Characterization of 30 low cryocrit cryoglobulins in patients with antibodies against hepatitis C virus

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ABSTRACT

Mixed cryoglobulinemia, i.e. the presence of IgG and/or IgM precipitating at temperature <37 °C and dissolving in serum on reheating, is strongly associated with hepatitis C virus (HCV) infection. We studied by immunofixation electrophoresis (IFE) monoclonal and/or polyclonal patterns of cryoglobulins in 30 patients with low cryocrit and positive anti-HCV test. Ten out of 30 IFEs presented an unexpected pattern, characterized by tiny monoclonal components and more than one polyclonal immunoglobulin of different isotype. Two patients showed two monoclonal components of different immunoglobulin classes and in a single case, a monoclonal IgM was associated with 3 different monoclonal IgG and, therefore, these cryoglobulins could be classified as type II, subtype IIb. Twelve cases showed polyclonal immunoglobulins (type III) and 5 cryoprecipitates were classified as type IIa, consisting of polyclonal IgG and monoclonal IgM. The results show the IFE role to accurately identify cryoglobulin patterns, mainly some specific cryoglobulin subtypes, improving thus the patient management and outcome.

INTRODUCTION

Cryoglobulinemia is the presence of immunoglobulins (Ig) precipitating in serum at temperature <37 °C and dissolving on reheating specimen. Reversible cryoprecipitability of proteins may be observed as a concomitant feature of immunocomplex formation. The relevance of the cryoprecipitation phenomenon became apparent when clinical associations with vasculitis and nephritis, resembling those seen in experimental serum sickness, were described. Cryoglobulins were classified by Brouet et al. (1) into three types according to the characteristics of the constituting Ig: type I refers to the presence of a single monoclonal Ig; type II is formed by two Ig, one monoclonal and the other one polyclonal, frequently by a monoclonal IgM-rheumatoid factor (RF) complexed with polyclonal IgG; type III is composed by polyclonal IgM-RF and the corresponding antigen (usually polyclonal IgG) immunocomplex. The two last types are referred as mixed cryoglobulins.

Cryoglobulins in vivo precipitate in the thin vessels of the extremities and the skin, where the blood temperature can drop well below 37 °C, and in the kidney as well. Type I cryoglobulins are found in patients with disorders such as Waldeström's macroglobulinemia, monoclonal gammopathies of undetermined significance (MGUS) and other monoclonal B-cell lymphoproliferative disorders, like multiple myeloma and lymphoma, producing symptoms of vasculitis until distal gangrene and necrosis. In these cases, the cryocrit is usually high. Type II and type III cryoglobulins are detected in patients with a wide variety of diseases, including viral and bacterial infections, autoimmune disorders, lymphoproliferative and chronic liver disease, with the typical symptoms of purpura, arthralgia and Raynaud's phenomenon. In these cases the precipitate is usually low. Type II and type III cryoglobulins were defined "essential" as they were found in patients without any clinically apparent disease (2).

Essential mixed cryoglobulinemia is a systemic vasculitis, affecting small arteries and veins, associated with the presence of large amounts of mixed IgM and IgG cryoglobulins showing RF activity. It is frequently associated with hepatitis C virus (HCV) infection (3-6). HCV may circulate in the blood in two forms, as free HCV and as antibody-complexed virus (7). Anti-HCV antibodies and HCV are found in patients with essential mixed cryoglobulinemia, suggesting that cryoglobulins may be related to the pathogenesis of HCV-associated liver and kidney disease (5). In 1992, Musset et al. observed in patients with cryoglobulinemia a microheterogeneity represented by the presence of two or more small monoclonal IgM or IgG (8). Similar results were described by Tissot et al., who characterized low amount cryoglobulins as polyclonal IgG associated with a mix of polyclonal and monoclonal IgM (9). They were defined as type II-III cryoglobulins. In 1997, Pontet et al. (10) described a new pattern of cryoglobulinemia in a patient with Gougerot-Sjögren syndrome showing a biclonal IgMκ and polyclonal IgG. Authors suggested a modification of cryoglobulin classification, dividing type II into subtype IIa for monoclonal Ig and subtype IIb for two or more Ig clones (10). In this study, we describe the immunoelectrophoretic pattern found on 30 low amount cryoglobulins detected in HCV positive patients and we correlate the low cryocrit type with HCV infection and clinical progression.

MATERIALS AND METHODS

Patients

From patients with anti-HCV positive test and cryo-

globulin presence evaluated in our laboratory, we selected 30 subjects (11 males and 19 females; mean age 59.6 years) with low cryocrit, i.e. <0.9%. Four (13%) patients had associated hepatitis B virus (HBV) infection, 13 (43%) were affected by extrahepatic pathologies, like neuropathy, renal failure, and low grade B cell lymphoma, 7 (23%) presented Meltzer's triad (purpura, arthralgia, and weakness). Six (20%) patients did not show laboratory signs of liver dysfunction as histologically documented (Table 1).

Methods

Using prewarmed equipment, 20 mL of blood were drawn, immediately transferred at 37 °C and allowed to clot for 2 h; then, blood was spun and serum surnatant placed in a Wintrobe tube for cryocrit determination. Serum specimen was incubated at 4 °C for 7 days. Cryoprecipitate was separated from serum proteins by 3 centrifugations, each followed by washing with ice-cold phosphate buffer saline (PBS) containing polyethylene glycol 6000; the PBS volume was approximately five time the quantity of cryoprecipitate. Finally, the cryoprecipitate was dissolved in a dithyothreitol (DTT) solution (DTT 0.5 g in 100 mL of barbital buffer pH 8.9) and incubated 1 h at 37 °C (11). 20 mL of blood sample were enough to find and immunotype also low amount cryoglobulins. Agarose gel electrophoresis was performed on

Table 1
Characteristics of studied patients

ID	Gender	Ethiology	Age, y	Liver disease	Extrahepatic pathology
1	F	HCV	76	Chronic hepatitis	Neuropathy
2	M	HCV	55	Chronic hepatitis	None
3	M	HCV	71	Cirrhosis	None
4	F	HCV	72	Chronic hepatitis	Low grade B-cell lymphoma
5	F	HCV/HBV	73	Cirrhosis	None
6	F	HCV/HBV	62	Cirrhosis	None
7	F	HCV	62	No	Glomerulonephritis, Meltzer's triad
8	M	HCV	56	No	None
9	M	HCV	79	Hepatocarcinoma	Meltzer's triad
10	F	HCV	51	No	None
11	F	HCV	51	Chronic hepatitis	Meltzer's triad
12	F	HCV	53	Chronic hepatitis	None
13	F	HCV	35	No	Neuropathy
14	F	HCV	41	No	None
15	F	HCV	55	Chronic hepatitis	Neuropathy
16	M	HCV	71	Chronic hepatitis	Hashimoto's thyroiditis
17	M	HCV	42	Chronic hepatitis	None
18	F	HCV	69	Chronic hepatitis	Neuropathy
19	M	HCV	63	Chronic hepatitis	Neuropathy
20	M	HCV	77	Chronic hepatitis	Neuropathy
21	F	HCV	58	Chronic hepatitis	Raynaud's phenomenon
22	M	HCV	59	Chronic hepatitis in liver transplant	Renal involvement
23	F	HCV	72	Cirrhosis	None
24	F	HCV/HBV	70	Chronic hepatitis	Meltzer's triad
25	F	HCV/HBV	36	Chronic hepatitis	Neuropathy
26	F	HCV	70	Chronic hepatitis	Neuropathy, renal involvement
27	F	HCV	39	Chronic hepatitis	Meltzer's triad
28	M	HCV	66	No	Meltzer's triad
29	F	HCV	57	Chronic hepatitis	Low grade B-cell lymphoma
30	M	HCV	48	Chronic hepatitis	Meltzer's triad

HCV, hepatitis C virus; HBV, hepatitis B virus.

the automatic system Hydrasys (Sebia), providing a specific program for cryoglobulins electrophoresis, warming the plate at 37 °C during the application and migration steps through the device based on Peltier effect. Application time was modified to 3 min for the small amount of cryoglobulins. Immunofixation was performed according to Alper and Johnson (12) using antisera against γ, α, μ, κ, and λ Ig isotypes supplied by Sebia (Hydragel). Purity degree of the cryoprecipitate was checked by using an additional antiserum against total human serum proteins. A stained band in the immunofixation electrophoresis (IFE) represents a reaction between an antiserum and the specific protein on the specimen (13).

The IgM-RF was measured by rate nephelometry (Siemens Health Diagnostics). In anti-HCV positive patients with IgM-RF negative, IgG-RF was determined by an ELISA technique (Arnika). Antibodies anti-HCV were determined by a 2nd generation ELISA test (Arnika) and confirmed by a 2nd generation recombinant immunoblot assay (RIBA II, Ortho Clinical Diagnostics). The presence of HCV-RNA was detected by reverse transcriptions-nested polymerase chain reaction PCR; viremia was determined by branched DNA probe assay (bDNA) and measured in equivalent genomes/mL. This procedure was performed with simple quantitative PCR.

RESULTS

According to Brouet et al. (1), Musset et al. (8) and Pontet et al. (10), cryoglobulins were classified as 12 type III, 5 type IIa, 3 type IIb, and 10 microheterogeneous. The IFE patterns of the last groups were: 4 patients with IgG oligoclonal, 4 patients with IgG oligoclonal and IgM polyclonal, and the remaining 2 patients with IgG polyclonal and IgM oligoclonal. The mean cryocrit was 0.68 ± 0.30 % for type III, 0.62 ± 0.42 % for type IIa, 0.38 ± 0.35 % for type IIb and 0.43 ± 0.20 % for microheterogeneus patterns. In 4 patients with an IgG pattern, we also found IgG-RF. In all 30 cryoprecipitate samples we detected the HCV RNA and we found different levels of viremia between supernatant and cryoprecipitate: 3.8 ± 1.90 meq genomes/mL in the cryoprecipitate versus 2.4 ± 2.06 meq genomes/mL in the supernatant. We classified 3 cryoglobulins as subtype IIb for the presence in two samples of two monoclonal components and in an other sample of 4 monoclonal components (Figure 1).

DISCUSSION

Our results show some differences when compared to previously published papers reporting a higher prevalence of type II cryoglobulinemia in HCV patients (6,8,14). We found different cryoglobulin patterns, possibly due to low protein amount of the analyzed cryoprecipitates. Many patients affected by chronic HCV hepatitis, whether symptomatic or not, have low concentrations of circulating cryoglobulins (4,6). Moreover, the presence of HCV RNA in all cryoprecipitates suggests a high affinity between cryoglobulins and HCV. Tissot et al. (9) typed 12 low amount cryoglobulins in asymptomatic HCV patients, detecting type III and type II-III cryoglobu-

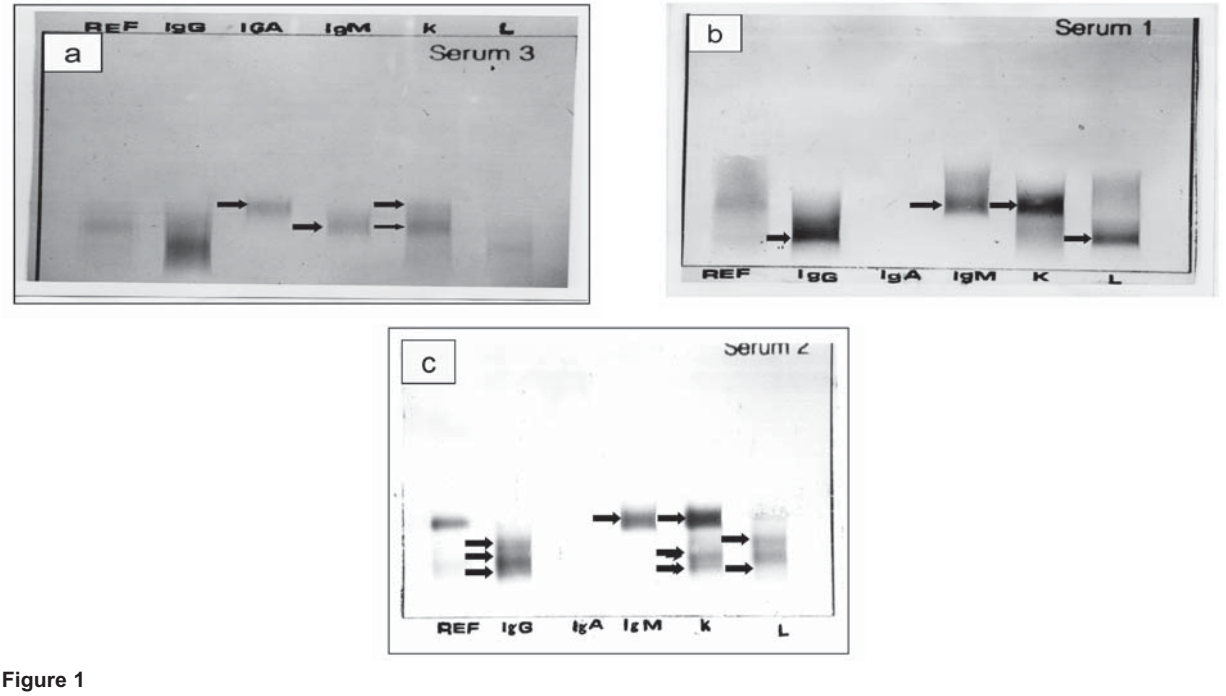

Figure 1
Low amount cryoglobulins immunofixation electrophoresis. a) type IIb cryoglobulin showing two monoclonal components, IgAκ and IgMκ, together with polyclonal IgG; b) type IIb cryoglobulin showing two monoclonal components, IgGλ and IgMκ, together with polyclonal IgM; c) type IIb cryoglobulin showing 4 monoclonal components, two IgGκ, one IgGλ and one IgMκ, together with polyclonal IgG.

lin patterns and postulated that type III cryoglobulin, even in small amount, may represent the earliest stage of cryoglobulinemia. The persistence of HCV in the host may induce continuous B-cell stimulation, overproduction of polyclonal Ig and RF and formation of type II cryoglobulins. Transformation from polyclonal to oligoclonal and, finally, to monoclonal RF may also be induced by HCV as a direct or indirect consequence of peripheral mononuclear cells infection and the mitogenic stimulation. This hypothesized pathogenic mechanism is rather similar to that proposed for the extranodal lymphomas of marginal-zone peripheral B-cells (MALT lymphomas) (15).

In our study, some patterns of the 30 low amount cryoglobulins may indeed represent the early phase of the cryoglobulinemic syndrome. In fact, we detected 12 type III and 10 microheterogeneous cryoglobulins that could be the transient stage of cryoglobulinemia, though the pathophysiological role and the findings of mixed cryoglobulinemia are still unclear (16). Detection of oligoclonal IgG cryoglobulins and relative IgG-RF in 4 patients may suggest that cryoglobulins are a sign of the HCV infection at early stage. The following stage is represented by the same microetherogeneus pattern, with the presence of IgM polyclonal and IgG oligoclonal, and finally the cryoglobulin pattern shift to type III (10). In 5 type IIa cryoglobulinemias we found a pattern of transformation from polyclonal to monoclonal RF after a clonal B cell selection, as described by Tissot et al. (9). We also found 3 cases of subtype IIb with the presence of two monoclonal components in two samples and 4 monoclonal components in another sample. They could represent a consequence of virus interaction with the host, specifically characterized by a genetic background modulating the immune response. The different amount of HCV RNA between cryoprecipitate and supernatant could demonstrate the clinical relevance of viremia determination in fresh serum without cryoprecipitate to improve the accuracy of the results, since high serum HCV RNA concentrations correlate with a poor response to interferon therapy.

There was no correlation between pattern of cryoglobulins and clinical manifestation, except for the neuropathy always observed in patients showing a microheterogeneous pattern. The four patients with IgG oligoclonal and related IgG-RF cryoglobulins presented neuropathy as extrahepatic clinical manifestation. Our hypothesis is that IgG-RF can pass through the hemato-encephalic barrier and then form immunocomplexes. Thus, the neuropathy related to mixed cryoglobulinemia could have an immune-mediated pathogenesis. Therapeutic approach of these clinical form is based on immunosuppressive drugs.

In conclusion, results of the study support the pathogenesis of HCV-related cryoglobulinemia and the molecular mechanism suggested for cryoglobulins generation. The presence of low concentrations cryoprecipitate and their immunotyping patterns, together with symptoms, could help patient management, with regard to the persistence of antigenic stimulation and the possible changes in cryoprecipitate patterns during the cryoglobulinemic syndrome follow-up. The evaluated patients, all with antibodies against HCV, showed a considerable predominance in type III and microheterogeneous cryoglobulin patterns, usually in the absence of other clinical signs. The HCV patients, mainly when chronic liver disease is active, prevalently show these two immunochemical patterns, evolving then into type II during the course of the infection. The presence of two or more monoclonal components is rare in HCV-positive patients and might depend on infection sustained by specific HCV genotypes or on individual immunogenic characteristics. In our study cryoglobulins appear as anti-HCV antibodies considering that we found HCV RNA in cryoprecipitates of all the patients, while in the supernatant the viremia was low or undetectable.

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