

Diagnostics and Treatment of Cryoglobulinaemia: It Takes Two to Tango

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Abstract Cryoglobulins are immunoglobulins that reversibly precipitate in the cold. They come in different flavours and, as such, are differentially associated with lymphoproliferative diseases (type I), or systemic autoimmune diseases, and/or infectious diseases (type II/III). The clinical manifestations of cryoglobulinaemia result from either hyper-viscosity or small vessel vasculitis. Hepatitis C virus (HCV) is a well-known factor in the aetiology of cryoglobulinaemia, but substantial geographical differences exist in the association between cryoglobulins and HCV. In the absence of any underlying disease, cryoglobulinaemia is referred to as ‘idiopathic’ or ‘essential’. Detection of cryoglobulins in the laboratory is hampered by several pitfalls, in particular in the pre-analytical stage as well as in the quantification. In addition, our personal experience reveals that the detection of rheumatoid factor, most often present in high concentrations in patients with mixed cryoglobulinaemia, relies on the choice of the test system. Hence, interpretation of the laboratory results in relation to the clinical manifestations requires a partnership between the clinician and the laboratory specialist in order to make a correct diagnosis. Treatment options are primarily directed by identification of underlying diseases, i.e. infections or systemic autoimmune diseases. Idiopathic cryoglobulinaemia is treated with corticosteroids and immunosuppression, or B cell depleting anti-CD20 biologicals. In this overview, the recent literature on current laboratory and

clinical practice of cryoglobulinaemia is discussed from a personal perspective.

Keywords Cryoglobulin · Vasculitis · Rheumatoid factor · Hepatitis C virus

Introduction

Cold precipitable serum immunoglobulins were first coined cryoglobulins in 1947 by Lerner and Watson [1]. Before, the phenomenon was already associated with Raynaud’s phenomenon by Wintrobe and Buell [2]. It lasted, however, until the 1960s of the twentieth century before the clinical profile of the immune complex-mediated disease was recognized [3]. Cryoglobulins may cause severe clinical manifestations due to hyperviscosity of the blood within the extremities, or due to immune complex deposition along the vessel wall within the skin, joints, peripheral nervous system and/or kidneys [4, 5]. Typically, both complications primarily affect the small blood vessels, predominantly capillaries, venules or arterioles [6]. Medium- and small-sized arteries, however, may be affected as well.

Cryoglobulins have three typical characteristics: (1) They precipitate at temperatures below 37 °C, (2) they re-solubilize at 37 °C and (3) they consist of immunoglobulins. Apart from immunoglobulins, also other proteins, such as complement factor C1q and C-reactive protein, may be contained within the cryoprecipitate. Also fibrinogen may precipitate in the cold and is referred to as cryofibrinogenemia (reviewed in [7, 8]). Both cryoglobulinaemia and cryofibrinogenemia share clinical manifestations like severe cutaneous involvement (necrosis and ulcers). However, in cryofibrinogenemia, the precipitating protein is fibrinogen, and not immunoglobulins, and detection of a precipitate thus requires plasma instead of serum. Cryofibrinogenemia is not further discussed in this

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review. Cryoglobulinaemia can be subdivided in three types as based on the immunoglobulin content of the cryoglobulin [9]. Firstly, cryoglobulins may consist of monoclonal immunoglobulins, most often of the IgM isotype, only. This is referred to as type I cryoglobulin. Secondly, the precipitate may reveal a combination of a monoclonal and a polyclonal immunoglobulin. This type II cryoglobulin most often consists of a monoclonal IgM and a polyclonal IgG. In type II cryoglobulin, the monoclonal IgM has rheumatoid factor (RF) activity and therefore co-precipitates the polyclonal IgG. Thirdly, the cryoglobulin may contain polyclonal antibodies of distinct isotypes. As in type II cryoglobulins, also in type III cryoglobulins, one component has RF activity which results in co-precipitation of the other component. Type III cryoglobulins may consist of polyclonal IgM, IgG and/or IgA in different combinations, or of only polyclonal IgG, where part of the IgG has RF activity and therefore co-precipitates another part of the IgG fraction. Obviously, both type II and type III cryoglobulins form immune complexes and therefore are referred to as mixed cryoglobulins. It has been suggested that type III cryoglobulins are a transition state between mere hypergammaglobulinemia and type II cryoglobulins [10].

Cryoglobulinaemia is defined as the presence of cryoglobulins in the blood. Cryoglobulinaemia, as such, is, just like monoclonal gammopathy, a laboratory finding not necessarily associated with any clinical manifestation. Due to the lack of standardization and widely accepted reference values, clinical interpretation of the laboratory results is not always straightforward [11]. In the current review, we have summarized the current clinical practice in combination with our personal experience with respect to cryoglobulinaemia. We conclude that optimal care of patients with cryoglobulinaemic disease requires a close collaboration between the clinician and the laboratory specialist. Hence, it takes two to tango.

Detection of Cryoglobulins

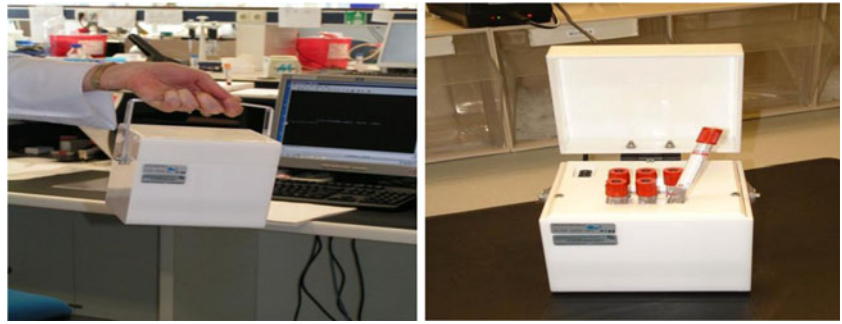
Since cryoglobulins are the hallmark of a clinical diagnosis of cryoglobulinaemic vasculitis, appropriate detection of cryoglobulins in the laboratory is essential [12, 13]. In this respect, the pre-analytical conditions are probably the most important. Indeed, if the temperature of the pre-analytical conditions is not controlled to prevent cooling down, the cryoglobulins may already precipitate during this stage. If so, the cryoprecipitate is already lost during centrifugation of the blood to separate the serum from the blood cells. To avoid this, the blood should be collected in pre-warmed tubes (37 °C) and transported at 37 °C. Also, the blood coagulation should occur at 37 °C, and since the coagulation process is delayed at this temperature, the coagulation should be extended to at least 1 h. Finally, also the centrifugation step requires a temperature of 37 °C. Interestingly, for a critical appraisal of

current practice in the detection of cryoglobulins, a questionnaire was distributed to 140 laboratories participating in the UK National External Quality Assessment Service (UK-NEQAS) quality control program. It was concluded that amongst the responding participants ($n=127$), only 35 % of the laboratories took all these standard precautions for maintaining the appropriate temperature during the pre-analytical stage [11]. Insufficient temperature control is considered as the single most important cause of a false negative result for the detection of cryoglobulins. In our University Medical Center, the technical service has devised a small, portable container, with a re-chargeable battery, that keeps the content at 37 °C (Fig. 1). Blood collection tubes, pre-warmed in this device, can be transported to the site of blood collection and back to the laboratory. As such, the first critical steps in the detection of cryoglobulins are effectively controlled. Indeed, by using an alternative device, a substantial increase in the number of samples (35→95 %) that reaches the laboratory at ambient temperature has been reported [14]. However, data are lacking on a possible increase in the occurrence of cryoglobulin-positive samples as associated with improved pre-analytical conditions.

The appropriately collected serum should be divided in two tubes which are stored at 4 °C for 3–7 days. Type I cryoglobulins tend to precipitate rather fast, i.e. within 3 days, but mixed cryoglobulins may take a longer time span for precipitation, i.e. after 3–7 days. Presence of precipitation is scored qualitatively (Fig. 2a), preferentially independently by two observers. If a precipitate is observed, one tube is placed at 37 °C in order to examine reversibility of precipitation (Fig. 2b). If this is the case, the precipitate in the second tube is washed whilst taking care that the precipitate remains at 4 °C.

Next, the precipitate can be used for typing and quantification of the cryoprecipitate. Both steps are highly recommended as best practice in cryoglobulin evaluation [12]. Typing of the cryoglobulin is normally performed by immunofixation or immuno-electrophoresis. The need for further characterization, however, has been disputed because of lack of evidence that this characterization influences patient management [15]. Based on results obtained in the UK-NEQAS questionnaire, up to 20 % of the participating laboratories do not immunotype the cryoprecipitate [11]. This is surprising since cryoglobulin typing enables identification of the cryoprecipitate as immunoglobulin, one of the typical characteristics of cryoglobulin, and because the clinical associations and possible complications are distinct for the three types of cryoglobulins [16]. Indeed, as will be discussed below, a type I cryoglobulin is suggestive for a lymphoproliferative disease, whilst type II or type III cryoglobulin may be associated with Sjögren's disease and/or hepatitis C infection, both requiring a different approach for dealing with the cryoglobulin-associated clinical manifestations.

Fig. 1 Precipitation and re-solubilization of cryoglobulin. Carrier system enables transport of pre-warmed tubes to the patient for blood collection and subsequent transport on ambient temperature back to the laboratory

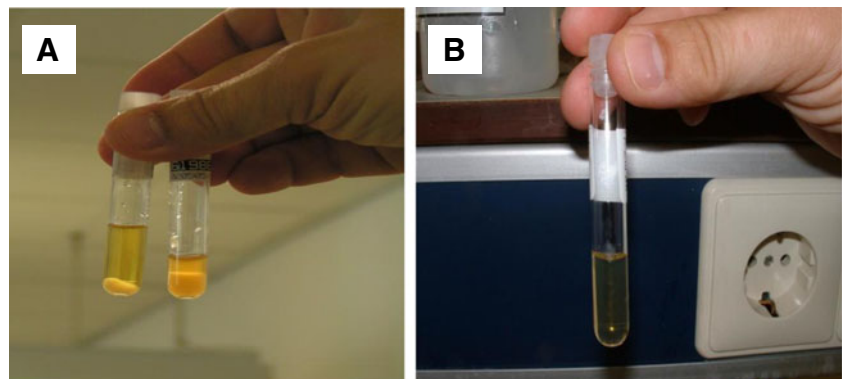


Quantification of the cryoglobulin in the precipitate can be performed in so-called Wintrobe tubes which have markings on their side that are used to quantify; the result is referred to as cryocrit, i.e. the packed volume of the precipitate relative to the original serum volume. The cryoglobulin can also be quantified by measuring the total protein content. Both methods quantify both the immunoglobulin fraction as well as the proteins that co-precipitate in the cryoglobulin. These contaminating proteins may be either proteins, like C1q and C-reactive protein, that specifically interact with the immunoglobulin fraction, or proteins, like albumin, that remain attached to the cryoprecipitate due to insufficient wash procedures. It has been reported that about 2/3 of the total protein content in the cryoprecipitate of patients with rheumatoid arthritis consisted of immunoglobulins, whilst the immunoglobulin content of cryoprecipitates of control subjects was less than 1/3 [17]. Concomitant agarose gel electrophoresis of the cryoprecipitate may help to estimate the presence of other, contaminating proteins. Alternatively, direct quantification of immunoglobulins in the cryoprecipitate can be performed. This quantification, however, may be hampered by the relatively high lower limit of detection of the respective assays preventing appropriate quantification. Since healthy individuals regularly present with low, but detectable amounts of cryoglobulin [18], the use of a normal range has been advocated. As concluded from the UK-NEQAS questionnaire mentioned above, only three of the 26 laboratories that report total protein content in the cryoprecipitate provided cryoprecipitate-specific reference

values for total protein content (ranging from 0–10 to 0–80 mg/L) [11]. Some laboratories used reference values for serum proteins and/or immunoglobulins, evidently being inappropriate for the cryoprecipitate [11]. Importantly, also cryoprecipitate-specific reference values for immunoglobulin isotypes have been established [19]. The cutoff values, i.e. the 97.5th percentile of a diseased control population, for IgM, IgG and IgA were set at 26, 11 and 2 mg/L, respectively, whilst the cutoff value for total immunoglobulin was set at 33 mg/L. Whatever the method of choice for quantification is, it should be kept in mind that there is no strict relationship between the cryoglobulin concentration and the severity of the clinical manifestations [18]. Type I cryoglobulins, though, tend to present in higher concentrations than type II and III cryoglobulins. For follow-up of a patient, it is obvious that the same method should be used for quantification.

Alternative techniques for the analysis of cryoglobulins have been reported over the years. Some case reports have revealed surrogate markers of cryoglobulins in blood smears. Cryoglobulins may appear as amorphous deposits between red blood cells as well as within white blood cells [20, 21]. Especially in neutrophils they reveal multiple round vacuoles with a particular pale blue colour [20, 22]. Also, the presence of bluish crystals, disappearing on warming the blood to 37 °C, has been described in a peripheral blood smear of a patient with clinical manifestations of cryoglobulinaemia [23]. Although such findings in blood smears may hint at the presence of cryoglobulins, detection by conventional methods

Fig. 2 Transport device for collection of blood for cryoglobulin detection. Cryoprecipitate after 4-day storage at 4 °C (a) and re-solubilized cryoprecipitate after incubation at 37 °C (b)



remains the golden standard for making a final diagnosis. Another alternative technique for analysis of cryoglobulins is based on gel diffusion. This procedure is based on cooling agarose plates [24]. Whilst pre-analytical conditions are similar to conventional cryoglobulin detection, the gel diffusion procedure allows 20 μ L of serum to precipitate at 4 °C. After 48 h incubation, a precipitation ring may have formed. After washing out other proteins by immersing the agarose plate in physiological saline, the precipitate can be re-dissolved at 37 °C to confirm that it involves cryoglobulin. The detection limit of this method was estimated at concentrations of 50 mg/L. Interestingly, the gel diffusion procedure can be combined with the Ouchterlony method of double diffusion in order to type the cryoglobulin [24]. Quantitative scanning analysis of the cryoglobulin ring, on the other hand, enables quantification by calculation from the area under the curve [25]. Interestingly, cryoglobulin quantification by conventional total protein measurement and by the area under the curve of the precipitation ring obtained by the cooling gel diffusion method is poorly correlated. This has been attributed to higher contamination with other serum proteins in the precipitate obtained by conventional methods [26]. Finally, cryoglobulin detection by flow cytometry has been described [27]. As compared with visual inspection, detection of cryoglobulin by flow cytometry for small diffracting particles appeared to be equally specific, as defined in 33 healthy controls, but much more sensitive, as defined in 91 patients with cryoglobulin associated symptoms. Obviously, all these alternative techniques for the analysis of cryoglobulins await further validation in larger multicentre studies.

Clinical Features of Cryoglobulinaemia

The clinical manifestations and the respective epidemiology of the distinct types of cryoglobulinaemia have been reviewed extensively before [4, 5, 28]. Clinical features will only be discussed briefly, especially in light of more recently published case series.

Type I cryoglobulinaemia is characterized by Raynaud's syndrome, acrocyanosis, peripheral gangrene and livedo reticularis. This type is typically associated with the presence of an M-protein in the circulation, in particular in combination with a diagnosis of multiple myeloma and/or Waldenstrom's macroglobulinemia. Type I cryoglobulins, however, can also occur in monoclonal gammopathy of unknown significance (MGUS) or other lymphoproliferative diseases. The typical clinical signs are caused by reduced blood flow in the extremities and can be attributed to locally reduced temperature and subsequent hyperviscosity of the blood. Whilst vascular occlusion is more common in type I cryoglobulinaemia, systemic vasculitis may also occur. The French multicenter CryoVas

survey focused in particular on those type I cryoglobulin patients that had clinical manifestations of systemic vasculitis in the absence of infection with hepatitis B/C virus (HBV/HCV) and/or human immunodeficiency virus (HIV) [29]. A case series of 64 patients with type I cryoglobulinaemia revealed that the major characteristics were severe cutaneous involvement (necrosis and ulcers) and high serum cryoglobulin levels. Glomerulonephritis was observed less frequently (30 % of cases) than expected from previous studies [30, 31]. Survival rates were 97 % (1 year) to 87 % (10 years). Type I cryoglobulin-associated vasculitis tended to be associated with a worse prognosis if related to lymphoproliferative diseases as compared to MGUS.

Type II cryoglobulins are associated with clinical manifestations typical for small vessel vasculitis, like cutaneous vasculitis (purpura and/or leg ulcers), glomerulonephritis and (peripheral) neuropathy. Renal biopsies of patients with cryoglobulinaemia with renal involvement most often show a histologic pattern of a membranoproliferative glomerulonephritis (MPGN). Recently, it was suggested that patients with cryoglobulin-associated MPGN most often have IgM-dominant depositions as found by immunofluorescence studies of these renal biopsies [32, 33]. Cryoglobulinaemic vasculitis is the result of immune deposits predominantly in the capillaries, venules or arterioles. Type II cryoglobulins may be associated with systemic autoimmune diseases, in particular Sjögren's syndrome, as well as with viral infections. An association with HCV is most frequently described, but also many other chronic viral infections such as HBV or HIV have been associated with occurrence of mixed cryoglobulinaemia. Also exotic viruses like the Chikungunya virus, an arbovirus, may result in persisting mixed cryoglobulinaemia [34]. If there is no apparent underlying disease identified, the term 'idiopathic' or 'essential' is used (mixed essential cryoglobulinaemic vasculitis). Otherwise, the underlying cause can be designated in the diagnosis, e.g. Sjögren's syndrome-associated cryoglobulinaemic vasculitis or hepatitis C-associated cryoglobulinaemic vasculitis [6].

Type III cryoglobulins are often asymptomatic. However, since type III cryoglobulins form immune complexes that may deposit within the small vessels, they also can give rise to clinical features as observed in type II cryoglobulinaemia. As compared to type II cryoglobulinaemia, they are more frequently associated with chronic infections (HCV, HBV, HIV, herpes viruses and/or mycobacteria) and autoimmune diseases (not only Sjögren's syndrome but also rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease and other).

As part of the French CryoVas survey, prognostic factors and survival rates were identified in a cohort of 242 patients with non-infectious mixed cryoglobulinaemic vasculitis [35]. Age above 65 years, pulmonary and gastrointestinal involvement and renal failure (glomerular filtration rate <60 mL/min)

were identified as main prognostic factors. Survival rates were 91 % (1 year) to 65 % (10 years). The main causes of death were severe infections and vasculitic activity. Although cryoglobulinaemia may also occur in children, clinical and epidemiological data are limited to only one small case series of 18 patients from Southeast Asia [36]. As compared to 96 adults with cryoglobulinaemia, children had a significantly higher prevalence of prolonged fever, arthralgia, arthritis and cutaneous involvement. Unfortunately, in this series, typing of the cryoglobulin was not performed.

Pathogenesis of Cryoglobulinaemia

The exact mechanisms that cause reversible precipitation of immunoglobulins in the cold remain elusive. Evidently, this mechanism will be different for type I cryoglobulins and mixed cryoglobulins. For type I cryoglobulins, being associated with lymphoproliferative diseases resulting in monoclonal expansion of B cells and concomitant production of M-proteins, the biochemical basis for cold insolubility probably is intrinsic to the characteristics of the monoclonal immunoglobulins. These characteristics may include primary structure and subsequent steric conformation, hydrophobicity, size and charge [12]. Indeed, increased presence of hydrophobic amino acids and relative shortage of tyrosine and sialic acid residues have been implicated [37].

In case of mixed cryoglobulins, infectious agents are most likely involved in the mechanisms of cold insolubility. It should be noted, however, that it has recently been suggested that, even within the regular immune response to infections, cryoprecipitation of immunoglobulin can be possible [38]. Since mixed cryoglobulinaemia is strongly associated with HCV infection, most studies have involved HCV in the search for mechanisms of immunoglobulin precipitation in the cold [39]. The association of cryoglobulins with HCV infection appears to show geographic variation. Within Europe, the prevalence of HCV infection in patients with mixed cryoglobulinaemia is the highest in the Mediterranean area and the lowest in the Northern countries [4]. Also in the Netherlands, the prevalence of HCV is low, and hence, most patients with mixed cryoglobulins test negative for HCV [40]. This emphasizes that HCV is not the only precipitating factor for cryoglobulinaemic disease. Also other infections, such as *Toxoplasma gondii* and cytomegalovirus, may cause disease manifestations, possibly due to polyclonal B cell activation [41].

The presence of RF, either monoclonal or polyclonal, is detrimental for generation of cold-dependent insoluble immune complexes in mixed cryoglobulinaemia. The RF present in these immune complexes has a restricted idiotype [42], possibly due to restricted expression of germline genes, and is in particular linked to IgG with anti-HCV reactivity [43]. Especially the HCV core protein has been identified as the

relevant ligand [44]. The formation of these three-molecular complexes, RF–anti-HCV IgG–HCV core protein, subsequently harbours cold-dependent insolubility [43]. This implies that the RF activity is probably not just directed to the Fc part of the IgG molecules but also to the antigen-binding part of the IgG molecule. Indeed, it has been noticed that the IgM molecules in cryoprecipitates display rheumatoid factor activity capable of reacting with intact IgG and/or its F(ab)2' fragment [45]. Whereas the mechanism described above is specific for HCV, the possible mechanism(s) involved in systemic autoimmune diseases like Sjögren's syndrome await further elucidation.

In infectious diseases, as well as in systemic autoimmune diseases, the humoral immune response results in a (transient) hypergammaglobulinemia due to a polyclonal B cell response. If the immune response becomes chronic, as in HCV infection and systemic autoimmune diseases, there is formation of tertiary lymphoid tissue in the target organ [46]. These structures are characterized by B cell follicles with active germinal centres, surrounded by T cells, and drive the local immune response of these chronic inflammatory conditions. This will result in oligoclonal expansion of B cells clones and, in case of cryoglobulinaemia, selection for RF activity. In particular, HCV induces outgrowth of oligoclonal B cells expressing a restricted pool of selected immunoglobulin variable gene subfamilies that confer RF activity (reviewed in [47]). These B cells have the phenotype of marginal zone-like IgM⁺CD27⁺ B cells [48]. Not only the HCV core protein but also the E2 envelope glycoprotein and the nonstructural protein 3 (NS3) may generate IgM RF due to molecular mimicry. Indeed, monoclonal IgM RF in mixed cryoglobulins share high sequence homology with antibodies to the HCV E2 envelope glycoprotein [49], whilst IgM molecules that recognize immunodominant epitopes on NS3 cross-react with an epitope in the CH3 domain of IgG [47]. Alternatively, autoreactivity for human IgG, i.e. RF activity, may result from antigen-dependent somatic hypermutation, as opposed to non-deletion of autoreactive germline immunoglobulins cross-reacting with HCV antigens [50]. The initial oligoclonal IgM RF response, revealing type III cryoglobulins, will eventually evolve into a monoclonal IgM RF response due to selection of a dominant clone and subsequent transition to type II cryoglobulins. This selection via an interphase oligoclonal transition stage is often referred to as type II/III or type IIa cryoglobulins. The monoclonal B cells characteristic for type II cryoglobulins at first do not yet display malignant features, but 5–10 % of patients with cryoglobulinaemic vasculitis eventually develop a malignant lymphoproliferative disease, in particular B cell non-Hodgkin lymphomas (reviewed in [47]). The sequence of events starting from a regular immune response towards development of a malignant lymphoma, in association with the development of cryoglobulins, is depicted in Fig. 3. Each consecutive step in this scheme becomes manifest in a subset of individuals, due to specific genetic and environmental

requirements, eventually resulting in a prevalence of clinically significant cryoglobulinaemia of approximately 1:100,000. Since B cell activating factor (BAFF) is increased in patients with chronic HCV infection and mixed cryoglobulinaemia [51], BAFF may play a crucial role in the development of autoreactive B cell clones [52]. BAFF is not only involved in the development, maturation and activation of B cells but also inhibits B cell apoptosis and thus in the development of monoclonal B cells.

For many diseases, animal models have provided detailed insight in the underlying pathogenesis. For cryoglobulinaemia, the number of available animal models is rather limited (reviewed in [53]). The first model is based on the overexpression of thymic stromal lymphopoietin (TSLP). In mice, this results in type III cryoglobulinaemia with associated inflammatory disease in multiple tissue including skin, liver, lungs, kidneys and spleen [54]. The skin ulcerations, typically involving the extremities, reveal histological characteristics of vasculitis. The second model is induced by the transfer of hybridoma cells that produce a murine IgG3 monoclonal antibody exhibiting cryoglobulin activity [55]. This model was further improved by combining both RF and cryoglobulin activity within the IgG3 monoclonal antibody, resulting in cutaneous leukocytoclastic vasculitis and glomerular injury [56]. More recently, a third model has been described as induced by *Capillaria hepatica* infection in mice [57]. The infection results in development of type II cryoglobulins, composed of monoclonal IgM RF and polyclonal IgA, revealing glomerulonephritis due to immune complex deposition in the capillaries. In the first two models, the role of complement and Fc γ receptors has been studied in the inflammatory response as induced by the cryoprecipitating immune complexes. Remarkably, with respect to the complement system, the components of the alternative pathway of complement activation appear the most important. However, it is not the lytic part of this pathway (C5–C9) that is held responsible for tissue injury, but merely the anaphylactic breakdown product C5a which is important for the influx of neutrophils [58]. Interestingly, C5a has also been identified as a critical factor in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis [59] and therefore reveals a common mechanism in both types of small vessel vasculitis. The engagement of either inhibitory or activating Fc γ receptors results in more or less similar effects, i.e. amelioration of disease [60, 61]. For the inhibitory Fc γ receptors, this protective role might have been expected, but for the activating receptors, this is counter-intuitive. For the latter, it has been suggested that the activating Fc γ receptors are important in the clearance of immune complexes [53]. Besides studying the role of complement and Fc γ receptors, the TSLP model has also revealed an important role for platelet-derived growth factor and transforming growth factor- β in glomerular sclerosis (reviewed in [53]). Both molecules share profibrotic characteristics and are typically upregulated in fibrotic diseases like systemic sclerosis [62]. Therefore, it is tempting to speculate that, although rare, combined occurrence of systemic

sclerosis and mixed cryoglobulinaemia exhibits synergistic activity of typical scleroderma microangiopathy and cryoglobulinaemic vasculitis [63].

Laboratory Findings in Cryoglobulinaemia

Next to the detection, typing and quantification of cryoglobulins, other laboratory parameters may be abnormal in patients with cryoglobulinaemia [43, 64]. Especially in case of mixed cryoglobulinaemias, the formation of immune complexes will result in activation and thus consumption of the classical pathway of the complement system. This will be apparent in functional assays for measuring complement activation by the classical pathway. Measuring single components of this pathway, in particular C4, will result in better quantifiable abnormalities. Detection of C4 is often combined with C3, but one should keep in mind that C3 also acts as an acute phase protein. Therefore, consumption of C3 may be partially compensated for by increased production in the liver, and therefore, normal C3 levels may be found. To distinguish between complement deficiency from complement consumption, the breakdown product C3d can be analysed. Increases indicate consumption rather than deficiency.

Since types II and III cryoglobulins are defined by the presence of immunoglobulins with RF activity, detection of IgM RF may also reflect disease activity. Indeed, in patients with mixed cryoglobulinaemia, the level of IgM RF may be extremely high and may clearly outpace the levels normally observed in patients with rheumatoid arthritis (RA). The appropriate detection of elevated RF in mixed cryoglobulinaemia, however, seems to be assay dependent (Damoiseaux and Hamann, unpublished). In search of a novel assay for IgM RF, because the assay in use (A) was taken out of the market, we tested two commercially available alternatives (B and C). Whilst all the assays similarly detected IgM RF in RA patients, one assay (B) readily detected IgM RF in the cryoglobulin samples that were positive in the original assay (A), but the second assay (C) revealed positivity in only few samples. In contrast to assays A and B, assay C was incubated at room temperature instead of 37 °C. Incubation at 37 °C, however, did not improve the results (Table 1). Another difference between the tests appeared to be the antigen: total IgG in assays A and B, but IgG Fc portion in assay C. Using an in-house IgM RF assay, coated with IgG Fc portion, we observed that only half of the cryoglobulin samples tested positive. The other half, though, tested positive up on titration, suggesting some kind of ‘pro-zone effect’. This might, however, not be restricted to assays based on IgG Fc portion, since we encountered in another commercially available assay (D) for IgM RF based on total IgG also a low sensitivity for IgM RF in cryoglobulin samples (Table 2). Altogether, it can be concluded that the choice of an appropriate assay for IgM RF should not only rely

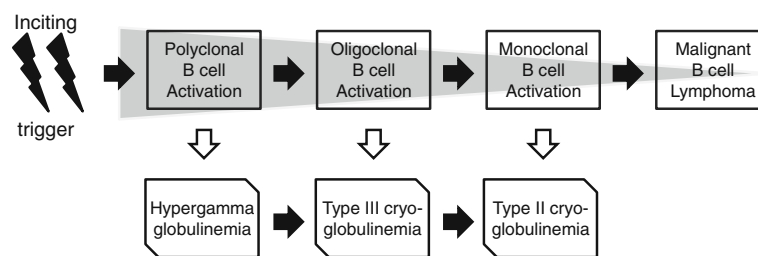


Fig. 3 Schematic overview for the development of mixed cryoglobulinaemia. Inciting factors, like infections or autoimmune diseases, may cause polyclonal B cell activation, eventually resulting in hypergammaglobulinemia. In part of these patients, some B cell clones with specificity for IgG become dominant and may give rise to type III

cryoglobulinaemia. If subsequently a single B cell clone out-competes the others, type II cryoglobulins can be detected in the circulation. In a minority of patients, this monoclonal B cell may become malignant. The *grey triangle* in the background represents decrease in prevalence of the consecutive events

on the test characteristics for RA but also on the correct detection of IgM RF in mixed cryoglobulinaemia. In addition to detection of IgM RF in the peripheral blood, it is also advocated to analyse IgM RF in the cryoprecipitate [65]. This is considered positive if the concentration is higher in the cryoglobulins than in the serum. De Rosa et al. found that this phenomenon is associated with the occurrence of vasculitic manifestations in patients with HCV infection, but not in uninfected patients with cryoglobulins.

Finally, there are multiple other laboratory abnormalities that hint at the possible presence of cryoglobulins [12]. These include gelling of blood, pseudo-thrombocytosis or pseudo-erythrocytosis, rouleaux formation of erythrocytes on the blood film and failure of protein migration on electrophoresis. These phenomena are most often found in case of type I cryoglobulins due to the relatively high cryoglobulin concentration. Cryoglobulins may associate with other circulating proteins and, upon cryoprecipitation, may result in reduced levels of these proteins (such as albumin) as a laboratory artefact [66]. In a recent study on the prevalence of clinically significant cryoglobulinaemia, 83 patients were identified by laboratory artefacts on protein electrophoresis. Out of 52 patients, subsequently evaluated for cryoglobulins, 14 patient tested positive. Findings indicative of clinically significant cryoglobulinaemia were observed in eight of ten patients in whom clinical data were available [67].

Classification Criteria

The definition of cryoglobulinaemic vasculitis was recently defined at the 2012 International Chapel Hill Consensus Conference on the nomenclature of vasculitis as ‘vasculitis with cryoglobulin immune deposits affecting small vessels (predominantly capillaries, venules or arterioles) and associated with serum cryoglobulins’ [6]. In addition, although there are no widely accepted classification criteria for cryoglobulinaemic vasculitis, several preliminary criteria have

been proposed [68, 69]. Ferri and Mascia proposed criteria for the classification of mixed cryoglobulinaemia patients based on serological, pathological and clinical manifestations [68]. Major criteria are confined to the presence of mixed cryoglobulins with or without low C4 levels (serological), leukocytoclastic vasculitis (pathological) and purpura (clinical). Minor criteria are the presence of RF, HCV and HBV (serological); clonal B cell infiltrates in liver and/or bone marrow (pathological); and chronic hepatitis, membranoproliferative glomerulonephritis, peripheral neuropathy and skin ulcers (clinical). Definite cryoglobulinaemic vasculitis is classified as such if all major criteria are met, or if mixed cryoglobulinaemia is associated with two minor clinical symptoms and two minor serological/pathological symptoms.

More recently, preliminary classification criteria for cryoglobulinaemic vasculitis were designed based on a

Table 1 IgM rheumatoid factor in mixed cryoglobulinaemia

	A (37 °C)	B (37 °C)	C (RT)	C (37 °C)
Cryo 1	neg	13	6	1
Cryo 2	neg	12	8	5
Cryo 3	neg	13	9	6
Cryo 4	130	150	7	<1
Cryo 5	480	1372	65	60
Cryo 6	215	307	35	36
Cryo 7	120	134	10	7
Cryo 8	125	296	7	1
Cryo 9	265	366	13	11
Cryo 10	588	3799	16	6

Results are presented in bold if positive according to the cutoff provided by the manufacturer; only samples positive in the screening dilution as indicated by the manufacturer were further diluted when levels were above the highest standard. Tests A and B are based on total IgG and are advised to be incubated at 37 °C; test C is based on IgG Fc portion and is advised to be incubated at room temperature

Table 2 IgM rheumatoid factor in mixed cryoglobulinaemia

	A (titration)	Fc (1:100)	Fc (titration)	D (1:100)
Cryo 11	150	23	33	8
Cryo 12	115	5	>50	1
Cryo 13	438	7	>100	ND
Cryo 14	510	>50	229	8
Cryo 15	420	1	5	ND
Cryo 16	160	9	198	0
Cryo 17	50	>50	84	8
Cryo 18	560	>50	481	1
Cryo 19	445	2	37	3
Cryo 20	141	>50	155	7

Results are presented in bold if positive according to the cutoff provided by the manufacturer. As indicated, samples were titrated to the end-point (titration) or only incubated in the screening dilution as indicated (1:100). Tests A and D are based on total IgG; the Fc test is based on IgG Fc portion

questionnaire, in combination with clinical and laboratory items [67]. The three questions involved are focused on the self-reported presence of vasculitic lesions and viral hepatitis; four clinical items include constitutional, articular, vascular and neurological involvement and three laboratory items are monoclonal gammopathy, RF and low serum C4. A patient is being classified as having cryoglobulinaemic vasculitis when, next to the presence of cryoglobulins in at least two determinations at ≥ 12 -week interval, at least two of the three items (questionnaire, clinical, laboratory) are positive. To be regarded positive for the questionnaire, at least two out of the three questions should be answered positive, for the clinical item at least three out of the four have to be present (or past) and for the laboratory item at least two out of the three have to be met.

Thus far, no independent validation of these sets of classification criteria has been performed. In the latter study, though, application of the criteria in an independent patient cohort revealed a sensitivity of 88.5 % and a rather low specificity of 93.6 %. It should be mentioned that this patient cohort was predominantly included in the HCV endemic Mediterranean region. The authors therefore correctly conclude that the criteria should also be tested in HCV-unrelated, ‘essential’ cryoglobulinaemic vasculitis patients [69]. This was more recently established by the same research group [70]. In a cohort of 117 HCV negative patients with serum cryoglobulins, either with ($n=42$) or without vasculitis ($n=75$), the classification criteria revealed a sensitivity of 89.5 % and a specificity of 90.3 %. The somewhat lower specificity (90.3 vs. 93.6 %) was attributed to the fact that some clinical manifestations included in the classification criteria were more frequently present in HCV-negative compared to HCV-positive controls without cryoglobulinaemic vasculitis.

Therapy

The therapy of cryoglobulinaemic vasculitis is according to the underlying cause. When the cause is not known or when it is not possible to treat the underlying disorder, therapy will be individualized to the severity of the disease. In these cases, the goal of therapy is to prevent organ damage as much as possible.

The therapy of idiopathic or essential mixed cryoglobulinaemia has yet to be defined. It is, however, not very likely that prospective randomized therapeutic trials will be performed within the next coming years. Therefore, therapy of this rare disorder is mostly ‘eminence-based’.

Patients with mild-to-moderate symptoms and signs of vasculitis such as purpura and arthralgias can be treated with nonsteroidal anti-inflammatory drugs, colchicine, dapsone and/or short-term corticosteroids. In addition, patients should be advised to avoid cold temperatures. In contrast, patients with severe vasculitic involvement (e.g. with renal, pulmonary and/or gastrointestinal involvement) should be treated aggressively with a combination of high-dose corticosteroids, cyclophosphamide and plasmapheresis [71]. After a remission is induced (generally after 3–6 months), cyclophosphamide can be stopped and azathioprine or mycophenolate mofetil maintenance therapy started and subsequently continued for 18–24 months. Mycophenolate mofetil may also be used as an alternative of cyclophosphamide for induction therapy. Complete clinical and immunologic responses are, however, only obtained in 60–70 % of patients [71]. Furthermore, when patients relapse and induction therapy is re-instituted, response rates are below 40–50 %.

Recently, cyclophosphamide has been successfully replaced by rituximab (reviewed in [72]), a chimeric monoclonal antibody specifically directed to CD20 expressed on B cells and resulting in B cell depletion. Importantly, since rituximab infusion is sometimes associated with an exacerbation of cryoglobulin-related symptoms due to increased complex formation of rituximab with RF-positive IgM/kappa [73], it is advised to use the lymphoma dose: four weekly infusions of 375 mg/m², and not the rheumatoid arthritis dose (two weekly infusions of 1,000 mg). Results obtained in 85 patients with essential mixed cryoglobulinaemic vasculitis treated with rituximab are comparable to the results obtained with cyclophosphamide. Patients with relapses, however, do better during rituximab as compared with cyclophosphamide [71]. Importantly, rituximab in patients with cryoglobulinaemia is much more toxic than in patients with rheumatoid arthritis, and almost half of the patients suffer from side effects. Of special note is the frequency of severe infections in these patients, especially when patients are older than 70 years or have renal involvement. As in ANCA-associated vasculitis [74], these side effects are probably due to simultaneously used high-dose corticosteroids.

Patients with hepatitis C-associated cryoglobulinaemic vasculitis should be treated with an optimal antiviral regimen with at least pegylated IFN- α and ribavirin [75]. With this regimen, now considered the standard of care for HCV management, a virologic response is obtained in 50–70 % of patients. In addition, cryoglobulins disappear and complement levels normalize in the majority of patients [75]. For patients with a hepatitis C genotype 1, a protease inhibitor should probably be added (e.g. Telaprevir or Boceprevir) [76–78]. Other drugs with pangenotypic properties, such as sofosbuvir, are currently tested and will probably be combined with interferon and ribavirin to treat hepatitis C genotypes other than genotype 1 in the nearby future [76, 77]. Finally, other interesting new drugs are currently tested as therapy for hepatitis C [77]. One of them is Miravarsen, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide that sequesters mature miR-122 in a stable heteroduplex thereby inhibiting the stability and propagation of hepatitis C [79].

Antiviral therapy should not be combined with high-dose corticosteroids and/or immunosuppressives in patients with mild-to-moderate disease [5]. In patients with severe disease and/or renal involvement, rituximab should, however, be used during the first month of therapy before anti-virals are started. To control severe and/or life-threatening manifestations in these patients, a short course of corticosteroids in combination with plasmapheresis may be used.

Two randomized controlled trials of rituximab have been performed for the treatment of cryoglobulinaemic vasculitis [80, 81]. Both studies primarily included patients with HCV-associated cryoglobulinaemic vasculitis in whom antiviral therapy had failed or was not indicated. The first trial used the lymphoma rituximab dose in 12 patients, and results were compared with 12 patients receiving conventional treatment [80]. After 6 months, ten patients in the rituximab group were in remission versus one patient in the control group. In the second trial, the rheumatoid arthritis regimen of rituximab was applied in 28 patients, whilst 29 patients were on conventional treatment. The primary end point was the proportion of patients who continued their initial treatment at 12 months, and this was significantly higher in the experimental group (64.3 %) as compared to the control group (3.5 %) [81]. Overall, rituximab was well-effective and well-tolerated in both studies, and no apparent differences between groups in serious side effects, like severe infections or accelerated cryoprecipitation, were reported. From previous case series, a major concern was raised with respect to a possible increased viremia upon rituximab treatment. However, in the study of Sneller et al., it is explicitly reported that no adverse effects of rituximab on HCV plasma viremia were observed [80].

Refractory patients may also be treated with low-dose interleukin-2 as was demonstrated in ten patients with refractory HCV-associated cryoglobulinaemic vasculitis [82]. Together with clinical recovery, these patients showed a

recovery of their regulatory T cell responses. So, the authors postulated that low doses of interleukin-2 induced the activation of Tregs without activating other T cells resulting in clinical recovery without adverse events related to immune activation, such as vasculitis flare.

Conclusion

Cryoglobulins may cause severe clinical manifestations due to hyperviscosity (type I) or vascular inflammation (type II and type III cryoglobulins). In order to define cryoglobulins as the causative agent in these manifestations, good laboratory practice is essential for appropriate detection of cryoglobulins. In particular, the pre-analytical phase of sample collection, requiring conditions at 37 °C throughout the procedure, is considered a prerequisite. The long time required for precipitation at 4 °C (3–7 days) seems, however, at odds with the stringent conditions prescribed for the pre-analytical phase. Nevertheless, by taking care of the correct pre-analytical conditions, a discussion on possible false-negative results can be prevented.

There is a clear association between the occurrence of cryoglobulinaemia and HCV infection, and it is suggested that this relation is causal. The transition from a polyclonal immune response towards an oligoclonal and eventually a monoclonal response with RF activity, however, is both present in patients with systemic autoimmune diseases and infectious diseases such as HCV. The positioning of HCV infection in the preliminary classification criteria for cryoglobulinaemic vasculitis therefore causes a dichotomy in the usefulness of these criteria in HCV-endemic areas versus non-endemic areas where most cryoglobulinaemic vasculitis patients do not suffer from HCV. Indeed, a reduced specificity of these classification criteria is found in cryoglobulinaemia patients that are not HCV infected. Also, the use of RF, sometimes referred to as the ‘poor man’s cryo’, in the preliminary criteria requires attention since commercial assays do not unequivocally detect RF in cryoglobulinaemic sera. Whether this also holds for RF detection in the cryoprecipitate remains to be determined.

Appropriate diagnosis requires close collaboration between the clinician and the laboratory specialist. Obviously, the clinician should be alarmed by the clinical manifestations that might be caused by cryoglobulinaemia. Next, the laboratory specialist should take care of the proper blood analysis, with special attention for the pre-analytical stage. After detection and characterization of the cryoglobulin, the clinician will be able to perform a search for a possible underlying disease and to choose an effective therapy. Besides the use of rituximab, either alone or in combination with plasmapheresis, novel therapies appearing on the horizon, like IL-2 treatment, are promising.

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