Original article

Prevalence of ANCA in mixed cryoglobulinemia and chronic hepatitis C virus infection

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ABSTRACT

Objective. *To determine the prevalence, target antigens and clinical associations of antineutrophil cytoplasmic antibodies (ANCA) in chronic hepatitis C without extrahepatic manifestations and in chronic hepatitis C virus (HCV)-associated mixed cryoglobulinemia (MC) in two European centers.*

Methods. *50 sera from patients with chronic hepatitis C and 116 sera from HCV-associated MC were tested for cytoplasmic or perinuclear pattern (C-ANCA/P-ANCA) by indirect immunofluorescence test (IFT). ANCA target antigens were determined by enzyme-linked immunosorbent assay (ELISA).*

Results. *Clinical characteristics of the patients were not different between the two centers. Cryoglobulinemic vasculitis (CV) was biopsy-proven in about 90% of the MC patients. Two patients with HCV-associated MC and 1 patient with chronic hepatitis C had a P-ANCA. A C-ANCA was detected in 1 patient with HCV-associated MC. Eight patients with a HCV-associated MC and 5 patients with chronic hepatitis C had an ANCA either directed against bactericidal/permeability increasing protein (BPI) or cathepsin G (CG). BPI- or CG-ANCA positivity was not associated with a more severe disease course. The C-ANCA titer followed disease activity in one C-ANCA positive HCV-associated MC patient. The subspecifity of the C-ANCA was not determinable in that patient.*

Conclusion. *Two new target antigens of ANCA have been identified in HCV-associated MC and chronic hepatitis C in this study. BPI-ANCA and GC-ANCA were present in about 10% of patients with HCV-associated MC or chronic hepatitis C. ELISA proved to be more sensitive in the detection of ANCA than IFT. The present study on chronic HCV infection adds to various reports on the induction of CG- and BPI-ANCA in chronic infections.*

Introduction

Soon after the identification of the hepatitis C virus (HCV) as the principle cause of chronic non-A, non-B hepatitis (1) several authors reported on the association of formerly so-called "essential" mixed cryoglobulinemia (EMC) with HCV infection in 80-90% of the cases (2-6). Purpura and rheumatic complaints are the most common symptoms found in HCV-associated MC (7-10). Leukocytoclastic vasculitis is the hallmark of cutanous manifestations of MC. The pathogenesis of vasculitis is secondary to the deposition of immune complexes, mainly cryoglobulins [cryoglobulinemic vasculitis (CV)], and complement in small and – less often – medium-sized blood vessels (8).

Autoantibody production and phenomena of autoimmune diseases have been reported in patients with chronic HCV infection (7-10). Rheumatoid factor (RF) is seen in more than 75% of the patients. In type II (monoclonal IgM and polyclonal IgG) MC cross-reacting idiotypes on monoclonal IgMk rheumatoid factors, e.g. the WA idiotype, indicate an antigen independent proliferation of autoreactive B-lymphocyte clones (11, 12). Ten percent to 30% of patients with MC have low-titer antinuclear antibodies (ANA) (10). Autoantibodies against extractable nuclear antigens such as anti-Ro or anti-La are detected in less than 5% of patients with chronic hepatitis C (8). Sicca syndrome is seen in about 20-30% of patients with chronic hepatitis C (8-10). Whether autoantibodies are found more frequently in HCV sequelae such as mixed cryoglobulinemia (MC) is still a

matter of debate (8).

The detection of antineutrophil cytoplasmic antibodies (ANCA) has been reported in chronic hepatitis C and HCV-associated MC in few cases so far (13-18). Cacoub *et al.* (14) did not find ANCA using IFT and MPO-ANCA ELISA in 36 patients with "essential", i.e. HCV-negative, MC or HCV-associated MC. They detected P-ANCA in 2 of 15 patients with HCV infection without cryoglobulinemia and concluded, that ANCA do not play a role as a marker or mediator of disease in MC. The target antigen of ANCA was reported not to be myeloperoxidase (MPO). However, other target antigens were not mentioned (14).

In this study, we sought to determine the prevalence of ANCA and association with disease manifestations in a much larger group of patients consisting of 166 patients with HCV-associated MC or chronic hepatitis C without extrahepatic manifestations. The study was conducted in 2 European centers with extensive experience in the diagnosis and treatment of patients with chronic hepatitis C and HCV-associated MC. An array of different target antigens of ANCA was determined in these patients. Several of these target antigens have not been tested in chronic hepatitis C or HCV-associated MC so far.

Patients and methods

Clinical evaluation

Sera from patients with HCV-associated MC and from patients with chronic hepatitis C without clinical symptoms of MC were analyzed. HCV-associated MC was diagnosed between January 1996 and January 2002 on the basis of the revised criteria of the GISC (Italian Group for the Study of Cryoglobulinemias) (19,20). In particular, MC was diagnosed in patients with either:

a) mixed cryoglobulinemia ± low complement factor C4, purpura, and biopsy-proven leukocytoclastic vasculitis or

b) mixed cryoglobulinemia ± low C4, 2/4 of clinical manifestations (chronic hepatitis, membrano-proliferative glomerulonephritis, peripheral neuropathy, skin ulcers), and 2/4 of serological/pathological findings

(RF, HCV RNA, HBV DNA, clonal B-cell infiltrates in the liver and/or bone marrow).

In all cases the coexistence of autoimmune, lymphoproliferative or other infectious diseases (except HCV or HBV infection) was excluded (19, 20). In addition, histological proof of cryoglobulin immune deposits affecting small vessels was sought in order to fulfill the Chapel Hill Consensus definition of CV (21). Diagnosis of chronic hepatitis C infection was based on standard criteria, e.g. history, laboratory abnormalities, serology and liver histology as described elsewhere (22). Detection of HCV antibodies and demonstration of HCV-RNA by the polymerase chain reaction (PCR) were required for the diagnosis of HCV-infection in all patients. The disease extension and vasculitis activity were described by the Disease Extension Index (DEI) and Birgmingham Vasculitis Activity Score (BVAS) as outlined elsewhere (23, 24). In brief, the DEI is the equivalent of organ involvement attributable to active vasculitis (23), whereas the BVAS considers clinical features and laboratory data to give a measure of vasculitis activity (24).

Study population

A total of 166 sera from 116 patients with HCV-associated MC and from 50 patients with chronic hepatitis C without clinical symptoms of MC were analyzed. The group of 50 patients with chronic hepatitis C without extrahepatic manifestations was from the Italian center. 100 patients from the Italian center and 16 patients from the German center had HCV-associated MC.

There were no statistically significant differences between the patient groups, when patients with chronic hepatitis C without extrahepatic manifestations and patients with HCV-associated MC were compared with regard to age, ESR, CRP, and complement factor C3. There was a trend towards lower complement C4 values in patients with HCV-associated MC (11 ± 6 resp. 12 ± 7 mg/dl in Italian resp. German center) compared with patients with chronic hepatitis C without extrahepatic manifestations (28 ±12 mg/dl, P= 0.06, Italian center). Trace amounts of cryoglobulin were detected in 14% of the patients with chronic hepatitis C. However, these patients had no symptoms attributable to MC such as purpura, arthralgia or signs of organ dysfunction attributable to immune complex-mediated disease, e.g. CV.

There were no statistically significant differences for age, ESR, CRP, complement factors C3 and C4 when the patient groups from the Italian and the German center were compared (Table I).

Table I. Patient characteristics were not significantly different for patients with chronic hepatitis C without extrahepatic manifestations and patients with HCV-associated mixed cryoglobulinemia (MC) between the two centers from Italy and Germany. There was a trend towards lower complement C4 between chronic hepatitis C and HCV-associated MC. Trace amounts of cryoglobulin were detected in 14% of patients with chronic hepatitis C without clinical symptoms of MC or cryoglobulinemic vasculitis.

	Chronic hepatitis C Italy	MC Italy	MC Germany
No.	50	100	16
Age (years)	59 ± 12	58 ± 9	54 ± 13
ESR (mm/h)	15 ± 12	21 ± 19	22 ± 16
CRP (mg/dl)	3 ± 2	4 ± 3	2 ± 2
Cryocrit (%) / cryoglobulin (mg/l)	Trace in 14%	5.5 ± 10.0%	476 ± 907 mg/l
Type II cryoglobulinemia[1]	-	65%	100%
Type III cryoglobulinemia[2]	-	35%	0%
C3 complement (mg/dl)	75 ± 30	66 ± 26	88 ± 27
C4 complement (mg/dl)	28 ± 12	11 ± 6	12 ± 7
Creatinine (mg/dl)	0.91 ± 0.23	1.2 ± 0.51	0.86 ± 0.17

[1]Type II cryogl.: mixed monoclonal IgM and polyclonal IgG cryoglobulinemia. [2]Type III cryogl.: mixed polyclonal IgG cryoglobulinemia. Type II and Type III mixed cryoglobulinemia according to (25).

All patients of the German group had HCV genotype 1, patients from the Italian center were not routinely tested for the genotype.

Laboratory studies
In both centers, patient sera were routinely tested for serum transaminases, GT, AP, concentrations of serum bilirubin, total protein, albumin, IgG, IgA, IgM, creatinine, complement factors C3c and C4, rheumatoid factor (RF), and anti-nuclear antibodies (ANA) by standard techniques. Cryoglobulins were detected and classified according to Brouet *et al.* (25). In brief, venous blood is kept at 37°C for 2 hours for complete coagulation. Thereafter, the serum is kept at 4°C for 96 hours for precipitation of cryoproteins. Visible precipitates are quantified either as the cryocrit (percent per volume) or in the form of protein concentration (i.e. mg/l). For classification according to Brouet *et al.* (25) cryoprecipitates are washed four times and assayed by immunofixation for polyclonal and monoclonal components (22, 25).

Detection of ANCA and ANA
ANCA were detected by established and evaluated techniques in the laboratory of the German group (26). All serum samples had been stored at –20°C prior to analysis. Cytoplasmic pattern (C-ANCA) and perinuclear pattern (P-ANCA) were detected by indirect immunofluorescence test (IFT). Briefly, air-dried, ethanol-fixed cytospin preparations of purified neutrophils were incubated with the test serum diluted with phosphate buffered saline (PBS). Antibody binding was detected with fluoresceinisothiocyanate-conjugated F(ab')$_2$ fragments of rabbit anti human IgG (Dako, Copenhagen, Denmark). Cytoplasmic or perinuclear fluorescence was denoted.
For the differentiation of P-ANCA from antinuclear antibodies, samples with perinuclear fluorescence on ethanol-fixed neutrophils were also tested on formalin-fixed neutrophils. These were prepared by incubation of cytospin preparations of purified neutrophils with 0.5% formalin at room temperature for 5 minutes. Perinuclear fluores-

cence on the former was taken to indicate the presence of P-ANCA only if associated with exclusively cytoplasmic fluorescence on formalin-fixed cells. If this procedure did not yield a clear-cut discrimination between ANA and P-ANCA, sera were further tested on Hep-2 cells. Results were accepted as P-ANCA-positive, if the titer on neutrophils was at least 4-fold higher than that on Hep-2 cells.
Target antigens of ANCA were determined by solid phase enzyme-linked immunosorbent assay (ELISA) as described earlier (27,28). Target antigens were highly purified myeloperoxidase (MPO), proteinase 3 (PR3), human leukocyte elastase (HLE), cathepsin G (CG), lactoferrin (LF), bactericidal permeability increasing protein (BPI) and lysozyme (LZ). The optical density (OD) mean value + 3 x sd of 140 healthy blood donors sera served as the cut-off point, as described earlier (29). Detection of antinuclear antibodies (ANA) was performed by the immunofluorescence technique on HEp-2 cells.

Statistical analysis
Patient characteristics are presented as the mean± standard deviation (SD). Clinical and serological parameters were tested for significant differences between both centers with the Mann-Whitney U Test.

Table II. Prevalent symptoms of HCV-associated mixed cryoglobulinemia (MC) were purpura, weakness, arthralgia or arthritis, polyneuropathy and glomerulonephritis in the German and the Italian center.

Clinical manifestation	German group	Italian group
no.	16	100
Purpura	88%	91%
Arthralgias	56%	83%
Weakness	75%	89%
Polyneuropathy	81%	40%
Glomerulonephritis	19%	31%
Gastrointestinal involvement	19%	8%
Cardiac involvement	13%	10%
Central nervous system manifestation	6%	3%
Raynaud phenomenon	19%	34%
Sicca syndrome	19%	38%
DEI	4.3 ± 3.7	6.4 ± 2.2
BVAS	8.5 ± 7.2	15.0 ± 4.8
Biopsy-proven cryoglobulinemic vasculitis	94%	90%

DEI = disease extension index according to (23). BVAS = Birmingham vasculitis index according to (24).

Results

Clinical manifestations of HCV-associated MC
Within the group of 116 patients with HCV-associated MC, the presence of cryoglobulinemic vasculitis was biopsy-proven in 90% of the patients from the Italian center and in 15 of 16 (94%) patients from the German center. Most prevalent symptoms and manifestations of MC were palpable purpura, arthralgia or arthritis, weakness, polyneuropathy, glomerulonephritis, sicca syndrome and Raynaud`s phenomenon (Table II).

Antinuclear antibodies in patients with chronic hepatitis C and HCV-associated MC
Antinuclear antibodies (ANA) were detected in 7 patients with chronic hepatitis C without extrahepatic manifestations. Twenty-two patients from the Italian group and 6 patients from the German group with HCV-associated MC had ANA. Thus, ANA were more prevalent in HCV-associated MC compared with chronic hepatitis C without extrahepatic manifestations (Table III). ANA were usually fine speckled.

Antineutrophil cytoplasmic antibodies (ANCA) in patients with chronic hepatitis C and HCV-associated MC
ANCA were detected by IFT in 1

Table III. BPI-ANCA and CG-ANCA were detected by ELISA in about 10% of patients with either chronic hepatitis C or HCV-associated mixed cryoglobulinemia (MC), whereas other target antigens were not detected. C- or P-ANCA were detected less often by IFT. ANA were more prevalent in HCV-associated MC and cryoglobulinemic vasculitis (CV) than in chronic hepatitis C.

	Chronic hepatitis C	HCV-associated MC
no.	50	116
C-ANCA (IFT)	0	1 (1:128)
P-ANCA (IFT)	1 (1:32)	2 (1:32, 1:64)
BPI-ANCA (ELISA)	2 [50, 66 (U/ml)]	4 [30, 37, 48, > 128 (U/ml)]
CG-ANCA (ELISA)	3 [118, 120, 120 (U/ml)]	4 [20, 32, 41, > 128 (U/ml)]
ANA	7 (1:64 – 1:512)	28 (1:64 to > 1:2048)

Antineutrophil cytoplasmic antibodies: ANCA; cytoplasmic pattern ANCA: C-ANCA; perinuclear pattern ANCA: P-ANCA; IFT: indirect immunofluorescence technique; enzyme-linked immunosorbent assay: ELISA.
Target antigens of ANCA determined in this study: Proteinase 3 (PR3), myeloperoxidase (MPO), human leukocyte elastase (HLE), lactoferrin (LF), lysozyme (LZ), bactericidal permeability increasing protein (BPI), and cathepsin G (CG). Titers of the IFT results and the ELISA tests are given in parenthesis. Cut-off of IFT 1:2. Cut-off of ELISA: 10 U/ml.

patient with chronic hepatitis C without extrahepatic manifestations and in 3 patients with HCV-associated MC. The patient with chronic hepatitis C had a P-ANCA. One patient with HCV-associated MC had a C-ANCA and 2 patients a P-ANCA. Moreover, BPI-ANCA and CG-ANCA could be detected by ELISA in about 10% of patients with either chronic hepatitis C without extrahepatic manifestations or HCV-associated MC. Two patient with chronic hepatitis C had a BPI-ANCA and 3 had a CG-ANCA. Four patients each had BPI-ANCA or CG-ANCA within the group of patients with HCV-associated MC.

The target antigen of the C-ANCA was CG in the patient with chronic hepatitis C. The target antigen of the P-ANCA in the patients with HCV-associated MC was BPI. The target antigen of the C-ANCA in the other patient with HCV-associated MC was not determinable. Of note, the C-ANCA titer followed disease activity in this patient. Disease extension was followed by the Disease Extension Index (DEI) (23) and disease activity was followed with the Birmingham Vasculitis Activity Score (BVAS) (24) in this patient similar to other patients with HCV-associated cryoglobulinemic vasculitis as described earlier (30). There was no ANCA detectable with IFT in the other patients, who were shown to have either a CG-ANCA or BPI-ANCA by ELISA. ANCA directed against other subspecifities, i.e. PR3, MPO, HLE, LF or LZ, were not detected by ELISA in the patients (Table III).

Comparison of CV patients with and without ANCA
No statistically significant differences were found for the laboratory and clinical parameters between patients with either BPI- or CG-ANCA with chronic hepatitis C without extrahepatic manifestations and HCV-associated MC.

Discussion
This study analyzed the prevalence of ANCA in the largest group of patients with either chronic hepatitis C without extrahepatic manifestations or HCV-associated MC tested for ANCA so far. Since HCV-associated cryoglobulinemic vasculitis is an immune complex-mediated vasculitis and does not belong to the group of ANCA-associated vasculitides, i.e. Wegener's granulomatosis, microscopic polyangiitis and Churg-Strauss syndrome (21), it was of particular interest whether ANCA can occasionaly be detected in this patient group and whether there was any relation to the disease course. We found two new target antigens antigens of ANCA in HCV-associated MC and chronic hepatitis C in this study. BPI- or CG-ANCA were detected by ELISA in 10% of the patients with either chronic hepatitis C or HCV-associated MC. Furthermore, IFT was less sensitive in the detection of ANCA. Thus, ANCA detection by IFT may underestimate the prevalence of ANCA in HCV related diseases. ANCA directed against other target antigens, i.e. PR3, MPO, HLE, LF or LZ, were not detected by ELISA within both patient groups. ANA, usually of the fine speckled type, were more prevalent in HCV-associated MC than in chronic hepatitis C.

Our data confirm earlier single-case reports and smaller case series (Table IV) in which ANCA have been detected in chronic hepatitis C without extrahepatic manifestations and in HCV-associated MC (13-18). MPO was found to be the target antigen of P-ANCA in two earlier case reports (13, 17). P-ANCA and C-ANCA were reported in two other cases of chronic hepatitis C without extrahepatic manifestations (14) and one case of biopsy-proven HCV-associated CV (16, 18). The only two cases, where a C-ANCA with PR3 specificity has been reported in biopsy-proven CV so far, had an "essential", i.e. HCV-negative, CV and an endocarditis-associated CV (16, 28, 31). PR3 specificity was also proven in these patients by an inhibition assay and by immunobloting (16, 18). Interestingly, PR3-ANCA and/or C-ANCA were shown to follow the disease course in these patients (16, 18, 31) similar to the one patient with C-ANCA positive HCV-associated MC in this study, whose C-ANCA titer followed disease activity as determined by the BVAS (24, 30).

In the present study, systematic testing for various target antigens revealed that about 10% of patients with chronic hepatitis C without extrahepatic manifestations and patients with HCV-associated MC had either BPI-ANCA or CG-ANCA. No other target antigens were detected. In contrast to the above mentioned rare cases of cryoglobulinemic vasculitis with PR3-ANCA and/or C-ANCA, where the ANCA followed disease activity, we could not find an association of BPI- and CG-ANCA in chronic hepatitis C without extrahepatic manifestations and in HCV-associated MC with more severe disease courses in this study. However, detection of BPI- and CG-ANCA in chronic hepatitis C and in HCV-associated MC may

Table IV. Previous reports on the detection of ANCA in chronic hepatitis C without extrahepatic manifestations, HCV-associated MC or biopsy-proven CV. C-ANCA have also been reported in "essential", i.e. HCV-negative, MC.

Author	Report	Number of patients	Clinical manifestation	ANCA (IFT/ELISA)	Comment
Warny et al. (13)	Case series	1 out of 34	Chronic hep. C	P-ANCA/MPO-ANCA	Patient also positive for GS-ANA
Cacoub et al. (14)	Case series	2 out of 15	Chronic hep. C	2 P-ANCA/	No ANCA detected in 36 patients with EMC or HCV-ass. MC
Malnick et al. (15)	Case series	32 (66.7%) of 48*	Chronic hep. C and HCV-ass. MC	C-ANCA/ in 11 (22.7%) of 48*	No specification of target antigen and patient characteristics
Lamprecht et al. (16, 18)	Case report	2	1 HCV-ass. CV and 1 ECV	1 C-ANCA/ and 1 C-ANCA/PR3-ANCA	Target antigens systematically tested
Papi et al. (17)	Case report	1	HCV-ass. CV	P-ANCA/MPO-ANCA	

MC: mixed cryoglobulinemia; CV: biopsy-proven cryoglobulinemic vasculitis; antineutrophil cytoplasmic antibodies: ANCA; cytoplasmic pattern ANCA: C-ANCA; perinuclear pattern ANCA: P-ANCA; IFT: indirect immunofluorescence technique; enzyme-linked immunosorbent assay: ELISA.
Target antigens of ANCA by ELISA: Proteinase 3: PR3; myeloperoxidase: MPO; : no target antigen identified; EMC: essential mixed cryoglobulinemia; HCV-ass. CV: HCV-associated cryogloblinemic vasculitis; ECV: essential cryoglobulinemic vasculitis; GS-ANA: granulocyte specific antinuclear antibodies.
*Malnick et al. (15) mention a "high incidence of ANCA (66.7%)" in their patient group. They only mention the pattern to be cytoplasmic in 22.7%, but give no further details with regard to the rest of their group.

be of value in the analysis and dissection of mechanisms leading to autoantibody formation against different target antigens in ANCA-associated autoimmune diseases, other chronic diseases and in chronic infections (32-34).

CG-ANCA have been reported in refractory ulcerative colitis (32), whereas BPI-ANCA have been detected in various conditions such as reactive arthritis, and chronic inflammatory bowel disease or cystic fibrosis, which are complicated by secondary infections (33, 34). BPI-ANCA have been detected in such patients, but often no ANCA immuno-fluorescence was seen on IFT (33, 34). The common feature of CG-ANCA and BPI-ANCA in chronic hepatitis C, HCV-associated MC and the above mentioned disorders may be their induction by chronic infection or autoimmune diseases complicated by infections.

Since no uniform disease definition of MC and CV exists, we used the GISC criteria (19, 20) to define HCV-associated MC and additionally applied the CHC nomenclature (21) for cases, where CV was biopsy proven as well. As not all symptoms, such as arthritis, may be a consequence of MC or CV in chronic HCV infection (8), we tentatively denominated those patients who did not have biopsy-proven CV as having HCV-associated symptomatic MC.

However, as most patients had purpura and other clinical manifestations, the patient group with MC or CV virtually had the same clinical disease manifestation of chronic HCV infection.

We did not find any significant differences for age, ESR, CRP, clinical manifestations, complement factors C3 and C4 between the patient groups with HCV-associated MC from the Italian and the German center. However, this does not exclude regional differences with regard to certain manifestations and the suspected, but not survey proven higher prevalence of "essential", i.e. HCV-negative, MC or CV in northern Europe (35). The prevalence of characteristic symptoms of MC was in agreement with earlier studies (7-11). The high prevalence of polyneuropathy within the German and Italian group of patients with HCV-associated MC may result from high clinical suspicion and extensive electrophysiological investigation in both centers, as reported previously (16, 18, 36). Polyneuropathy may prove to be resistant to antiviral therapy and require plasmapheresis or immunosuppressive therapy in some patients, especially when motor neuropathy is found (36, 37). Interestingly, the age of both patient groups, i.e. chronic hepatitis C and HCV-associated MC and CV, was not significantly different as may have

been expected from previous observations.

Other factors, such as genotype or virus load may also influence the prevalence of MC in chronic HCV infection (38). Lower complement factor C4 levels were seen in HCV-associated MC compared with chronic hepatitis C without extrahepatic manifestations. Complement consumption is a common feature of MC and CV. Complement factor C4 generally tends to be low, whereas C3 may take an intermittent disease activity related course (30, 39). Moreover, clinically symptomatic MC or CV was mainly seen in the presence of type II cryoglobulinemia. This finding is in agreement earlier studies and pathophysiologic considerations (40, 41).

In conclusion, this study identified two new target antigens of ANCA in HCV-associated MC and chronic hepatitis C. BPI-ANCA and GC-ANCA were present in about 10% of patients with HCV-associated MC or chronic hepatitis C. BPI-ANCA and GC-ANCA were not associated with a more severe disease course. ELISA proved to be more sensitive in the detection of ANCA than IFT.

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