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IgG cryoglobulinemia

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Abstract. – OBJECTIVE: Mixed Cryoglobulinemia is the most well-known Hepatitis C Virus (HCV)-associated extrahepatic manifestation. MC is both an autoimmune and B-lymphoproliferative disorder. Cryoglobulins (CGs) are classified into three groups according to immunoglobulin (Ig) composition: type I is composed of one isotype or Ig class. Type II and type III mixed CGs are immune complexes composed of polyclonal IgGs acting as autoantigens and mono, polyclonal or oligoclonal IgM with rheumatoid factor activity. IgG1 and IgG3 are the predominant subclasses involved. This study shows the simultaneous presence of IgG-RF and IgG3, supporting the hypothesis of an involvement of this subclass in the initiation of early stages of CGs.

PATIENTS AND METHODS: We describe a case series of six HCV-positive patients, all of whom had peripheral neuropathy and transient ischemic attacks, presenting cryoprecipitates formed by IgG3 and IgG1. Cryoprecipitate IgG subclass research was carried out by immunofixation electrophoresis by using antisera against IgG1, IgG2, IgG3, and IgG4.

RESULTS: Our six patients presented with an immunochemical pattern characterized by the mere presence of IgG1 and IgG3 subclasses with probable RF activity and one of these six patients exhibited monoclonal IgG3 in his cerebrospinal fluid.

CONCLUSIONS: We can hypothesize that the IgG passage through the blood-brain barrier could have contributed to the cause of TIAs, through a mechanism involving the precipitation of circulating immune complexes formed by the two subclasses in the intrathecal vessels.

Key Words:
Cryoglobulin, IgG3, Hepatitis C Virus.

Abbreviations

MC: Mixed Cryoglobulinemia; HCV: Hepatitis C Virus; NHL: Non-Hodgkin's Lymphoma; CG: Cryoglobulin; Ig: Immunoglobulin; RF: Rheumatoid Factor; TIA: Transient Ischemic Attack.

Introduction

Mixed Cryoglobulinemia (MC) is the most well-known Hepatitis C Virus (HCV)-associated extrahepatic manifestations, but most often it can be due and associated with immunological diseases and disorders or, rarely can be idiopathic. MC is a vasculitis caused by the presence of circulating cryoprecipitate immune complexes. MC is both an autoimmune and B-lymphoproliferative disorder, which is clinically benign but can evolve into frank malignancy in about 8-10% of cases[1,2].

According to different studies[1], many patients affected by chronic HCV infection present with MC whose clinical manifestations are purpura, fatigue and arthralgia. The different symptoms arise from the involvement of different organs and systems. Hence, the multiple symptoms defining full-blown MC can be so severe as to determine a rather poor quality of life for the patient[3,4].

The relationship between Non-Hodgkin's Lymphoma (NHL) and HCV has now been confirmed in a large number of studies with the most convincing evidence resulting from the reduced prevalence of NHL in patients after successful HCV eradication and hematological response to antiviral therapy[4].

Cryoglobulins (CGs) are usually classified into three groups according to immunoglobulin (Ig) composition: type I is composed of one isotype or

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Ig class. Both type II and type III mixed CGs are immune complexes composed of polyclonal IgGs acting as autoantigens and mono, polyclonal or oligoclonal IgM (microheterogeneous type II-III) with rheumatoid factor (RF) activity[5,6]. The expansion of B-cells contributes to the production of autoantibodies, such as anti-IgG RF that constitutes IgG-IgM immune complexes, commonly found in CG precipitates.

Three serological types of MC, Type III, Type II-III and Type II, may represent the different stages of an evolving autoimmune disorder, beginning with polyclonal B-cell activation and leading to final oligo-monoclonal lymphoproliferative disorder. MC could, thus, be viewed as a marker for antigen-dependent lymphoproliferation[7,8], with Type II MC usually being quite stable, compared to the unpredictable Type III MC[6].

IgG molecules generate an immune response by binding Fc γ receptors on target cells and/or by activating the complement system. Functional differences among the IgG subclasses stem from structural differences in the hinge and heavy chain constant regions[9].

IgG1 and IgG3 are the predominant subclasses involved in response to protein antigens. IgG2 is involved in the response to polysaccharide antigens. IgG4 has the minimal ability to activate effector cells or to fix complement[9].

In a case report, Bellotti et al[10] showed a particular CG constituted of polyclonal IgM and a monoclonal IgG3 λ, with IgM representing the antibody of the CG complex[10].

Another study[11] showed the simultaneous presence of IgG-RF and IgG3, supporting the hypothesis of an involvement of this subclass in the initiation of early stages of CGs. Furthermore, a recent study[12] ascertained that CGs were originally composed of only two classes of IgG: IgG1 and IgG3 with RF activity, and that their presence might be interpreted as an early stage of MC. Moreover, murine CGs has shown selective enrichment of a particular IgG subclass: IgG3. IgG3 monoclonal CGs with RF activity induces extensive extra-hepatic manifestations. Human and murine IgG3 have an equal tendency to self-assemble, although there are structural and functional differences, demonstrating a role in the molecular mechanism of the formation of cryoprecipitate[13,14].

This observational study aims to investigate the presence and characteristics of the subclasses in IgG CGs in patients infected with HCV, to show a possible relationship between their occurrence in HCV-positive patients and MC evolution.

Patients and Methods

Patients

The patients underwent a liver biopsy and the histological features of liver specimens were analyzed with the METAVIR group scoring system. The METAVIR scoring system was assessed by a specialist physician, who was unaware of the patients characteristics.

Here, we describe a case series of six HCV-positive patients, all of whom had peripheral neuropathy and transient ischemic attacks (TIA) (Table I), presenting cryoprecipitates formed by IgG3 and IgG1. These patients cannot be completely placed in the classification criteria for MC as proposed by the Italian Group for the Study of Cryoglobulinemias in 1989, which was later revised in 2002[15]. They were enrolled in this study having given their informed consent (in accordance with the Principles of the Declaration of Helsinki) between January 2013 and January 2016. The inclusion criterion was normal complement, HCV-RNA detectable in the serum and positive cryocrit; exclusion criterion was current antiviral therapy or HIV or HBV co-infection.

Methods

Samples were left to clot for ≥1 h (until complete clotting) prior to centrifugation at 37°C at 1700 x *g* for 5 minutes. Separated serum was transferred into Wintrobe tubes, which were immediately incubated at 4°C for 7 days to allow CG precipitation and subsequently centrifuged for 10 minutes at 1700 x *g* (at 4°C). Cryoprecipitate was reported as a percentage of the total serum volume.

The supernatant was then removed and stored for further analyses; the remaining cryoprecipitate was washed 3 times using 2 mL of a 4% polyethylene glycol solution, obtained by mixing 4 g of polyethylene glycol (PEG 6000) with 100 ml of phosphate-buffered saline (4% p/v). After each wash, the cryoprecipitate sample was centrifuged at 1700x *g* for 5 minutes at 37°C. Cryoprecipitates were resuspended in an appropriate volume of 3% PEG 6000 solution, and re-solubilized for 30 min at 37°C. Immunoglobulins were typed and characterized by immunofixation gel electrophoresis, optimized for the Easyfix G26 fully-automated system (Interlab, Rome, Italy), according to the manufacturer's instructions. Immunofixation electrophoresis was performed using antisera against γ, α, μ, κ, and λ (supplied by Interlab, Rome, Italy).

Cryoprecipitate IgG subclass research was carried out by immunofixation electrophoresis by using antisera against IgG1, IgG2, IgG3 and IgG4 (supplied by Binding Site, Birmingham, England). The purity of cryoprecipitates was checked by running the samples on immunofixation gel electrophoresis with total protein antiserum. Immunofixation electrophoresis was carried out on one of these patients with anti IgG3 on cerebrospinal fluid.

All patients showed variations in IgG levels at 37°C and of supernatant due to the precipitation of IgG alone (Table I).

HCV-RNA was assessed in both supernatant and cryoprecipitate and was determined through real-time polymerase chain reaction, transcription-mediated amplification, and multi-probe reverse hybridization of the 5′ untranslated region of the HCV genome. The HCV genotype was determined for each sample by using the line probe assay Versant HCV genotype LiPA 2.0 (Siemens Healthcare Diagnostics, Munich, Germany). Genotypes and subtypes detectable from this test include 1, 1a, 1b, 2, 2a/2c, 2b, 3, 3a, 3b, 3c, 4, 4a, 4b, 4c/4d, 4e, 4f, 4h, 5a, and 6a.

The following serum parameters were assessed: IgG, IgA, IgM, C3, C4 (determined at 37°C, following cryoglobulin separation) and IgG, IgA, IgM supernatant by Nephelometric measurement with BNII (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Rheumatoid Factor was tested in duplicate at 37°C, using ELISA kits for IgG-RF (INOVA Diagnostics, San Diego, CA, USA), and nephelometric assays for IgM-RF IU/ml (Siemens Healthcare Diagnostics, Marburg, Germany). All specimens were analyzed at the same time following the manufacturer's instructions (Table I).

Results

The main baseline characteristics of the case series with chronic HCV infection are reported in Table I.

Our six patients presented with an immunochemical pattern (Figure 1a-b) characterized by the mere presence of IgG1 and IgG3 subclasses with probable RF activity.

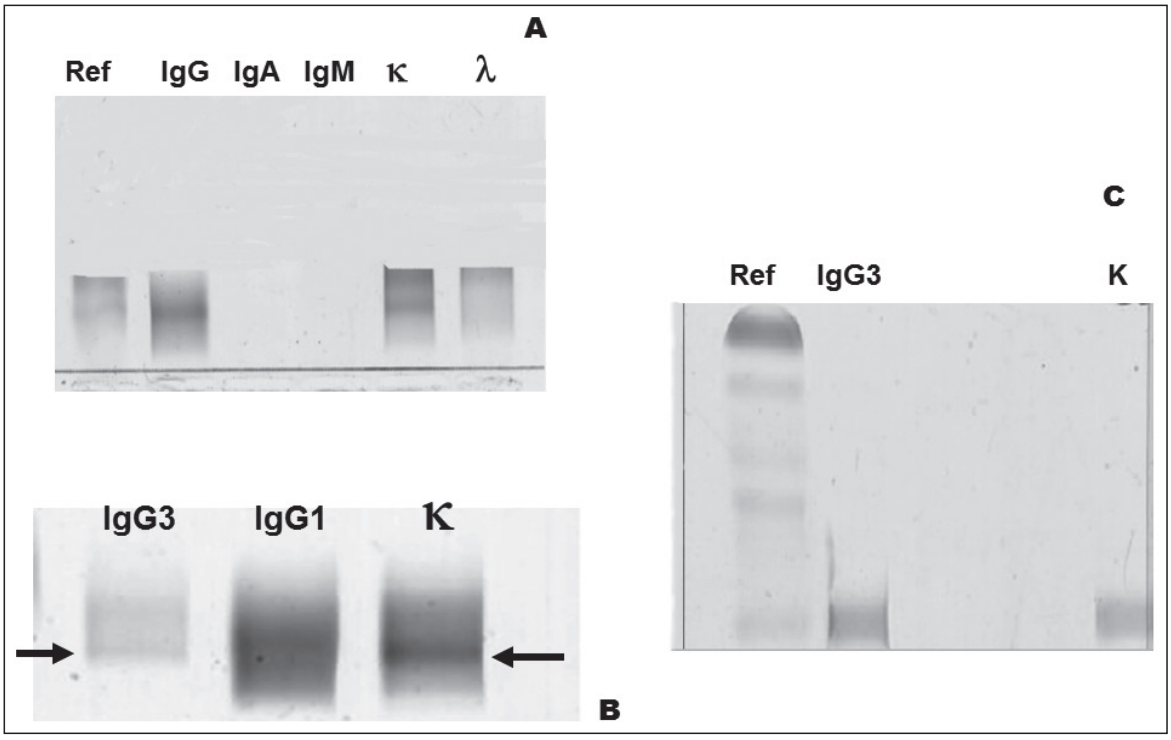

Figure 1. Presence of cryoglobulins in serum and liquor with immunofixation methods. ***A)*** Serum Cryoprecipitate Immunofixation with Antisera Anti-IgG, Anti-IgA, Anti-IgM,Anti- Light Chain Kappa and Anti-Light Chain Lambda. ***B)*** Serum Cryoprecipitate Immunofixation With Antisera Anti-Subclasses IgG1,IgG3 and Anti- Light Chain Kappa. Arrows show monoclonal component IgG3 Kappa. ***C)*** cerebrospinal fluid Immunofixation with Total proteins fixative (Ref), Antisera Anti-Subclasses IgG3 and Anti- Light Chain Kappa.

Table I. Hepato-virological, clinical and biochemical characteristics of the six patients.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Gender	F	F	M	F	M	F
Age	55	76	55	55	77	68
Pathology	No Liver disease	No Liver disease	No Liver disease	No Liver disease	No Liver disease	No Liver disease
Etiology	HCV	HCV	HCV	HCV	HCV	HCV
Extrahepatic Manifestation	TIA -Neuropathy	TIA -Neuropathy	TIA -Neuropathy	TIA -Neuropathy	TIA -Neuropathy	TIA -Neuropathy
ALT limit 45 UI/l	87	24	126	42	46	58
IgG 37°C g/l	26.4	12.7	13.7	8.50	19.40	13.60
IgA 37°C g/l	3.64	1.99	1.5	1.00	1.05	2.31
IgM 37°C g/l	1.02	0.79	1.79	0.92	1.10	1.20
C3 37°C g/l	1.09	1.25	0.84	0.96	1.75	1.12
C4 37°C g/l	0.15	0.16	0.19	0.29	0.30	0.21
IgG sup. g/l	25	11	12.2	7.21	18.50	12.24
IgA sup. g/l	3.7	1.95	1.54	0.98	1.07	2.34
IgM sup. g/l	0.98	0.78	1.75	0.97	1.05	1.18
Cryocrit%	3.5	1.2	1.3	2.1	0.5	0.9
METAVIR SCORE	F0	F0	F0	F0	F0	F0
Immunochemical pattern	IgG1poly+IgG3kmono	IgG3Lmono+IgG1poly	IgG1poly+IgG3kmono	IgG1poly+IgG3oligo	2IgG3kmono + IgG1+IgG2 poly	IgG1poly+IgG3kmono
IgGFR a 37°C [U/mL]nv<20	59	48	52	56	69	63
FR [IU/mL]nv<15	10	12	10	12	4	5
Genotype	2a	1b	1b	2a-3c	1b	1b
Viral titer (IU/mL x 106) Cryoprecipitate	2	5	7	4	1	2
Viral titer (IU/mL x 106) Supernatant	1	3	2	3	0.5	1

°TIA: Transient ischemic attack. Data is expressed as mean ± standard error; *Alanine Aminotransferase (ALT) normal range: 12-45 U/l; IgG normal scores: 7-16 g/l, IgA normal scores: 0.7-4 g/l, IgM normal scores: 0.4-2.3 g/l; #C3 normal scores: 0.8-1.9 g/l, C4 normal scores: 0.1-0.5 g/l. ^Based on liver stiffness assessed by FibroScan; "IgG Rheumatoid Factor (RF) normal scores: < 20 U/mL, IgM Rheumatoid Factor (RF) normal scores: < 15 IU/ml

Notably, one of these six patients exhibited monoclonal IgG3 in his cerebrospinal fluid (Figure 1C). Since HCV infection is a progressive disease, we used the Metavir Score as a surrogate indicator of infection staging (Table I).

Discussion

HCV is a heterogeneous virus that may trigger several immunological mechanisms responsible for the development of different autoimmune and neoplastic disorders[6].

The activation of B-lymphocytes is responsible for the production of immune complexes, including CGs and various autoantibodies[11]. Low temperatures and IgG3 seem to trigger a reversible cryoprecipitation, possibly by inducing steric modifications of Ig molecules. The biological activities of each subclass of IgG are poorly understood and their presence suggests early immune system activation. We have described a clearly differentiated immunological pattern in cryoglobulinemic patients with HCV infection. Patients who are MC HCV positive show hypocomplementemia and Ig RF, suggesting that the stimulation of the immune system by continuous antigenic exposure to HCV would drive a predominant physiopathologic mechanism based on immunocomplex deposition (related to RF activity) and the *in situ* complement activation, but in patients in early stage IgG CG the complement is still normal.

We can hypothesize that the IgG passage through the blood-brain barrier could have contributed to the cause of TIAs, through a mechanism involving the precipitation of circulating immune complexes formed by the two subclasses in the intrathecal vessels. All six patients were treated with antiviral therapy, consisting of Pegylated Interferon and Ribavirin[16], and experienced a progressive improvement in symptoms, concomitantly with a decrease in viral replication and viremia, leading to complete viral eradication. Although a more thorough analysis is needed to confirm our speculations, this clinical data could support the involvement of IgG3 immune complex in causing TIA (Figure 1C).

During the early stages of HCV infection, increased production of circulating CGs comprised of IgG3 and IgG1, related to systemic inflammation, were detected[12]. The hypothesis that IgG3 could play an important role both in the induction and persistence of cryoglobulinemia[12], as well as the assumption of an evolution of such a condition from type III CGs to type II CGs, has been previously confirmed[6].

Since immunotherapy can cause changes in these possible inflammatory mediators and induce HCV liver damage, the presence of IgG3 should be considered when evaluating the clinical status of HCV-infected patients. When HCV-infected patients have extrahepatic manifestations with the possibility of coexisting autoimmune disorders, or more rarely TIA and neuropathies, circulating antibodies need to be evaluated.

Conclusions

The present study, despite the limited amount of samples, suggests that IgG3 subclasses could play a key role in extrahepatic manifestations[12], and shows the interest for further studies investigating this hypothesis. It is important to quantify patients eligible to the new anti-HCV therapies in Italy. However, there are no reliable sources of information on hepatopathic patients prevalence at regional and national level[17].

The risks of morbidity and mortality are frequently underestimated because they do not take into account non-liver consequences of chronic HCV infection. Numerous extrahepatic manifestations have been reported in up to 74% of patients, from perceived to disabling conditions.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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