Serological and clinical markers of autoimmune disease in HCV-infected subjects with different disease conditions

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Abstract

Objective

To investigate whether the serological markers of autoimmunity and the clinical features of autoimmune disease which occur in hepatitis C virus (HCV)-infected subjects are correlated to each other and/or to the clinical pattern of the disease.

Methods

Seventeen symptom-free, anti-HCV antibody positive subjects, 17 patients with chronic hepatitis C, 21 patients with mixed cryoglobulinemia (MC), and as controls 17 anti-HCV negative patients with dyspepsia were enrolled in a prospective study. A patient history, clinical examination, self-administered questionnaire and laboratory investigations (hepatic enzyme levels, serum HCV-RNA and anti-HCV antibody testing, and serum autoantibody profile) were performed to detect liver and/or autoimmune disease.

Results

Serological markers of autoimmunity and clinical findings of autoimmune disease were found to be more frequent in the HCV-infected patients considered as a whole than in controls. However, rheumatoid factor and clinical findings of autoimmune disease were more frequent in MC patients, while anti-smooth muscle antibodies not linked to symptoms or signs of autoimmune disease were detected in all groups of HCV-infected individuals, including healthy carriers and subjects who had recovered from a previous HCV infection.

Conclusion

Anti-smooth muscle antibodies, a serological marker of autoimmunity, are detectable in HCV-infected subjects whatever their clinical status. Clinical findings of autoimmune disease prevalently occur in patients with mixed cryoglobulinemia.

Key words

Autoimmunity, anti-smooth muscle antibodies, hepatitis C virus, mixed cryoglobulinemia.

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Introduction

In the last few years, hepatitis C virus (HCV) has been shown to play a pathogenetic role in "essential" mixed cryoglobulinemia (MC) (1) and has been associated with a number of other autoimmune conditions (2). In addition, the prevalence of serological markers and/or clinical findings of autoimmunity has been reported to be significantly high in patients with chronic hepatitis C (3-5), as well as in HCV-positive patients with either lymphoproliferative or connective tissue disorders (6). This evidence strongly suggests a link between HCV and autoimmunity.

Three aspects of this relationship, however, have yet to be addressed:

(1) Is the prevalence of serological markers of autoimmunity also increased in symptom-free HCV-infected individuals and in anti-HCV positive patients with no evidence of on-going infection ?

(2) Does the pattern of autoimmune response differ among HCV-infected patients with different clinical conditions, i.e. chronic hepatitis C patients, MC patients and symptom-free individuals ?

(3) Are serological markers of autoimmunity associated with any of the signs or symptoms of systemic autoimmune disease in HCV-infected subjects without MC ?

Materials and methods

Patients

During the period January 1 to December 31, 1996, 30 symptom-free individuals (SFI) (14 M, 16 F; age 27 - 54 years; median 47) with persistently (i.e. > 6 months) normal ALT and AST and anti-HCV positivity as detected by a second generation enzyme-linked immunosorbent assay (ELISA-II, Ortho Diagnostic Systems, Raritan, NJ, USA) were referred to the Outpatient Clinic of the Hepatology Division at the Second University of Naples. They were all invited to participate in the present study, whose protocol included a complete medical history, a complete physical examination, a self-administered questionnaire designed to elicit any symptoms/signs of systemic autoimmune disease, routine liver function tests, confirmation testing for serum anti-HCV antibody by recombinant immunoblotting assay (RIBA -II), serum HCV-RNA testing, a serum organ/non-organ specific autoantibody profile and an optional percutaneous liver biopsy. Seventeen subjects (8 M, 9 F; age 27 to 52 years; median age 45) gave their informed consent for the clinical and serological study; eleven (5 M, 6 F; aged from 27 to 52 years; median age 44) agreed to undergo the percutaneous liver biopsy. No differences in the age and sex distribution were found among the 11 biopsied subjects, the 17 enrolled in the clinical and serological study and the original group of 30 symptom-free anti-HCV positive individuals.

Seventeen previously untreated chronic hepatitis C (HC) patients (7) and 17 anti-HCV negative dyspeptic controls, admitted to the same out-patient clinic during 1996 and matched for sex and age with the 17 anti-HCV positive SFI, were recruited for the clinical and serological study. In addition, the 17 chronic HCV patients agreed to undergo the percutaneous liver biopsy.

In the same year, 21 patients with type 2 MC (8) (4 M, 17 F; age 37 - 72 years, median age 52) were admitted to the Outpatient Clinic of the Rheumatology Division of the Second University of Naples for a first visit (n = 6) or returned for a follow-up visit (n = 15) and were invited to participate in the present study. They were all enrolled for the clinical and serological study and 8 of them agreed to undergo percutaneous liver biopsy. All 21 MC patients were being treated with low dose steroids (5 - 10 mg prednisone equivalent/die), 11 with α-interferon 3 x 10^6 U/die, and none with cytotoxic drugs.

The presence of any other liver virus infection or HIV was ruled out in all of the subjects investigated.

Methods

On admission, 30 ml of peripheral venous blood was collected from each subject in anticoagulant-free tubes and kept at 37°C until completely coagulated. Sera from 20 ml of blood were stored in graduated glass tubes at 4°C for eight days to detect and classify the cryoglobulins and evaluate the cryocrit, as previously reported (8). The sera from the remaining 10 ml of blood were divided into 8 aliquots of 0.5 ml each and stored

at -20°C without thawing [optimal conditions according to Davis *et al.* (9)] for the subsequent determination of HCV-RNA, anti-HCV antibodies and the serum autoantibody profile. In the MC patients and in the other patients with detectable serum cryoglobulins, all of the tests described below were carried out on serum re-warmed to 37°C after thawing to solubilize the cryoprecipitates.

Serum anti-HCV antibodies were determined by a recombinant immunoblotting assay (RIBA II, Ortho Diagnostic Systems, Raritan, NJ, USA).

On the first of the two aliquots set aside for HCV-RNA determination, a reverse trascription polymerase chain reaction (RT-PCR) was carried out with primers from highly conserved 5' UTR (Amplicor Roche; minumum levels of sensitivity 1,000 genomes/mL). On the second aliquot HCV-RNA levels were evaluated using a branched DNA signal amplification (b-DNA) assay (Chiron HCV-RNA, Chiron Corporation, Emeryville, CA; minimum levels of sensitivity 3.5 x 10^5 genomic Eq/mL). To confirm the results, the RT-PCR assay was repeated 3 months later.

The following autoantibody specificities were investigated: IgM-rheumatoid factor (RF) (Latex agglutination, RF Latex, ND Biolab Labs, Italy); antinuclear antibodies (ANA) (Indirect Immunofluorescence on HEp-2 cells, ANA test, Immunoconcepts, Sacramento, CA, USA); anti-dsDNA antibodies (by indirect immunofluorescence (IF) on Crithidia luciliae, nDNA test system, Immunoconcepts, Sacramento, CA, USA); anti-mitochondrial antibodies (AMA), anti-smooth muscle antibodies (ASMA), type 1 anti-liver/kidney microsome antibodies (anti-LKM 1), anti-parietal cell antibodies (APCA) and anti-thyroid antibodies (ATA) (by indirect IF on tissue sections of rat stomach, kidney and liver, and of ape thyroid, ANA System Test, Promesan srl, Italy). The following starting sera dilutions were used: 1:10 for anti-dsDNA; 1:20 for AMA, ASMA, anti-LKM 1, APCA and ATA; and 1:40 for RF and ANA on HEp-2 cells. The presence of each antibody (except for rheumatoid factor) on IF was detected by fluoresceinated goat serum anti-human gammaglobulins. The IF pattern was observed by two independent observers using a Leitz Orthoplan microscope at 400x.

Liver biopsy was performed using a Surecut® needle (Ø 1.8 mm). All specimens were examined by the same experienced pathologist, who was blinded to the clinical, serologic and biochemical status of the donors. Patients were classified in the following categories: 1 = no lesion; 2 = minimal changes (i.e., specimens showing inflammatory infiltration of some, but not all portal spaces and some sinusoidal or lobular inflammation); 3 = mild, moderate or severe chronic hepatitis with or without cirrhosis (10).

Results

Consistent with the enrollment process, no difference in age or sex distribution was found between the 17 symptom-free anti-HC positive subjects and the 17 chronic HCV patients (8 M, 9 F; age 30 - 56 years, median 44.5). On the contrary, the mean age at enrollment was significantly higher in the MC patients (53 ± 8.1 SD) than in either the chronic HC patients or the SFI ($p < 0.01$); the difference in sex distribution did not reach statistical significance ($\chi^2 = 22$; $p > 0.05$). The disease duration from the appearance of the first symptom/sign or the detection of a significant laboratory finding was significantly longer in MC patients (10.5 months ± 7.6) than in chronic HC patients (4 ± 2.2) ($p = 0.0002$).

All 17 chronic HC and all 21 MC patients tested positive on the RIBA II assay. Four of the 17 chronic HC patients and 3 of the 21 MC patients were HCV-RNA negative. As already stated, none of the chronic HC patients were undergoing any treatment. The 3 HCV-RNA negative MC patients were all on α-interferon therapy.

Table I shows the results of the liver biopsy study. The 11 biopsied SFI included: 3 HCV-RNA positive subjects with no lesions or minimal changes at the liver biopsy study; 3 HCV-RNA positive patients with asymptomatic chronic hepatitis C; and 5 HCV-RNA negative, anti-HCV positive subjects with no lesions or minimal changes on liver biopsy.

Table II shows the prevalence of signs and symptoms of systemic autoimmune disease in the controls and in the anti-HCV positive patients, who were subdivided according to their clinical status. The prevalences of skin vasculitis, arthralgia/arthritis, xerostomia, xerophthalmia and renal disease were found to be significantly higher in the HCV-infected patients considered as a whole than in the controls. However, when the single groups of patients were considered separately, only the MC patients showed a high prevalence of each of the above listed manifestations, as well as of Raynaud's phenomenon and cardiovascular disease.

Table III shows the prevalence of cryoglobulins and of each of the organ/non-organ specific autoantibodies investigated in the controls and in the anti-HCV positive patients subdivided according to their clinical status. The frequency of the autoimmune response (whatever the serological marker) was significantly higher in anti-HCV infected patients than in controls, both when the patients were

Table I. Findings of the liver biopsy study in anti-HCV positive subjects differentiated according to the clinical condition and serum HCV-RNA status.

	Number of pts.	No lesions	Minimal changes	Chronic hepatitis w/out cirrhosis	Chronic hepatitis w/ cirrhosis
Symptom-free individuals (n = 11)					
HCV-RNA +	6	1	2	3	0
HCV-RNA -	5	2	3	0	0
Chronic hepatitis C (n = 17)	17				
HCV-RNA +	13	0	0	10	3
HCV-RNA -	4	0	0	4	0
Mixed cryoglobulinemia (n = 8)	8				
HCV-RNA +	8	0	0	3	5
HCV-RNA -	0	0	0	0	0

Table II. Prevalence of previous and/or present findings of systemic autoimmune disease in controls and in anti-HCV positive subjects distinguished according to their clinical condition (as detected by their history, a clinical examination and a self-administered questionnaire).

	Controls	Anti-HCV positive patients			
		Symptom-free individuals (n = 17)	Chronic hepatitis C (n = 17)	Mixed cryoglobulinemia (n = 21)	All (n = 55)
Fever	0	0	2	3	5
Raynaud's phenomenon	0	0	0	8**	8
Skin rash	0	0	0	3	3
Skin vasculitis	0	0	0	21***	21***
Arthritis/arthralgia	2	1	2	18***	21*
Xerostomia	0	1	3	12***	16**
Xerophthalmia	0	1	2	13***	16**
Lung disease	0	0	0	1	1
Cardiovascular disease	0	0	0	9*	9
Renal disease	0	0	0	11***	11*

Statistical significance vs controls: *p < 0.05; **p < 0.01.

Table III. Prevalence of serological markers of autoimmunity in controls and in anti-HCV positive patients subdivided according to their clinical condition at presentation.

		Anti-HCV positive patients			
Serological markers of autoimmunity	Controls (n = 17)	Symptom-free individuals (n = 17)	Chronic hepatitis C (n = 17)	Mixed cryoglobulinemia (n = 21)	All (n = 55)
Cryoglobulins	0	1	5*	21***	27***
RF	0	3	3	20***	26***
ANA	2	2	5	5	12
Anti-dsDNA	0	3	1	1	5
ASMA	2	12**	12**	17**	41***
AMA	0	0	2	0	2
Anti-LKM 1	0	0	0	0	0
APCA	0	1	3	0	4
ATA	0	1	1	0	2
Whatever marker	3	15***	16***	21***	52***

Statistical significance vs controls: * p < 0.05; ** p < 0.01; *** p < 0.001.

considered as a single group (52 out of 55 versus 3 out of 17; Fisher exact test; $p < 0.0001$) or when they were separated into the three clinical subgroups (15 of 17 SFI, $p < 0.0001$; 16 of 17 chronic HC, $p < 0.0001$; 21 of 21 MC, $p < 0.0001$ vs controls). No differences were detected in the prevalence of the autoimmune response (whatever the serological marker) among the 3 subgroups of HCV-infected patients (i.e. SFI, chronic HC, and MC patients) ($\chi^2 = 2.43$; $p = 0.29$).

Concerning the single autoantibody specificities considered, anti-smoth muscle antibodies were found to be more frequent in each group of HCV-infected patients than in the controls. Cryoglobulins were significantly more frequent in both the chronic HC and MC patients, but RF was detected in a significantly higher percentage of MC patients only. No differences were found in the autoantibody profile between MC patients undergoing interferon treatment and those who were not (data not shown).

The cryocrit was 1% in the SFI and ranged from trace amounts to 4.5% in the 5 chronic HC patients with detectable serum cryoglobulins and from 1% to 60% in the 21 MC patients. The anti-smooth muscle antibody titer ranged from 1:40 to 1:320, but did not show any difference among the 3 subgroups of HCV-infected patients. The RF titre ranged from 1:40 to 1:520, the higher titers being detected in patients with MC.

In the 11 biopsied symptom-free individuals, no correlation was found between the autoimmune response and the HCV-RNA status or the liver biopsy results.

Finally, no correlation was found between the prevalence of the autoimmune response and the level of viral replication in any of the clinical subgroups of HCV-infected patients.

Discussion

Our study demonstrates the high prevalence of serological markers of autoimmunity in patients infected by HCV. Such a serological feature has been found to characterize HCV-infected subjects independently of their clinical status, i.e. chronic HC, MC patients and SFI.

The occurrence of serological markers of autoimmunity in patients with chronic hepatitis C has already been reported (3-5). In these reports, however, chronic HCV patients with detectable serum cry-

oglobulins but without a cryoglobulinemic syndrome were not differentiated from patients affected by MC. We made such a distinction in our study and found that anti-smooth muscle antibodies and cryoglobulins were the only serological autoimmune markers which were more prevalent in chronic HC patients than in controls. On the contrary, RF was primarily detected in patients satisfying the diagnostic criteria for MC (8). In that regard, however, one must stress that RF positivity is an established diagnostic criterion for MC.

We first investigated anti-HCV positive symptom-free individuals and detected an increased prevalence of anti-smooth muscle antibodies. When the HCV-RNA status and liver biopsy findings were considered (Table I), the 11 biopsied SFI could be divided into 3 subgroups: 3 asymptomatic chronic HC patients who were HCV-RNA positive; 3 HCV-RNA positive subjects without any evidence of significant liver damage on liver biopsy, who could be considered healthy carriers (11); and 5 anti-HCV positive individuals who were HCV-RNA negative and did not show any evidence of significant liver damage. These latter individuals could be considered subjects who had recovered from a previous HCV infection (11). In them we cannot exclude the occurrence of HCV infection in target organs (i.e., lymphoid tissue). Nevertheless, the detection of serological markers of autoimmunity in both HCV-infected healthy carriers and in subjects who had recovered from a previous infection may indicate that HCV is able to trigger an autoimmune response that can persist even after recovery from the infection. Viruses have long been suspected to trigger the development of autoimmune disease in predisposed subjects. Our finding of anti-smooth muscle antibody positivity in subjects with a previous HCV infection might support the role of this virus in some cases of autoimmune hepatitis type 1 (12).

The relationship between HCV infection and clinical features of systemic autoimmune disease deserves to be discussed. Adinolfi *et al.* (3) and Pawlotsky *et al*. (4) detected a high prevalence of autoimmune clinical features in their patients with chronic hepatitic C. The results for our HCV-infected patients considered as a single group confirm this finding. However, when the HCV-infected patients were divided into subgroups, a higher prevalence was found for the MC patients only. Since our cryoglobulinemic patients were recruited from a rheumatological clinic, a selection bias could be suspected. However, the 17 correctly classified chronic hepatitis C patients were found to present a low prevalence of autoimmune clinical features.

In conclusion, we have demonstrated that some serological markers of autoimmunity are detectable in all HCV-infected patients whatever their clinical status, but differences in the serological and clinical pattern occur in patients with different clinical conditions. Such differences among patients infected by the same virus do not appear to be due to the HCV genotype (13). The infection of lymphocytes and bone marrow (14, 15) can play a role. The chronic hepatitis C and MC patients from our series did also differ in age and disease duration and these differences might also be implicated.

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