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Hepatitis C: a possible etiology for cryoglobulinaemia type II

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SUMMARY

Out of 15 successive patients with mixed essential cryoglobulinaemia type II (monoclonal IgM kappa /IgG), 13 had serological evidence for hepatitis C infection as shown by specific enzyme immunoassays and immunoblot. RNA was purified from the serum of seven patients and hepatitis C sequences were identified in five following reverse transcription and DNA amplification. The liver histology showed chronic active hepatitis with or without cirrhosis in the 12 patients with hepatitis C who had a liver biopsy. The two patients without serological evidence of hepatitis C suffered from haematological malignancies. Hepatitis C may be a major etiological agent of cryoglobulinaemia type II.

Keywords cryoglobulinaemia hepatitis C chronic active hepatitis cirrhosis serology

INTRODUCTION

Cryoglobulinaemia type II is characterized by the presence of a circulating monoclonal immunoglobulin rheumatoid factor which precipitates with polyclonal IgG at a temperature below 37°C [1]. The vasculitis and nephritis appear to be secondary to the deposition of immunoglobulin aggregates in small vessels [2,3].

Liver abnormalities have been reported in a variable percentage of patients (16–78%) [4]. The histology reveals most often a chronic liver disease ranging from chronic hepatitis to cirrhosis [4–6]. However, hepatomegaly and high transaminase levels were in many cases the only evidence for liver disease. Some of the patients have serological evidence for chronic infection with hepatitis B virus, which has been considered as the agent responsible for the liver disease and the trigger for the cryoglobulinaemia [4]. However, subsequent epidemiological analysis indicated that only a limited number of cases can be attributed to hepatitis B infection [4,7,8]. More recently Fiorini *et al.* have suggested that the Epstein–Barr virus (EBV) might induce cryoglobulinaemia type II [9].

Recent data have suggested an association between hepatitis C and cryoglobulinaemia type II [10–14]. To explore this hypothesis further, we assessed liver histology, transaminase levels and viral parameters in the 15 patients with cryoglobulinaemia type II seen at our hospital over the last 5 years.

SUBJECTS AND METHODS

The cryoglobulins were quantified by the folin assay after precipitation for 4 days at 4°C. The purified cryoglobulin was dissolved in acetate buffer 0·1 M pH 4·5. The components were separated by gel filtration on Sephacryl-300 (Pharmacia-LKB, Uppsala, Sweden) and analysed for the presence of IgA, IgG, IgM, kappa and lambda light chains using monospecific antibodies. IgG was purified from serum supernatant by Protein-A Sepharose (Pharmacia-LKB). $F(ab)_2$ fragments of IgG were obtained by digestion with pepsin and separation by gel filtration on Sephacryl-200 followed by protein-A Sepharose to eliminate contaminant IgG. The $F(ab)_2$ fragments were pure as assessed by SDS–PAGE.

The presence and specificity of anti-HCV antibodies were evaluated using two enzyme immunoassays (EIA), a neutralization assay and an immunoblot. The two EIA and the neutralization assay are distributed by Abbott (Abbott Laboratories, North Chicago, IL). The first EIA (Abbott HCV EIA) allows for the detection of antibodies against a non-structural HCV recombinant protein: c100–3 antigen. The corresponding neutralization assay is based on the principle that HCV antigen in solution can block binding of antibodies to the HCV antigen coated on the beads of the EIA. Recently a second generation EIA (Abbott HCV EIA second generation) was introduced. In this EIA beads are coated with the c100–3, c22–3 and c33c recombinant antigen (R. Sutherland, Manager R&D, Abbott GmbH, Wiesbaden, Delkenheim, Germany, personal communication); this test may therefore detect antibodies directed against additional epitopes but there is no neutralization assay available. The sera positive on the EIAs were also tested on a second generation Chiron recombinant immunoblot assay (RIBA) (Ortho Diagnostics). This assay comprised recombinant HCV protein corresponding to non-structural peptides: 5–1-1, c100–3, c33c and HCV core associated antigen c22–3 [15].

To detect HCV in clinical samples, 150 μl aliquots of serum were frozen at −75°C within 3 h. RNA was extracted and

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Table 1. Fifteen patients with cryoglobulinaemia type II-IgM-kappa/polyclonal IgG

Origin	Sex	Age (years)	Cryoglobulin (g/l)	Clinical findings	Associated pathologies
1. Swiss	M	73	2·0	P, nephritis*	Myelodysplastic syndrome
2. Swiss	F	55	9·1	P, nephritis*	Sjögren's syndrome, B cell lymphoma
3. Spanish	M	70	6·2	P, SU, stroke	LN of BM, diabetes, silicosis
4. Hungarian	F	59	1·1	P, SU	None
5. Spanish	F	57	4·0	P, neuropathy, nephritis*	LN of BM, lymphadenopathy, diabetes
6. Swiss	M	45	5·0	P, arthritis, NS	None
7. Spanish	M	47	14	P, SU, arthritis, neuropathy	LN of BM
8. Italian	M	54	+	P, neuropathy	Diabetes with nephropathy†
9. Swiss	M	45	0·8	P, SU	None
10. Italian	M	50	22·5	P, SU, arthralgia, nephritis*	LN of BM
11. Swiss	M	35	1·9	P	LN of BM, lymphadenopathy
12. Swiss	F	66	6·8	P, NS*, stroke	None
13. Italian	F	60	0·8	P, stroke, arthritis, NS*	LN of BM
14. Swiss	F	75	1·3	P, Raynaud	LN of BM
15. Erythrean	F	56	1·2	P, nephritis*, stroke	Lymphadenopathy, Sjögren's syndrome, diabetes, breast cancer

P, Purpura; SU, skin ulcers; NS, nephrotic syndrome; LN of BM, lymphoid nodules of bone marrow.
Renal biopsy: * glomerulonephritis of cryoglobulinaemia; † diabetic nephropathy.

cDNA synthesis was performed using 200 units Moloney Murine Leukemia Virus Reverse Transcriptase (Life Technologies, Gaithersburg, MD) in a buffer containing 75 mM KCl, 55 mM Tris-HCl, 6 mM $MgCl_2$, 1 mM each dNTPs, 5 μM random hexamers (Boehringer Mannheim, Germany) and 25 U RNase-Inhibitor (Boehringer Mannheim). Polymerase chain reaction (PCR) was performed using 24-mer oligonucleotide primers derived from the 5′ non-coding region of HCV [16] (sense 5′-TGA GGA ACT ACT GTC TTC ACG CAG-3′; anti-sense 5′-GCT CAT GGT GCA CGG TCT ACG AGA-3′). In brief, 40 cycles of amplification were carried out in a buffer containing 60 mM KCl, 10 mM Tris-HCl, 3 mM $MgCl_2$, 200 mM of each dNTPs, 400 nM of each primer and 2·5 U Taq polymerase (Ampli Taq, Perkin Elmer Cetus, Norwalk, CT). Cycling parameters were as follows: 1 min at 93°C, 1 min 30 s at 60°C, 1 min 30 s at 71°C. PCR products were analysed on 3% sieving agarose gels, blotted on a nylon membrane (Gene Screen Plus, NEN Research Products, Boston, MA) and hybridized to a ^{32}P-labelled 20-mer probe (5′-CAC TAC TCG GCT AGC AGT CT-3′) at 50°C overnight. The membranes were washed three times at 50°C for 15 min with solutions of increasing stringency (third wash: 0·1 × SSC, 0·1% SDS), and autoradiographed.

Liver biopsies were examined for the presence of HBs and HBc antigen by staining deparaffinized sections with a peroxidase-antiperoxidase method using rabbit anti-HBs (Behringwerke, Marburg, Germany) and anti-HBc antibodies (Dako Corporation, Santa Barbara, CA).

RESULTS

All 15 patients had cryoglobulins containing polyclonal IgG and a monoclonal IgM-kappa which had rheumatoid factor activity. The clinical presentations included purpura ($n=15$), nephritis ($n=5$), nephrotic syndrome ($n=3$), skin ulcers ($n=5$), arthritis-arthralgia ($n=4$), stroke ($n=4$), diabetes ($n=4$), and Raynaud's phenomenon ($n=1$) (Table 1) and correspond to the percentages found in the literature [4]. A myelodysplastic syndrome was present in patient 1 at the time of diagnosis, and a malignant lymphoma developed in patient 2 four years after presentation with Sjögren's syndrome and vasculitis. Although eight of the other 13 patients had enlarged lymph nodes or lymphoid nodules of bone marrow, none developed a lymphoma.

Liver histology was obtained in 12 of these 13 patients. It showed chronic active hepatitis in all with a variable degree of fibrosis and, in some cases, cirrhosis (Table 2). Steatosis of variable intensity, lobular inflammation and occasional liver cell necrosis (acidophilic bodies) and bile duct lesions were also present. Nodular lymphoid infiltrates were observed in the portal spaces in four patients. Vasculitis was present only once. Immunoperoxidase staining for HBs and HBc antigen was negative in all. Seven had serological evidence for a past hepatitis B virus infection as shown by HBs, HBc antibodies (patients 3, 4, 5, 8, 9, 11, 15) but none had HBs antigen or HBe antigen. Transaminases were elevated in many patients (Table 2), including the only patient without a liver biopsy. All sera were negative for ANA (titres $\leq$ 1/80), and anti-smooth muscle antibodies.

Anti-hepatitis C antibodies were present in the 13 patients with liver disease. All were clearly positive in the second generation EIA and immunoblot (Table 2). Out of these 13 patients, nine were positive in the first generation EIA, and the specificity of the reaction was attested by the neutralization assay. These assays were not influenced by the presence of the monoclonal rheumatoid factor, since purified IgM kappa rheumatoid factor from two of these patients was negative. In addition, the $F(ab)_2$ fragments prepared from the IgG of four patients contained anti-HCV antibody activity (OD > 1·800 in all four using a final concentration of 125 μg/ml). To see whether there was an enrichment of specific anti-HCV antibodies in the cryoglobulins, i.e. evidence for HCV antigen/antibody complexes which would be precipitated by the monoclonal rheumatoid factor, we compared in one patient the $F(ab)_2$ fragments of the IgG purified from the cryoprecipitate and from the IgG

Table 2. Hepatitis C serology in the 15 patients with cryoglobulinaemia type II

Abbott 1st gen. OD 280	Per cent neutralization + if >50%	Abbott 2nd gen. OD 280	RIBA 2nd generation					Liver tests ASAT/ALAT*	Liver histology
			5-1-1	c100-3	c33c	c22-3	SOD		
1. −(0·01)	—	−(0·15)	−	−	−	−	−	26/40	Minimal fibrosis
2. −(0·07)	—	−(0·07)	−	−	−	−	−	34/31	ND‡
3. +(0·96)	100	+(>2)	+	+	+	+	−	40/54	CAH+extensive fibrosis§
4. +(>2)	93	+(>2)	+	+	+	+	−	71/58	CAH+cirrhosis
5. +(1·94)	96	+(>2)	+	+	+	+	−	215/181	CAH+cirrhosis
6. +(>2)	86	+(>2)	+	+	+	+	−	67/125	ND
7. +(1·91)	100	+(>2)	+	+	+	+	−	85/93	CAH+extensive fibrosis
8. +(>2)	97	+(>2)	+	+	+	+	−	61/66	CAH+cirrhosis
9. +(>2)	89	+(0·64)	+	+	+	+	−	28/43	CAH+extensive fibrosis
10. +(>2)	97	+(>2)	+	+	+	+	−	55/65	CAH+extensive fibrosis
11. +(1·34)	94	+(1·14)	−	+	+	+	−	58/94	CAH+extensive fibrosis
12. −(0·18)	—	+(>2)	−	−	+	+	−	47/25	CAH+minimal fibrosis
13. −(0·18)	—	+(>2)	−	−	+	+	−	49/52	CAH+fibrosis
14. −(0·37)	—	+(>2)	−	−	+	+	−	59/20	CAH+cirrhosis
15. −(0·15)	—	+(1·87)	−	−	−	+	−	165/215	CAH+minimal cirrhosis
Cut off 0·42		0·53							

SOD, Expression vector control; ND, not done; CAH, chronic active hepatitis.
* Normal values U/*l* female/male ASAT 9–40/14–50; ALAT 11–36/11–60.
‡ Normal by inspection during surgery.
§ One artery with fibrinoid necrosis.

remaining in the serum supernatant after cryoprecipitation. Although both provided positive results, there was no clear difference in the antibody titres. Thus HCV antigen/antibody complexes could not account for the cryoglobulinaemia.

Reverse transcription and DNA amplification were performed in the seven patients from whom fresh serum samples were available and five of them were positive.

Patient 6 had abnormal liver function tests and clinical nephritis. He was treated for several years with steroids and cyclophosphamide. Remissions were incomplete and not sustained. At the time of his last relapse, he was started on interferon-alpha (IFN-α) (3×2 MU/week) and went into complete remission (ASAT/ALAT from 71/145 to 25/25 U/*l*, proteinuria from 8·2 g/24 h to 1·2 g/24 h, creatinine from 270 to 119 μmol/*l*, and cryoglobulins from 5 g/*l* to less than 50 μg/ml).

DISCUSSION

The data provided here indicate that HCV infection with chronic liver disease is present in many patients with cryoglobulinaemia type II.

First, the serological evidence for hepatitis C infection in 13 out of the 15 patients is compelling and cannot be explained by an artifact related to the presence of a monoclonal IgM rheumatoid factor for several reasons. The inhibition of binding of the antibodies by the soluble peptide in the nine patients who were already positive in the first generation EIA, indicated that the positive signal was not due to the monoclonal component. In all 13 patients, the second generation EIA test was clearly positive, the results expressed in OD units were well above the cut-off point generally accepted. In addition, all sera were positive in the second generation immunoblot assay. The reactivity was due to the antigen recognition site of IgG since $F(ab)_2$ fragments of the patients' IgG were positive. Finally, control incubations with purified IgM kappa were negative in these assays. The two patients with haematological malignancies but no liver disease were negative in all these assays, which as suggested by others [17] is a further indication that the presence of a monoclonal rheumatoid factor in serum does not invalidate the serology for HCV.

Second, direct evidence of ongoing active hepatitis C infection was provided by the detection of viral RNA in the serum of 5/7 patients tested.

Third, histological evidence of liver disease was striking in the patients with positive HCV serology. The only patient without a biopsy had elevated transaminase levels. Although histological lesions cannot be specific, they were those expected for chronic HCV infection. Similar results were obtained by Dammacco & Sansonno [14] who found a positive serology for HCV in 7/11 patients with mixed essential cryoglobulinaemia (type II and III), and biopsy-proven hepatitis, and by Ferri *et al.* [13], who found HCV RNA in 18/19 such patients.

Several of the patients with severe liver disease on biopsy had only mild or no alterations of the liver function tests, which might explain why in previous series the proportion of patients having liver disease was underestimated [4–6,12,13]. A liver biopsy done systematically might be helpful to determine the extent of the liver disease and treatment.

A history of heavy alcohol ingestion was obtained in only one patient, and other causes of hepatic diseases were excluded, in particular autoimmune hepatitis (negative ANA and anti-smooth muscle antibodies). The most likely confounding disease is hepatitis B. Serological results suggested that some of the patients had had hepatitis B, but HBs and HBe antigens were not detected in serum, and HBs and HBc were absent in liver, suggesting that the infection was not active at the time of our

studies [18]. Fiorini *et al.* have suggested that patients with cryoglobulinaemia type II have ongoing EBV infection because of the presence of IgM antibodies against the viral capsid, and in some cases of the EB genome in the patients' lymphocytes [9]. Our patients have not been tested for persistent EBV infection. It is possible that the emergence of a clone of B cells producing an IgM rheumatoid factor is triggered by a combined effect of different viruses. A further observation suggesting a major etiological role for one or more of these viruses is that many of the patients described here came from the Mediterranean region, where HBV and HCV have a high prevalence.

Bonomo *et al* have treated patients with essential cryoglobulinaemia with IFN-α and have obtained a reduction in the cryoglobulin level and clinical remission in approximately half of them [19,20]. IFN-α suppresses HBV and HCV infections. Thus it is of interest that in the patient with HCV infection and cryoglobulinaemia described here, liver function tests normalized, the cryoglobulin disappeared and the vasculitis went into remission after IFN-α therapy. A similar observation has been made recently in two other such patients [21,22]. This suggests that successful treatment of the viral disease is accompanied by a reduction in the stimulus for the B cell clone to produce the IgM rheumatoid factor.

Finally, many of the patients with hepatitis C had bone marrow biopsies showing lymphoid nodules, and some had large lymph nodes. As reported by Dammacco [14], non-specific lymphoid aggregates were also present in the portal tracts in some liver biopsies. However, the development of lymphoma was not observed in contrast with the two patients without hepatitis C. Thus, the presence of hepatitis C might indicate that progression to lymphoma is unlikely.

Since submitting this paper, we have seen two additional patients with cryoglobulinaemia type II (IgMk/polyclonal IgG) having a positive serology for hepatitis C and chronic active hepatitis on liver biopsy.

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