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Incidence, character and clinical relevance of mixed cryoglobulinaemia in patients with chronic hepatitis C virus infection

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SUMMARY

Hepatitis C virus (HCV) infection has been implicated in the pathogenesis of mixed cryoglobulinaemia. Several studies have shown the presence of anti-HCV antibodies and HCV-RNA in both sera and cryoglobulins of such patients. However, the prevalence and clinical significance of cryoglobulins remain uncertain in patients with chronic HCV infection. We have studied 113 consecutive patients referred for assessment because of the presence of anti-HCV antibody in serum for the presence of cryoglobulinaemia and ascertained their clinical relevance and immunochemical properties. Twenty-one of 113 (19%) had detectable cryoglobulins with a mean protein concentration of 0·38 g/*l* (range 0·15–3·34 g/*l*). Most of these patients were asymptomatic. The cryoglobulins were of type III in 19 (91%) and of type II in two patients (9%). The latter two patients had the highest concentration of cryoglobulins, subnormal C4 and C1q levels suggesting classical pathway activation and vasculitis with renal impairment. The cryoglobulin IgG subclasses were mainly IgG1 and IgG3. HCV-RNA was detected more frequently in the sera of cryoglobulin-positive patients than in cryoglobulin-negative patients. This study showed that mixed cryoglobulinaemia is common in chronic HCV infection, and is predominantly type III. Evidence of systemic or renal disease was rare except in those with type II cryoglobulinaemia, and this may reflect either the concentration of the cryoprecipitate or the presence of a monoclonal complement-activating IgM paraprotein. The detection of HCV-RNA in the majority of the cryoprecipitates further supports the important role of HCV in the etiopathogenesis of essential mixed cryoglobulinaemia, although the mechanism is at present unclear.

Keywords hepatitis C virus HCV-RNA mixed cryoglobulinaemia complement

INTRODUCTION

Mixed cryoglobulinaemia is a systemic disorder characterized clinically by purpura, arthralgia and vasculitis which may also involve the kidney and other organs. The diagnosis is based on the detection of cryoglobulins in serum which contain at least two immunoglobulin isotypes. Cryoglobulins are usually classified on the basis of their immunoglobulin composition into three types according to Brouet *et al.* [1]. Type I consists of an isolated monoclonal component alone, type II of mixed cryoglobulins containing monoclonal and polyclonal immunoglobulins, and type III of polyclonal immunoglobulins only. Both type II and III often have rheumatoid factor activity [2]. Type I cryoglobulinaemia is frequently associated with immunoproliferative disorders such as Waldenstrom's macroglobulinaemia, while mixed cryoglobulinaemia may appear during the course of lymphoproliferative, autoimmune or non-viral infectious diseases [3,5]. However, in 30–55% reported cases no underlying cause has been found, and these are termed essential mixed cryoglobulinaemia [6,7]. Essential mixed cryoglobulinaemia is associated with liver dysfunction, and the role of hepatotropic viruses as a trigger has been investigated over many years. Although early studies [8–10] indicated a link with hepatitic B virus (HBV), numerous subsequent studies have not revealed any relationship between HBV infection and essential mixed cryoglobulinaemia [11,12].

Since the initial report of antibodies against HCV in patients with type II mixed cryoglobulinaemia in 1990 [13], several groups have found an increased prevalence of anti-HCV antibodies in patients with essential mixed cryoglobulinaemia ranging from 30% to 98% positivity in sera and 25% to 78% in cryoglobulins [14–22]. This suggests that HCV infection may be involved in the pathogenesis of cryoglobulinaemia. More direct evidence was provided by the detection of HCV-RNA by polymerase chain reaction

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(PCR) in these patients (63–86% in sera and 75–93% in cryoglobulins) [6,7,14,23,24]. These studies addressed the prevalence of HCV in patients with essential mixed cryoglobulinaemia. In contrast, the prevalence and clinical significance of cryoglobulinaemia in patients with chronic HCV infection are less certain. This study therefore examined the prevalence of cryoglobulinaemia in chronic HCV infection, to assess their clinical relevance, and to elucidate their immunochemical characteristics. In addition, the prevalence of anti-HCV antibodies and HCV-RNA was sought in sera, cryoglobulinaemia-free supernatants and cryoprecipitates.

PATIENTS AND METHODS

Patients

One hundred and thirteen consecutive patients referred from the East Anglian and surrounding regions with chronic HCV infection were studied in the hepatology clinic, Addenbrooke's NHS Trust. All patients were positive for antibody to HCV (by second-generation ELISA (ELISA II) or second-generation recombinant immunoblot assay (RIBA II)). Forty healthy blood donors were enrolled as controls. Full clinical details including routes of transmission and estimated duration of infection (dated from the time of exposure to the first risk factor) were documented. All patients had a liver biopsy and their histological findings were staged according to a modified fibrosis score of 0–5 (0 = normal, 1 = fibrosis confined to portal tract, 2 = fibrous spurs radiating from the portal tracts, 3 = some fibrous linkages between portal tracts, 4 = severe fibrous bridging linkages, 5 = cirrhosis) [25,26]. Other causes of liver dysfunction (such as HBV infection, alcohol, autoimmune and metabolic) were excluded by standard serology and histological features.

Blood donors

The control group comprised 40 healthy blood donors (25 men, 15 women, median age 32 years (range 20–56 years)). All were negative for antibody to HCV, hepatitis B surface antigen (HBsAg), antibody to HIV, and had normal serum alanine transaminases (ALT).

Methods

Detection, isolation and characterization of cryoglobulins. Venous blood was drawn from patients into pre-warmed tubes. Sera were separated from clotted blood by centrifugation at 37°C. Aliquots of sera were stored at –70°C for subsequent analysis. Cryoglobulins were precipitated at 4°C for 72 h. These were only considered to be positive when cryoglobulins were detected on at least two separate occasions. The cryoprecipitates were washed carefully four times at 4°C with PBS, to separate the cryoprecipitates from the remaining supernatant. The total protein concentration of each precipitate was measured by reading the optical absorbance at 280 nm. A purified human protein preparation (Nycomed, Oslo, Norway) was used as a standard. The immunoglobulin composition of the washed cryoglobulins was determined by an immunofixation electrophoretic technique (Dako, Ely, UK). Briefly, washed cryoglobulins (run neat or diluted 1:4 or 1:10 as appropriate) were separated by warm electrophoresis on an agarose gel in a 37°C tank with pre-warmed buffer. A template was placed over the surface of the gel and appropriate monospecific antibody (anti-IgG, anti-IgA, anti-IgM, anti-κ, anti-λ or protein fixative) was added to each trough and incubated for 15 min in a moist box. After incubation, non-precipitated proteins were removed by washing and pressing. Staining of the precipitated proteins was performed using coomassie blue. Confirmation of the identify of the two type II cryoglobulins was undertaken using a separate immunofixation technique in 1% agarose gels using different MoAbs. The presence of IgG subclasses was determined by radial immunodiffusion (The Binding Site, Birmingham, UK) at 37°C according to the manufacturer's instructions.

Immunological investigation. Immunological markers (anti-mitochondrial M2 antibody (AMA), anti-smooth muscle antibody (SMA), anti-liver kidney mitochondrial antibody (LKM), anti-parietal antibody and anti-reticulin antibody) were sought by standard routine indirect immunofluorescence using fixed sections of rat tissue as substrate (Dako). Antinuclear antibodies (ANA) were detected by indirect immunofluoresence on fixed HEp-2 cells.

Serum complement components C3 (reference range, 60–180 mg/*l*) and C4 (reference range, 10–30 mg/*l*) were measured at room temperature by nephelometry from all 21 cryoglobulin-positive patients and 30 cryoglobulin-negative age- and sex-matched patients. C1q levels (reference range 69–120%) at 37°C were measured by radial immunodiffusion (The Binding Site). Rheumatoid factor (RF) was detected by latex particle agglutination at 37°C and latex agglutination and nephelometry at room temperature using standard techniques.

Renal function. The presence or absence of renal disease in the cryoglobulinaemia-positive patients was determined by measurement of serum urea and electrolytes, creatinine, 24-h urinary creatinine clearance and protein excretion using standard methods. Microscopic examination of urine for erythrocytes and casts was also performed.

Virology

Anti-HCV antibody. The presence of anti-HCV antibodies in fresh sera (collected and separated at 37°C), cryoglobulin-free supernatants (i.e. after removal of cryoprecipitates) and washed cryoprecipitates was determined using a second generation ELISA (ELISA; Murex Diagnostics Systems, Dartford, UK, and Sandofi Diagnostics Pasteur, Guildford, UK) according to manufacturers' instructions.

HBV markers. Screening for HBsAg and hepatitis B core antibody (HBcAb) was undertaken using the Amerlite HBsAg and anti-HBc antibody systems, respectively (Johnson and Johnson Diagnostics, Amersham, UK). All these tests were carried out and interpreted according to the manufacturers' instructions.

Detection of HCV-RNA. The sera, supernatants and cryoprecipitates from the 21 patients with cryoglobulinaemia and the sera from the remaining 92 patients without cryoglobulinaemia were studied in parallel to detect HCV-RNA by PCR. This was carried out using nested primers specific for the 5′ untranslated region as previously described [27]. HCV-RNA was released from 200 μl of serum or cryoprecipitate by guanidinium isothiocyanate lysis, extracted in chloroform and precipitated in isopropanol. Reverse transcription was performed using AMV reverse transcriptase (Pharmacia, St Albans, UK) and cDNA was amplified by two-step PCR using Taq DNA polymerase (Promega Ltd, Southampton, UK). The first round PCR using primers NCR2 (5′-ATACTCGAGGTGCACGGTCTACGAGACCT-3′; sense) and OKA3 (5′-CTGTGAGGAACTACTGTCTT-3′; antisense) involved 25 cycles of denaturation (30 s at 94°C), annealing (60 s at 50°C)

Table 1. Clinical, biochemical, virological and histological features of chronic hepatitis C virus (HCV) patients with or without cryoglobulinaemia

	Total ($n = 13$)	Cryoglobulin-positive ($n = 21$)	Cryoglobulin-negative ($n = 92$)
Median range (years, range)	38 (23–78)	43 (23–78)	39 (29–77)
Sex (F:M)	23:90	9:12	24:68
Median duration of infection (years, range)	11 (5–30)	9 (3–30)	12 (5–28)
Abnormal ALT	66 (58%)	15 (71%)	51 (55%)
HCV-RNA-positive* (serum)	72 (64%)	18 (86%)	54 (59%)
Liver fibrosis			
Grade 0	5 (4%)	1 (5%)	4 (4%)
1 and 2	63 (56%)	11 (52%)	52 (57%)
3	25 (22%)	4 (19%)	21 (23%)
4	11 (10%)	2 (10%)	9 (10%)
5	9 (8%)	3 (14%)	6 (7%)
Clinical			
Asymptomatic	104 (92%)	16 (79%)	88 (96%)
Arthralgia	9 (8%)	5 (24%)	4 (4%)
Vasculitis	2 (2%)	2 (10%)	0

* The proportion of patients seropositive for HCV-RNA was higher in those with cryoglobulinaemia than in those without ($P < 0{\cdot}005$).

and extension (60 s at 72°C), followed by a single extension of 7 min at 72°C. In the second round 1 μl (out of 25 μl) of the first round product was amplified in a volume of 25 μl using primers NCR4 (5′-CACTCTCGAGCACCCTATCAGGCAAGT-3′; sense) and OKA1 (5′-TTCACGCAGAAAGCGTCTAG- 3′; antisense) for 25 cycles under the same conditions as described for round one. In positive samples a fragment of 287 bp size was detected under UV light after agarose gel electrophoresis and staining with ethidium bromide. The size of PCR products was estimated by comparison of mobility with DNA molecular size markers (GIBCO BRL, Paisley, UK). Appropriate negative serum controls and negative PCR controls were included in all experiments, and all PCR-positive results were confirmed in a second assay, as were most of the PCR-negative results. Careful precautions to prevent contamination were observed throughout [28].

Statistical analysis

Statistical analysis was performed using the χ^2 test and Fischer's exact test. Differences were considered to be significant when $P < 0{\cdot}05$.

RESULTS

Patient characteristics

The characteristics of the 113 HCV patients with and without cryoglobulinaemia are shown in Table 1. The median age was 38·5 years (range 23–78 years, 90 males and 23 females). The route of transmission was parenteral in 93 (81%), whilst 20 (19%) had no identifiable source (data not shown). The estimated duration of infection was 11 years (range 5–30 years), 58% had a raised ALT and 8% had cirrhosis at the time of the investigation.

Cryoglobulins, complement and C1q levels

In this series, 21 of the 113 (19%) patients with chronic HCV infection had detectable cryoglobulinaemia; mean protein concentration of the washed cryoprecipitates was 0·38 g/*l* (range 0·15–3·34 g/*l*). The other 92 patients and 40 healthy controls who were negative for cryoglobulinaemia had protein concentrations in the washed cryoprecipitates of < 0·06 g/*l*. The immunochemical characteristics of these patients are shown in Table 2. Of 21 cryoglobulinaemia patients, 19 (91%) had type III with polyclonal IgM and IgG components (Fig. 1). Small quantities of IgA were seen in 3/19 patients. Two patients had type II cryoglobulins with a monoclonal component (IgM κ and IgM λ) and polyclonal IgG

Table 2. The immunochemical characteristics of type II and type III cryoglobulins of chronic hepatitis C virus (HCV) patients

	Type II ($n = 2$)	Type III ($n = 19$)
Mean cryoglobulin concentration (g/*l*)	2·05	0·44
IgG subclasses		
IgG1	2	16
IgG2	1 (1)	6 (4)
IgG3	2	17
IgG4	1 (1)	3 (3)
IgM	2	19
IgA	0/2	3/19
Cryoprecipitable RF	1/2	12/19

Trace values are in parentheses. RF, Rheumatoid factor.

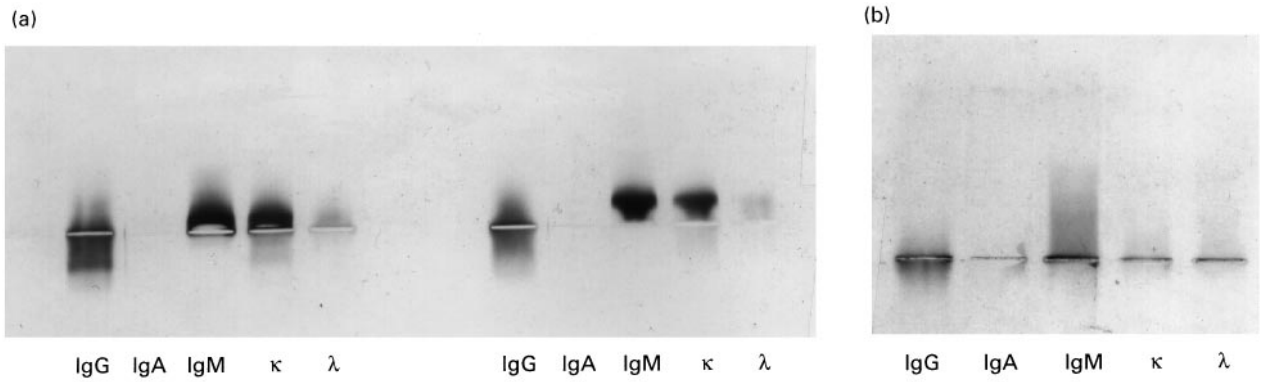

Fig. 1. (a) Type II (monoclonal IgM and polyclonal IgG) and (b) type III cryoglobulins (polyclonal IgM and IgG) visualized by immunofixation.

(Fig. 1). The mean cryoglobulinaemia level was significantly higher in type II (2·05 g/*l*) than type III cryoglobulinaemia (0·44 g/*l*) ($P < 0·001$). The IgG was mainly of the IgG1 (2/2 in type II and 16/19 in type III) and IgG3 (2/2 in type II and 17/19 in type III) subclass, although IgG2 and IgG4 were also detected in a minority of patients. IgG1 was usually the major component. RF activity was present in 1/2 of type II and 12/19 of type III cryoprecipitates. The distribution of the serum complement concentrates (C3 and C4) are shown in Figs 2a,b (normal ranges: C3 = 60–180 mg/*l* and C4 = 10–30 mg/*l*). The mean complement levels from the 21 cryoglobulinaemia-positive patients and 30 cryoglobulinaemia-negative patients were not significantly different ($P > 0·05$); C3 (96·3 mg/*l* *versus* 98·7 mg/*l*) and C4 (17·0 mg/*l* *versus* 20·2 mg/*l*), respectively. However, two patients had subnormal levels of C4 (as indicated by the filled circles in Fig. 2a), both of whom had type II cryoglobulinaemia. Further analysis revealed that the C1q levels of these two patients were also subnormal (see Fig. 3). Among the 21 patients with cryoglobulinaemia, 16/21 (76%) were asymptomatic, 5/21 (24%) had arthralgia. Two patients (both with type II cryoglobulinaemia) had vasculitis manifest by purpura, and both had renal impairment due to glomerulonephritis and low C4 and C1q levels. Both patients responded clinically to interferon-alpha (IFN-α) treatment, but the treatment was discontinued because of intolerance.

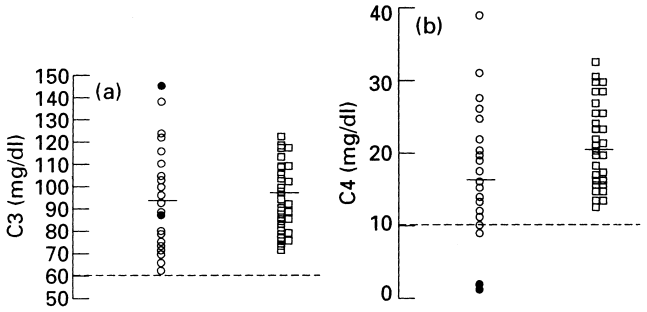

Fig. 2. (a) Serum C3 levels in chronic hepatitis C virus (HCV) patients with ($n = 21$, ○, ●) and without cryoglobulinaemia ($n = 30$, □), and (b) serum C4 levels in HCV patients with ($n = 21$, ○, ●) and without ($n = 30$, □) cryoglobulinaemia. Type II; ○, type III. Mean levels and lower limit of normal are indicated by black lines and dotted lines, respectively.

HCV markers

Anti-HCV antibodies by ELISA II were detected in the supernatants and cryoprecipitates of all the 21 anti-HCV-positive patients with cryoglobulinaemia. HCV-RNA was detected in serum of 18 (86%) and cryoprecipitates in 19 (90%) of those with cryoglobulinaemia, but less frequently in serum (59%) of those without cryoglobulinaemia; only 10 (48%) of the cryoglobulinaemia-depleted supernatants were positive for HCV-RNA.

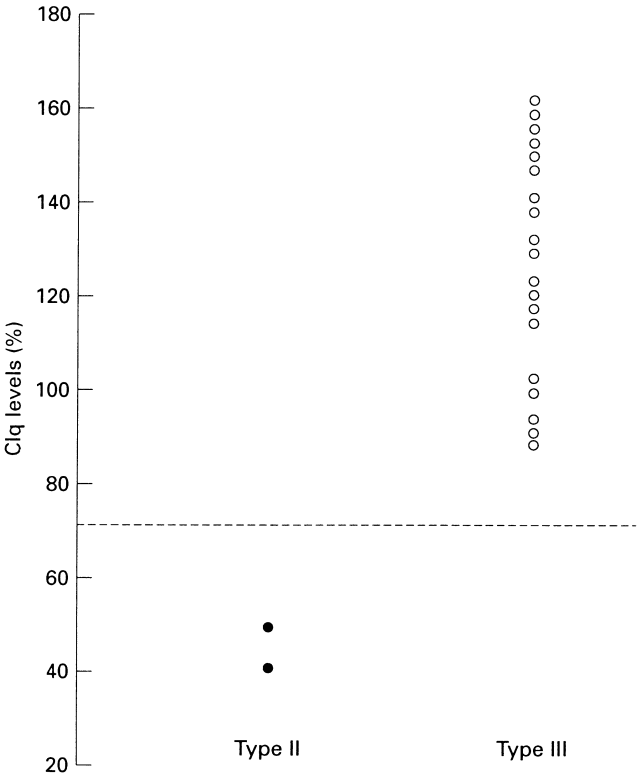

Fig. 3. Serum C1q levels in chronic hepatitis C virus (HCV) patients with type II cryoglobulinaemia (two patients, ●) and type III (19 patients, ○). The lower limit of normal range of C1q (69–120%) is indicated by the dotted line. The C1q levels from the two patients with type II cryoglobulinaemia were subnormal at 39% and 49% compared with the mean C1q level of the 19 patients with type III cryoglobulinaemia (132%).

HBV markers

All the patients were HBsAg- and anti-HIV-seronegative. Anti-HBc antibody was detected in five out of 21 (24%) and 30 out of 113 (27%) patients with and without cryoglobulins, respectively.

Immunological markers

Twelve out of 19 (63%) with type III and one out of two (50%) patients with type II cryoglobulinaemia had positive RF activity in their cryoglobulins. In contrast, most of the sera appeared negative for RF at room temperature (4% positive). Thus, the RF activity was predominantly cryoprecipitable. No autoantibodies (ANA, SMA, AMA, LKM, GPC or reticulin antibody) were found in either group of patients except for one patient with type III cryoglobulinaemia who had positive ANF antibody.

Renal function

Renal function was normal in all the patients with type III cryoglobulinaemia but abnormal in both patients with type II cryoglobulinaemia. Both had signs of chronic renal impairment (raised serum creatinine and impaired 24-h urinary creatinine clearance), and the renal biopsy from one patient showed diffuse proliferative mesangiocapillary glomerulonephritis, and immunohistochemistry revealed granular fluorescence of peripheral capillary loops for IgG, IgM, C3 and C1q and focal IgA.

Comparison between patients with and without cryoglobulinaemia

Table 1 summarizes the epidemiological, clinical, virological and histological data for both groups of patients. There were no significant differences with respect to age, sex, estimated duration of infection or median ALT levels between the two groups. HCV-RNA was detected more frequently in the sera of the cryoglobulinaemia-positive (18/21, 86%) than in the cryoglobulinaemia-negative group (59%) ($P < 0{\cdot}05$). There was no difference in the histological features between the two groups in terms of liver fibrosis. Cirrhosis was present in 3/21 (14%) and 6/92 (7%) of cryoglobulinaemia-positive and cryoglobulinaemia-negative groups, respectively. Among the 21 patients with cryoglobulinaemia, clinical features (arthralgia/arthritis, purpura) attributable to the cryoglobulinaemia did not differ significantly between those with type II or type III cryoglobulinaemia ($P \geqslant 0{\cdot}05$). The frequency of HBV markers (anti-HBc antibodies) was similar in both cryoglobulinaemia-negative and cryoglobulinaemia-positive groups.

Blood donors

Among the 40 healthy blood donors studied, none had cryoglobulinaemia, detectable autoantibodies or complement abnormalities.

DISCUSSION

Essential mixed cryoglobulinaemia has been tentatively linked to many viral infections, including hepatitis A, Epstein–Barr virus and HBV, but these associations were not seen in this series. Recently, HCV has been strongly implicated in the pathogenesis of essential mixed cryoglobulinaemia based on the finding of HCV antibody and HCV-RNA in sera and cryoprecipitates of patients with mixed cryoglobulinaemia [6,7,14–24]. In most of these series, patients were selected for the presence of mixed cryoglobulinaemia and all exhibited significant clinical manifestations related to the underlying cryoglobulinaemia. The prevalence and clinical relevance of these cryoglobulins is less well described in patients presenting with chronic HCV infection. This prospective study demonstrates that mixed cryoglobulinaemia in an unselected population of HCV patients is common. Most of the patients were young and had only mild to moderate liver fibrosis, suggesting that the association was not with liver disease *per se*. The observation that HCV-RNA could be found more frequently in the sera of cryoglobulinaemia-positive patients may indicate an increased viral load, suggesting that HCV infection and viral load may play a direct role in the pathogenesis of cryoglobulinaemia.

Lunel *et al.* [24] reported a higher prevalence of mixed cryoglobulinaemia in France in a series of 127 HCV patients (69/127, 54%), and associated this with the presence of cirrhosis and longer history of hepatitis [29]. There may be several explanations for this difference. It is noteworthy that there is a similar geographical distribution of mixed cryoglobulinaemia and HCV infection, both being more common in southern Europe than northern Europe and North America [30]. The population studied by Lunel *et al.* [24] was also slightly older (average age 50·4 years) and more had cirrhosis (43/127, 33·8%) than our study group (median age of years, 8% had cirrhosis (9/113)). It should be acknowledged that the absence of any correlation between chronic liver disease and cryoglobulinaemia in our study may be a result of the small number of our patients who had severe liver disease, but this is unlikely.

We found that type III cryoglobulinaemia was predominant in UK patients with chronic HCV infection, but in type II cryoglobulinaemia the mean concentration of cryoglobulins was much higher. This finding is in agreement with Lunel *et al.*, but differs from those with mixed cryoglobulinaemia secondary to lymphoproliferative diseases, where type II cryoglobulinaemia predominates. The major immunoglobulin isotypes in this study were IgG (mainly IgG1 and IgG3 subclasses) and IgM. Clinically, most of our patients were asymptomatic, but the two patients with typical systemic disease associated with cryoglobulinaemia had the highest concentrations of cryoglobulinaemia and both were of type II variety. In addition, only sera from these patients showed classical pathway complement activation.

Cryoglobulinaemia may be due to prolonged antigenic stimulation by either an exogenous or an endogenous antigen or by polyclonal activation of B cells. Why certain antibodies have the physiochemical property of cryoprecipitation is unknown. The murine IgG3 subclass (different from human IgG3) in MRL-*lpr/lpr* mice is capable of non-immunological Fc-Fc-mediated self-aggregation and may therefore provide a cryoprecipitate regardless of antibody specificity [32]. In addition, many cryoprecipitable RF autoantibodies in this mouse model are of the IgG3 isotype. Cryoprecipitation may also depend on specific antigen–antibody recognition mediated by the antigen binding site of the antibody, and this interaction may alter the cryoprecipitation properties of the immunoglobulin, perhaps as a result of conformational changes [33]. As in the lpr mouse, RF complexes may be precipitated in the cold, leading to the formation of large insoluble immune complexes, although interestingly some of both the type II and type III cryoglobulinaemia in this study had no detectable RF. It should also be noted that small quantities of cryoglobulinaemia are found in normal serum.

Our results suggest that the HCV genome is more readily located in the cryoprecipitate than in the supernatant, perhaps as a result of antibody–antigen binding. This suggests that viraemia is important in the development of cryoglobulinaemia and that anti-HCV antibodies are an important part of the cryoprecipitate. This suggestion is supported by other studies [6,24]. Polyclonal activation of B cells by chronic HCV infection or superantigen properties of HCV proteins may be involved in the formation of cryoprecipitable immunoglobulin and/or RF. The presence of HCV antibody and HCV-RNA in the cryoprecipitates may also be due to non-specific trapping of immunoglobulin, and may not necessarily favour a direct role for antibody–antigen interaction in the formation of cryoglobulinaemia. However, it is possible that HCV antigen–antibody binding may alter the cryoprecipitability of the complex, as described for positively charged conjugates in mice [33], but this does not necessarily involve the participation of RF activity. Cryoprecipitable RF was found in only 62% of the cryoglobulinaemia-positive population, yet all of the sera of HCV-infected patients (both with and without cryoglobulinaemia) contain anti-HCV antibody, and 64% contain HCV-RNA. Thus the majority of anti-HCV antibodies are non-cryoprecipitable, even in the presence of viral RNA.

A further possibility is that there is antigenic cross-reactivity between HCV and an unknown hepatic antigen. This may be supported by the finding that HCV infection can induce antibody responses to a microsomal antigen, including cytochrome p450 isoenzymes or other host proteins [31], although this observation would not exclude polyclonal activation of immunoglobulin production by HCV. It is interesting to note that the predominant IgG subclasses in the polyclonal cryoglobulinaemia component are IgG1 and IgG3, which constitute the major human IgG subclasses generated to T cell-dependent protein antigens and viral capsids, and these subclasses are potent activators of the classical pathway of complement.

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