis C virus proteins). A small fraction of CRs (5%) comprise pure monoclonal gammopathies, which have poor solubility at low temperatures because of their unique amino acid sequences. There are also other cryoproteins that precipitate in the cold, such as cryofibrinogen (in plasma) and fibronectin [1].

**History.** In 1933, Winthrobe and Buell [2] first observed the precipitation of protein upon cooling serum from a patient with Raynaud’s phenomenon and the subsequent dissolution of the protein upon sample re-warming. In 1947, Lerner and Watson coined the term “cryoglobulin” [3,4]. In the mid-1960s, Meltzer described the syndrome of purpura, arthralgia, asthenia, and renal disease in association with cryoglobulin and immune complex deposition [3,4]. In 1974, Brouet et al [5] popularized a classification of CRs according to their immunochemical composition. In the late 1980s, because chronic hepatitis, mainly hepatitis C (HCV), was frequently observed during the clinical course of mixed cryoglobulinemia, a role for hepatotropic viruses in the pathogenesis of the condition was suggested [6,8] and later confirmed [9]. Based on this finding, many cases of mixed cryoglobulinemia, that in the past were called “essential cryoglobulinemia,” are now recognized as being due to viral infections, mainly HCV [9,10].

**Classification.** CRs are divided into 3 types (Fig 1). Type I is composed of monoclonal immunoglobulins (large monoclonal bands detected in both the serum and the CR precipitate), which usually denote malignancy. Types II and II are composed of immune complexes (antigen-antibody complexes)
and thus called mixed cryoglobulins (mixed CRs). Type III comprises immune complexes containing polyclonal rheumatoid factor (RF). Type II also comprises immune complexes, but with monoclonal RF. The importance, frequency, and detection methods of the 3 types of CRs are described later.

Cryoprecipitate composition. Several components can be found in CR precipitates in addition to immunoglobulins, including RF, albumin, fibronectin, fibrinogen, viruses, and bacteria [1,4]. Some of these are contaminants or co-precipitants (eg, albumin and the normal immunoglobulins). In CR type I only a monoclonal immunoglobulin should be detected. However, in the mixed CRs (types II and III) many components can be detected including RF, which is composed of IgM K (monoclonal in type II, and polyclonal in type III). Because these complexes have rheumatoid factor activity they can cause immune-complex vasculitis in target organs such as skin, nerves, kidneys, liver, and joints. The RF is complexed with polyclonal immunoglobulins directed against the stimulating agent (viruses, bacteria, or specific immunogens) [11]. By itself, the RF does not precipitate in the cold, but it does so when bound to polyclonal or monoclonal immunoglobulins [11].

Pathogenesis. Mixed cryoglobulins, because of their decreased solubility in serum, tend to precipitate in the small vessels (venules, capillaries, arterioles) of the different tissues, causing a special type of vasculitis (cryoglobulinemic vasculitis). The histological hallmark of this condition is leukocytoclasic vasculitis secondary to vascular deposition of circulating CR immune-complexes and complement, together with leukocytes. Cryoglobulinemic vasculitis may involve many organs, particularly the skin, peripheral nervous system, and kidneys. The deposition of CR in and around the wall of the capillaries can lead to ischemia, infarction, and purpura. Not all patients with mixed CR develop clinical symptoms of vasculitis [1,4,10]. CR vasculitis may or may not be accompanied by inflammatory infiltrates, typically lymphocytes [10]. Purpura is more frequent in patients with a low cryocrit [10]. Type II cryoglobulinemia is more associated with vasculitis [10]. As discussed later, hepatitis C (HCV) is highly associated with type II cryoglobulinemia.

Rheumatoid factor. Low affinity rheumatoid factors (often K light chains) are natural polyreactive antibodies present in human serum that have specificity for IgG. The normal role of RF in the immune response is unclear but it probably aids in immune complex clearance by making complexes larger and activating complement. The RF cross-reacts with other autoantigens and binds to microorganisms covered with specific IgG antibodies, leading to agglutination and complement activation. 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-K RF, leading to type II mixed cryoglobulinemia [11]. As will be discussed later, CRs similar to those in the serum can be detected in white cells of the patients. HCV-RNA is found concentrated and bound to the CR precipitate [10,12]. Patients with HCV and cryoglobulinemia type II have been found to have a Bcl-2 rearrangement in peripheral blood leucocytes [13]. Occasionally, a small monoclonal protein (IgM RF type II CR) is present in the plasma of hepatitis C patients, which resembles the small paraprotein in monoclonal gammopathy of undetermined significance. IgM-K RF binds avidly to anti-HCV IgG or to the IgG-HCV immune complex leading to the presence of CR in the serum. These circulating immune complexes concentrate in capillaries of different tissues (eg, renal glomeruli), where they deposit in the subendothelium and mesangium and initiate cellular proliferation and leukocyte infiltration [11]. Chronic HCV infection may also produce autoantibodies to native renal antigens, which may account for some of the glomerular pathologic response that occurs in membranous glomerulonephritis. Elevated levels of RF factor seem to be associated with higher incidence of CR [12,14]. In many patients with type II cryoglobulinemia, the CR is not simply monoclonal RF, but an oligoclonal form [15,16] that is termed type II-III. This may represent a transition from type III to type II.
Clinical Aspects

Clinical manifestations. The common symptoms of CR are due to cutaneous ischemia, and include purpura, livedo reticularis, ecchymosis, ulcerations, ischemic necrosis, and, rarely, gangrene. Other manifestations of CR are chronic hepatitis, membranoproliferative glomerulonephritis, vasculitic neuropathy, dysesthesia, Raynaud’s syndrome, and secondary Sjogren’s syndrome. In one study, the typical clinical triad—purpura, weakness, arthralgia—was present in almost 80% of patients at the time of diagnosis [17]. Purpura remains the main (~80%) clinical sign of cryoglobulinemia. It is intermittent, with palpable lesions appearing on the lower limbs and less frequently on the buttocks, trunk, or face. Lower-limb purpura is usually preceded by paresthesia or local pricking sensations rather than frank pain. The eruptions vary greatly in frequency.

About 50-70% of symptomatic patients with cryoglobulinemia have liver involvement, arthralgia, and asthenia, and about 25% have renal involvement [4,10]. CR vasculitis affects the kidney usually with a membranoproliferative glomerulonephritis and affects the prognosis and the survival rate of the patients [10,17]. The incidence of nervous system involvement is 36% [10], affecting the peripheral nervous system and presenting as sensory-motor neuropathy, especially in the lower limbs, with painful paresthesias and loss of strength.

Diagnosis. Cryoglobulinemia denotes the presence of circulating immunoglobulins that precipitate at a temperature <37°C and re-dissolve on warming (Fig. 2). There are no generally accepted clinical diagnostic criteria for mixed cryoglobulinemia [18]. An Italian group has proposed major criteria for classification of patients with mixed cryoglobulinemia based on findings of circulating mixed cryoglobulins, decreased serum complement, and orthostatic skin purpura, which are hallmarks of the disease. The diagnosis is supported by secondary criteria that include positive tests for RF, HCV, HBV, chronic hepatitis MPGN, peripheral neuropathy, and skin ulcers [18]. Mixed cryoglobulinemia is classified among the systemic vasculitides in the subgroup of small vessel vasculitides [18]. Patients with orthostatic skin purpura, cutaneous vasculitis associated with systemic symptoms, especially in the presence of hypocomplementemia, and those with monoclonal gammapathy with cold-sensitivity or hyperviscosity, should be tested for CR. Cryoglobulinemia can be detected accidentally as a laboratory finding, sometimes without evident clinical relevance [4]. It can be very mild requiring no intervention, but it can also be associated with severe symptoms.
Pathophysiology of CR in viral infection. Mixed CRs (the majority of CRs) are associated with viral infections, including HIV, HBV, and HCV [5,6,19]. In acute viral hepatitis of various etiologies, CRs appear in the acute period of the disease triggered by the viruses. The CR-precipitate mostly is type II, containing monoclonal IgM (RF), and polyclonal IgG in addition to the viral proteins and viral RNA. Levo et al [7] were the first to implicate hepatitis B virus (HBV) as a causative agent of mixed cryoglobulinemia, and subsequent studies indicated a high prevalence of HBV surface markers in the sera and cryoprecipitates of patients with mixed cryoglobulinemia. However, the prevalence of CR is 2-4 times higher in HCV than in HBV (21-23). The association of hepatitis C (HCV) and cryoglobulinemia and the pathogenesis of HCV-induced cryoglobulinemia have been intensively studied [4,9,13,14].

Low levels of circulating mixed CRs can be detected in 40-66% of HCV-infected individuals, mostly asymptomatic. A meta-analysis of 19 studies involving a total of 2323 patients showed the prevalence of cryoglobulinemia to average 44% [24]. Overt cryoglobulinemia syndrome develops in about 5%. There are geographical differences in the prevalence. For example, the prevalence is much higher in Japanese patients than Egyptian patients [14]. The prevalence also, as expected, depends on the sensitivity of the analytical method [21].

Considering the high frequency of positive CR tests in patients with HCV, the percentage of those with severely symptomatic vasculitis appears low. Circumstances that predispose HCV-infected patients to develop mixed cryoglobulinemic vasculitis remain unclear, but the host’s immune response genes may play a role [25]. Based on multivariate analysis, four independent factors appear to be associated with the presence of CR; female gender, alcohol consumption, HCV genotype II or III, and extensive liver fibrosis [25]. In general, patients with cryoglobulinemia tend to have more severe arthralgia and elevated liver enzymes [20,26], compared to patients without cryoglobulinemia.

Pathogenesis of cryoglobulinemia in hepatitis and lymphoproliferation. HCV is considered to be both a hepatotropic and lymphotropic virus. About 5-30% of patients with CR type II eventually develop non-Hodgkin’s lymphoma [10,17]. Moreover, HCV-related proteins and replicative particles were detected on the peripheral lymphocytes [28]. The HCV envelope protein E2, which is able to bind to CD81 molecule expressed on B-lymphocytes, may be involved in the first steps of HCV-driven autoimmune and lymphoproliferative phenomena. Interestingly, translocation along with Bcl-2 activation has been demonstrated in B-lymphocytes of many HCV-related cryoglobulinemias [27-29]. Mixed cryoglobulinemia, renal syndromes, lymphoproliferative disorders, Sjogren syndrome, porphyria cutanea tarda, and neuropathies are all strongly associated with HCV infection [29]. The monoclonal CR in type II can be used as a marker to predict the development of lymphomas and ulcerative-necrotic vasculitis in other disorders (eg, HIV, Sjogren’s disease) [30]. For example, 20% of Sjogren’s disease patients have CR type II. After 10 yr, half of the patients with CR developed lymphoma and ulcerative-necrotic vasculitis, while only 5% of patients without CR developed lymphoma [30]. Careful clinical monitoring of patients with HCV-related CR is important, with particular attention to neoplastic complications.

Disorders associated with different types of cryoglobulins. Based on several reports [4,5,31], the disorders associated with CR can be grouped into lymphoproliferative, connective tissue (autoimmune), and infectious disorders (Table 1).

<table>
<thead>
<tr>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal gammopathy</td>
<td>Hepatitis C virus</td>
<td>Systemic lupus erythematosus (SLE)</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>Autoimmune disease</td>
<td>Biliary cirrhosis</td>
</tr>
<tr>
<td>Waldenstrom’s macroglobulinemia</td>
<td>Sjogren’s syndrome</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td></td>
<td>Chronic lymphocytic leukemia</td>
<td>Viral infections (CMV, Epstein-Barr, HIV, HBV)</td>
</tr>
<tr>
<td></td>
<td>Non-Hodgkin’s lymphoma</td>
<td>Bacterial infections (leprosy, spirochetes)</td>
</tr>
</tbody>
</table>

Table 1. Disorders associated with the different types of CR.
Experience at our institution regarding the frequency of various diagnoses and the concentrations of CR in 72 symptomatic patients is summarized in Table 2. Lymphoproliferative disorders represent a small fraction of the patients. On the other hand, the majority of the patients with CRs have infectious (especially HCV) or autoimmune disorders. Consistent with our data, mixed CRs are often detected in autoimmune rheumatic diseases [30,31]: systemic lupus erythematosus (SLE) (25%), Sjogren’s syndrome (17-37%), and rheumatoid arthritis (46%) [4]. Ferri et al [29] reported the frequency of renal involvement in cryoglobulinemia as 31%. At our institution 17% of patients with positive CR tests have renal involvement (Table 2). The renal manifestations range from isolated proteinuria to overt nephritis or nephrotic syndrome (see Case 1, discussed later). The most frequent histological picture of cryoglobulinemic glomerular nephritis (GN) is that of membranoproliferative GN with subendothelial deposits [32,33]. Examination by light microscopy shows aggregates of CR (hyaline thrombi) within capillary lumina.

**Table 2. Serum CR concentrations in 72 symptomatic patients at our institution (based on 1-wk precipitation).**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of subjects</th>
<th>% of subjects</th>
<th>Serum CR level (mean, mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple myeloma</td>
<td>1</td>
<td>1.4</td>
<td>2200</td>
</tr>
<tr>
<td>Skin purpura</td>
<td>9</td>
<td>12.5</td>
<td>443</td>
</tr>
<tr>
<td>Renal diseases</td>
<td>12</td>
<td>16.7</td>
<td>434</td>
</tr>
<tr>
<td>Infectious diseases</td>
<td>28</td>
<td>38.9</td>
<td>133</td>
</tr>
<tr>
<td>Rheumatoid arthritis/SLE</td>
<td>14</td>
<td>19.4</td>
<td>175</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>8</td>
<td>11.1</td>
<td>87</td>
</tr>
</tbody>
</table>

**Table 3. Characteristics of the three types of serum cryoglobulins.**

<table>
<thead>
<tr>
<th>Type of CR</th>
<th>Immunoglobulin (Ig) composition</th>
<th>Incidence (%)</th>
<th>CR concentration (mg/L)</th>
<th>Speed of CR precipitation</th>
<th>CR stability in frozen serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Monoclonal Ig (unmixed) (RF is absent)</td>
<td>5-15</td>
<td>&gt;2000</td>
<td>Rapid (30°C)</td>
<td>Stable</td>
</tr>
<tr>
<td>II</td>
<td>RF-IgM (monoclonal) + polyclonal IgG (mixed)</td>
<td>40-60</td>
<td>200-5000</td>
<td>Intermediate</td>
<td>Unstable</td>
</tr>
<tr>
<td>III</td>
<td>RF-IgM (polyclonal) + polyclonal Ig (mixed)</td>
<td>40-60</td>
<td>60-1000</td>
<td>Slow (3-7 days)</td>
<td>Unstable</td>
</tr>
</tbody>
</table>

**Importance of typing CRs.** There are 3 types of CR (Fig 1), and their characteristics are summarized in Table 3. The typing of CRs is based on immunological composition and is useful in differentiating cryoglobulinemia due to malignancy from that due to immune stimulation (ie, antigen-antibody binding). Consequently, CR typing is important for selection of the proper treatment. There are several ways to detect and classify the CR types, mainly electrophoresis and immunofixation. Capillary electrophoresis (CE) [34] is the most practical method for CR typing, as discussed later. CE is rapid, simple, and sensitive. It involves analyzing the CR precipitate and the warmed serum. Gel electrophoresis can also be used, but it requires a larger sample volume and longer time for analysis.

Type I cryoglobulinemia is generally associated with malignancy (multiple myeloma) or high serum viscosity (eg, Waldenstrom’s macroglobulinemia) and requires special and more aggressive treatment. This type, although not common, can be recognized easily. It is characterized by high serum CR concentration (>2 g/L) with clearly visible turbidity or precipitate. The CR precipitates (or gels) easily and rapidly, sometimes at room temperature. CR may be detected incidentally in the laboratory because of interference in other laboratory tests. Electrophoresis confirms the presence in the serum of a large monoclonal protein band that is also present in the precipitate, but at a higher proportion (relative to albumin) when compared to that in the serum (Fig. 1). Immunofixation is used to identify the type of the monoclonal protein for diagnosis and treatment (eg, Waldenstrom’s macroglobulinemia, which causes high serum viscosity, or multiple myeloma, which represents malignancy). From a
clinical perspective, any patient with monoclonal gammopathy and cold-sensitive symptoms should be examined for CR.

In cryoglobulinemias of types II and III, the warm serum does not contain any monoclonal peak (Fig. 1). In type II, the CR precipitate only contains a monoclonal peak, whereas in type III no monoclonal peak is present in the CR precipitate. Differentiating type II from type III has less immediate importance since both types indicate immunostimulation and/or infection. However, differentiating types II and III is important for prognosis, since type II may eventually develop into non-Hodgkin’s lymphoma. The serum CR concentration is usually very high in type I, so from CR quantitation a good idea about the typing can be deduced (Tables 3 and 4).

CR concentration and the severity of symptoms. Normal persons have very low serum concentrations of CR (0-60 mg/L) [34]. Because CRs are heterogeneous compounds that vary in chemical composition, typing, thermal properties, and ability to stimulate complement [35-37], it should not be expected that their serum levels would correlate well with the severity of symptoms.

Immune-mediated vasculitic lesions are responsible for the different clinical symptoms of cryoglobulinemia, including cutaneous and visceral organ involvement [10,18]. CRs of types II and III are accompanied by RF, activate complement, and thereby cause vasculitis [10,11]. Type III is not associated with development of malignancy and is less associated with vasculitis. Type II occurs with chronic infections but it can lead, as discussed later, to leukemia. Since type I CR is not accompanied

Table 4. Of 960 serum samples submitted for CR analysis at our institution during a 2-yr period, 194 samples gave positive tests for CR. This table lists the numbers (and percentages) of positive samples containing the three CR types, and the mean concentrations of serum CR in the respective types.

<table>
<thead>
<tr>
<th>Type of CR</th>
<th>CR-positive serum samples (N)</th>
<th>CR concentration (mean, mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>9</td>
<td>10,222</td>
</tr>
<tr>
<td>II</td>
<td>109</td>
<td>372</td>
</tr>
<tr>
<td>III</td>
<td>76</td>
<td>216</td>
</tr>
</tbody>
</table>

by RF or immune complexes, this type does not activate complement and causes less vasculitis but more hyperviscosity. Type I is associated with B-cell malignancy. Thus the different CRs act through different and complex mechanisms.

When HCV patients with cryoglobulinemia are compared as a group to those without cryoglobulinemia, the severity of disease is higher in the CR-positive group [23]. For example, patients with CR had increased fibrosis and increased incidence of cirrhosis compared to those without CR [38], but there was no significant correlation between the serum CR level and hepatic histological activity index [35]. Also, in patients with mixed cryoglobulinemic glomerulonephritis, correlation between serum cryoglobulin level and disease activity was not significant [37]. Similar findings were reported for the development of lymphoma in SD patients [20]. The fact that many patients have cryoglobulinemia without symptoms [1,4,10,29,35], even when serum CR levels are high [17], indicates that clinical symptoms and severity do not depend simply on the serum CR concentration but on other factors mentioned above [4,13,35-38].

Dammacco et al [10] observed that “there is an inverse relation between vasculitis and the cryocrit: the lower the cryocrit, the greater the frequency of signs of vasculitis, especially in type II mixed cryoglobulinemia. This is a still unexplained phenomenon, but it seems to be related to the intrinsic capacity of immune complexes to activate complement in situ” [10]. Because of the lack of correlation between serum CR concentration and severity of disease, low CR values should not be ignored. Sensitive methods for detection CR at low levels are important for patient diagnosis and prognosis. Furthermore, in patients with a positive CR test, the CR level is useful for following the response to treatment [4].

During the past 2 yr, 960 tests for serum CR were ordered in our institution, with 194 samples yielding positive results (Table 4). Of the positive samples, 109 were type II with an average CR concentration of 372 mg/L, and 76 samples were type III with an average CR concentration of 216 mg/L; there were 9 samples of type I with an average CR concentration of 10,222 mg/L. As shown in Fig. 3, the majority of the positive samples
had low serum CR concentrations (100-300 mg/L). As the method of CR detection becomes more sensitive the percentages of positive samples of types II and III tend to increase, but the percentage of positive samples of type I decreases.

Mechanisms of cryoprecipitation. The solubility of proteins depends on many factors, such as the protein concentration, hydrophobicity, size, and surface charge, as well as the solution temperature, pH, and ionic strength. Changes in the primary structure of the variable portion of Ig, and reduced concentrations of sialic acid and galactose in the Fc region of Ig, may be related to decreased solubility of CRs. Because of the high molecular weight of CR complexes (ie, mixed CR), they are less soluble and tend to precipitate at cold temperatures in vitro and in vivo. Production of IgM-RF complexes represents a crucial factor in the pathophysiology of cryoprecipitation [11,39]. The formation of cryo-aggregates upon exposure to cold may be the triggering factor for vasculitis; however this does not explain why tissues (eg, kidney, nerves) that are distant from the site of exposure to cold may be affected. One explanation, suggested by in vitro studies, is that alterations of chloride concentration may influence CR structure and aggregation [40].

Prognosis. Mixed cryoglobulinemia is generally a benign immunologic disorder. The patients usually have mild symptoms characterized by sporadic purpura and arthralgia. However, the prognosis is affected by several complications, in particular the development of malignancy. The 10-yr survival rate was significantly lower in 231 patients with cryoglobulinemic vasculitis compared to an age- and sex-matched general population, being significantly worse in men than women and in patients with renal involvement [41]. Membrano-proliferative glomerulonephritis significantly affects the prognosis and survival [41]. The impairment of renal function can be ameliorated or retarded with proper treatment as discussed later.

Analytical Aspects

Overview. Several factors should be considered before performing CR analyses. CR assays have not been adequately standardized. The precipitation step of CR is often incomplete, especially for type III. CR precipitates are generally contaminated with normal serum proteins [1]. The presence of albumin as measured by electrophoresis or microalbumin assay confirms this fact [1]. Most contamination of the CR precipitate comes from serum proteins that adhere to the walls of the tube. Washing steps can decrease the serum protein contamination but they also cause a loss of CR precipitate, especially when dealing with small volumes [3]. Based on our experience, about one-third of the CR precipitate is lost with each washing step. Hence it is wise to minimize the washing steps and to correct for serum protein contamination based on microalbumin assay.

Cryoglobulins vary substantially from one patient to another. CRs differ widely in the temperature and rate of precipitation (type I can precipitate in few minutes while type III may require a week). Some CRs dissolve easily in warm saline while others are difficult to redissolve. As shown in Table 3, the majority of CR are types II and III, which are unstable when frozen; they should be analyzed promptly. On the other hand, some CR samples become denatured and precipitate if left for an extended period (ie, a few days) at room temperature. Redissolution of the CR precipitate upon re-warming is important for CR confirmation. The differences in prevalence of CR in various studies may reflect differences in analytical sensitivity and techniques [21].
Several methods have been described for CR analysis, including electrophoretic and non-electrophoretic methods (e.g., cryocrit assays, immunoassays, protein assays by ultraviolet absorption, etc.) [1,3]. The electrophoretic methods have the advantage that they do not need sample washing to remove non-CR serum proteins. The results can be corrected for serum protein contamination by comparing the ratio of albumin/immunoglobulins in the CR precipitate to that in the serum. The electrophoretic methods also allow direct phenotyping. Most methods for CR quantification ignore the fact that the cryoprecipitate is heavily contaminated by normal serum proteins, as evidenced by the presence of albumin, unless the precipitate is washed repeatedly [1]. Measurement of RF has been suggested instead of CR [14,23].

In type I cryoglobulinemia, which is relatively rare, the blood sample should be collected and brought to the laboratory at 37°C. However, in patients with mixed CR (types II and III), blood samples can be collected and transported to the laboratory at room temperature.

**Visual screening and qualitative detection of CR.** Cryoglobulins cause serum to become turbid upon cooling; visual observation of turbidity is the easiest method of detecting CR. However, the turbidity (Fig. 2A) is neither specific nor quantitative, especially at low levels where lipids (especially chylomicrons) and fibrinogen may interfere. Visual inspection of the CR precipitate after centrifugation (preferably in the cold as a cryocrit) avoids these problems and is more sensitive, especially when a portion of the serum sample is kept at 37°C as a control (Fig. 2B). Different CRs produce different degrees of turbidity. In about half of the positive samples, serum CR concentrations are <300 mg/L (Table 4, Fig. 3) and such levels are difficult to detect visually based on turbidity. However, CR levels close to the upper reference range (~100 mg/L) can be detected after centrifugation of the cold and warm tubes (Fig 2C).

**Semi-quantitative detection of CR by cryocrit.** The cryocrit is a simple and widely used method [1,3]. A special conical tube (Wintrobe) is filled with 5 to 10 ml of serum and left in a refrigerator for 3-7 days. The tube is centrifuged and the volume of the CR precipitate is read from the tube markings [3]. The precipitate can then be washed and analyzed further [3]. This method has good sensitivity, but quantification of the CR precipitate as a cryocrit requires a large volume of serum, is affected by the speed of centrifugation, and assumes that different proteins sediment with equal volumes.

**Colorimetric assay of CR.** Serum (1 ml) in a conical centrifuge tube (similar to the cryocrit tube) is kept in an ice-water bath for 3 days and centrifuged for 5 min at 5000 rpm. The top layer is discarded. The tube is gently washed 3 times with 3 ml of ice-cold water, without disturbing the precipitate, followed by centrifuging for 2 min. The CR precipitate is dissolved in 1 ml of warm (37°C) saline solution (1%, w/v). The CR precipitate is measured by a urine protein assay (e.g., pyrogallol red or coomassie blue) and its contamination by albumin is measured by a urine microalbumin immunoassay. The albumin content is used to correct for contamination of the CR precipitate by normal serum proteins. However, if the albumin concentration is >100 mg/L, the correction is unreliable.

**Electrophoretic phenotyping and quantification of CR.** Electrophoretic methods quantify and phenotype CR at the same time. We have used both gel and capillary electrophoresis (CE) for CR quantification and typing [34]. The CE procedure is more rapid and accurate than gel electrophoresis for CR quantification [34]. The accuracy stems from avoiding dyes for measuring proteins and basing the calculation on ratio measurements [34]. The CR precipitate from the cryocrit or colorimetric assay is washed only twice and subjected to electrophoresis [34]. The increase of the immunoglobulin/albumin ratio in the CR-precipitate, relative to that in the serum, is used to calculate the CR. The reference range for serum CR concentration by the CE method is 0-60 mg/L [34].

**Other methods.** Other quantification methods for CR have been described but have not gained wide acceptance. These include laser nephelometry [42], diffusion of CR in gels [21,43], and light scattering [44]. For type 1 cryoglobulinemia, immunofixation
analysis is used to determine if the CR is due to multiple myeloma or Waldenstrom’s macroglobulinemia. For cryoglobulinemia of types II and III, the immunofixation technique is not helpful.

**Reporting results of CR tests.** Negative results are reported as such. When a serum sample is positive for CR, the concentration and phenotype of serum CR are reported, as well as the concentration of total gamma globulin. Since serum RF assays are positive in a majority of cryoglobulinemic patients and the concentrations of RF correlate with CR levels, serum RF concentration is routinely analyzed and the results are included.

**Interferences in CR analysis.** Hemoglobin precipitates as CR and interferes in the analysis. In electrophoresis, hemoglobin can be detected as a sharp peak that migrates ahead of the immunoglobulins. Hemolyzed samples should not be tested for CR unless they are to be analyzed by electrophoresis. Fibrinogen and heparin also interfere. It is difficult to solubilize fibrinogen, but the presence of fibrinogen in a precipitate can be confirmed by immunoassay. As discussed above, the main interference in CR assays is the contamination from normal serum immunoglobulins.

**Interference of CR in other tests.** Serum samples that contain CR sometimes gel or precipitate and clog analytical instruments. CR that precipitates during serum centrifugation or storage may elude detection; upon electrophoresis such samples can exhibit a normal serum pattern despite having a large monoclonal protein. The CR may precipitate at the application point on the electrophoretic membrane. When a CR precipitate traps other immunoglobulins, they may confuse the interpretation of the immunofixation pattern [1]. The presence of CR can cause false elevated cell counts in automated cell counters [45].

**Therapeutic Considerations**

The treatment of cryoglobulinemia depends on the severity of symptoms, the underlying disease, and the type of CR. The goal of therapy is to limit in vivo precipitation of cryoglobulins and the resultant inflammatory effects. Asymptomatic patients usually do not need treatment [17,43], even in the presence of high cryocrit levels [17]. Some patients with cryoglobulinemia suffer from mild, recurrent episodes of lower extremity purpura that require no specific therapy or only therapy with general anti-inflammatory drugs (eg, ibuprofen, aspirin) or low doses of steroids [25]. When there is evidence of organ involvement such as vasculitis, renal disease, progressive neurological findings, or disabling skin manifestations, especially in absence of HCV, cryoglobulinemia is treated by suppression of the immune response (eg, corticosteroid therapy, cyclophosphamide, azathioprine) [10,25,29,46]. In all cases, careful clinical monitoring of the disease, with particular attention to severe vasculitic or neoplastic complications, is mandatory [17].

**Treatment of type I cryoglobulinemia.** In multiple myeloma with type I CR, cytotoxic drugs (eg, melphalan, prednisone, cytoxan, chlorambucil) are prescribed. Approximately one-half of myeloma patients respond to biaxin with >50% reduction in monoclonal component, a response that is comparable to standard therapy with melphalan and prednisone [10]. Plasmapheresis is indicated for severe complications related to cryoprecipitation or serum hyperviscosity syndrome [10]. It removes circulating cryoglobulins and prevents their deposition in glomeruli [29,31]. Thalidomide has also been used to treat type I cryoglobulinemia [47].

**Treatment of type II cryoglobulinemia.** Acute nephritic or nephrotic flare-ups with rapid deterioration of renal function and systemic vasculitic episodes associated with CR are treated with corticosteroids and plasmapheresis [17,46]. Plasmapheresis alone is not very effective because although it removes circulating cryoglobulins, it does not suppress their production. Extensive vasculitis may respond to prednisone, cyclophosphamide, or both. Some have recommended concomitant cytotoxic medications and corticosteroids to reduce the rebound of CR phenomena that may develop after plasmapheresis [17,46]. These combined treatments can improve renal function substantially.
Interferon-alfa (IFN) and PEG-interferon-alfa have been effective in patients with cryoglobulinemia associated with hepatitis C and in patients with chronic myelogenous leukemias and low-grade lymphomas. However, the effects are temporary, and relapse may occur within a few months after discontinuation of the treatment [10,44]. IFN is contraindicated in acute flare-ups, since its immuno-stimulatory activity may aggravate acute flares of acute nephritis, nephrotic syndrome, and systemic vasculitis [48,49]. In the presence of acute cryoglobulinemic GN, IFN does not prevent the progression of renal damage. Combination therapy with cytotoxic and anti-inflammatory drugs, and sometimes plasma exchange, has been shown to improve renal function [50]. A study by Sarac and colleagues [49] demonstrated that high doses of IFN (10 million units, 3 times/wk) resulted in sustained improvement of renal disease in a few patients. This regimen may induce remission in patients who fail to respond to conventional doses of IFN.

In patients with chronic hepatitis C without renal involvement, combination therapy with IFN and ribavirin (Rebetron) has been shown to be superior to IFN alone in inducing longer remission and may eradicate HCV infection in a considerable number of subjects [51]. In patients in whom antiviral therapy is ineffective, contraindicated, or not tolerated, rituximab, a monoclonal chimeric antibody that binds to the B-cell surface antigen CD20 with selective B-cell blockade, may be a useful alternative to standard immunosuppression [32,48].

Treatment of type III cryoglobulinemia. This type is generally treated with anti-inflammatory drugs (eg, ibuprofen, aspirin) and steroids. Patients with low serum levels of CR are difficult to type accurately, which often influences the treatment strategy.

Case Studies

Case 1. A woman in her fourth decade was referred to our institution because of hypertension, edema of the lower extremities, and proteinuria. On admission, her urine protein concentration was 3-15 g/g creatinine (reference range 0-0.2 g/g creatinine) and her serum creatinine concentration was 2.2 mg/dl (reference range 0-1.5 mg/dl). A renal biopsy showed membranoproliferative glomerular nephritis, weakly positive for cryoglobulinemia. Based on the biopsy results, serum cryoglobulins were ordered and were found to range from 100 to 210 mg/L of type II (reference range 0-60 mg/L) (Fig. 4). Plasmapheresis was performed several times during a 2 mo period, during which the cryoglobulins transiently disappeared but returned to 100-200 mg/L within a few weeks. A small M protein corresponding to her CR M spike was detected in homogenates of the patient’s white blood cells (Fig. 4). Finally, the patient was treated with cytoxan. The cryoglobulins disappeared and remained within the reference range. After 2 yr, she is doing well with serum creatinine concentration

![Fig. 4. Cryoglobulins of Case 1 as detected by capillary electrophoresis.](image)
of 1.4 mg/dl, urine protein of 0.2-0.4 g/g creatinine, and negative tests for serum CR.

**Case 2.** A woman in her forties was referred to our institution because of systemic lupus erythematosus (SLE). At admission, she complained of arthralgia in the right hand, photosensitivity, oral ulcers, and purpura on the hands. The serum contained a type III CR level of 219 mg/L (reference range 0-60 mg/L) (Fig. 5). Tests were positive for anti-nuclear antibodies and negative for double-stranded DNA. The anti-Smith antibody level was 230 U (reference range <100 U) and the erythrocyte sedimentation rate was 66 mm/hr (reference range 0-30 mm/hr). She was treated with prednisone, methotrexate, and colchicine. After 6 mo, her CR was still type III, but the serum CR level dropped to 90 mg/L. The patient had no rashes, the joint pain improved, and the oral ulcers disappeared.

**Concluding Comments**

The case summaries underline the importance of CR analysis for diagnosis, treatment, and follow-up of patients, especially when serum CR levels are low. They illustrate that small amounts of CR can cause or be associated with severe symptoms. The first case showed that plasmapheresis alone was ineffective. The response to this treatment was temporary while the synthesis of the cryoproteins continued. However, when cytoxan therapy was added, better control of her renal function resulted. The second case illustrates that small CR levels can be associated with severe symptoms. Treatment with a combination of drugs decreased the serum CR level and improved the patient’s symptoms.

The overall prevalence of mixed cryoglobulinemia has not been adequately defined by epidemiologic studies [46]. It is probably underestimated due to the referral of patients to different specialists according to the dominant manifestation of the disease [46]. Cryoglobulinemia has a high incidence in many autoimmune and infectious disorders. For example, low levels of CR and the cryoglobulinemic syndrome occur in about 50% and 5%, respectively, of patients infected with HCV [17]. Given the prevalence of HCV of about 170 million individuals worldwide [52,53], it appears that many individuals have CR and that testing for CR is underutilized. A faster analysis time, better quantification, and improved sensitivity would encourage utilization of this test in routine patient care.

For many years, our laboratory used the visual test for serum CR. The number of samples submitted for analysis was about 10-20/yr, with only about 3-5 positive results. However, a decade ago we introduced CE for CR quantification and phenotyping. The number of samples submitted for analysis increased gradually and so did the positive results. During the past two years, 960 samples were ordered for CR testing with 194 positive results (Table 4). The increase in test volume suggests that when the laboratory offers a reliable and useful assay, physicians order it more often.

![Fig. 5. Cryoglobulins of Case 2 as detected by capillary electrophoresis (S = serum; CR = cryoglobulin precipitated from serum; A = albumin; G = globulin).](image-url)
This report provides an overview of cryoglobulins in patient diagnosis and management, and focuses attention on clinical and analytical aspects. Many questions remain to be answered. Why are some infections not accompanied by CR production? Do these infections cause CR production below the analytical detection limits? Why can some CR be present in a patient's serum and not elicit symptoms? Can more sensitive assays for serum cryoglobulins improve early disease detection and patient treatment? Further studies are needed to answer these important questions.

References