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Impact of parietal cell autoantibodies and non-organ-specific autoantibodies on the treatment outcome of patients with hepatitis C virus infection: A pilot study

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Abstract

AIM: Various side effects have been reported in patients infected with hepatitis C virus (HCV) who were treated with interferon-alpha (IFN-α), including the appearance or exacerbation of underlying autoimmune diseases and the development of a variety of organ and non-organ specific autoantibodies (NOSA). However, very few studies in adults have been strictly designed to address: whether the prevalence and the titre of organ and NOSA in serial samples of HCV-treated patients were affected by IFN-α, and the impact of these autoantibodies on the treatment outcome of HCV patients.

METHODS: We investigated whether parietal cell autoantibodies (PCA) and/or NOSA were related with treatment-outcome in 57 HCV-treated patients (19 sustained-responders, 16 relapsers, 22 non-responders). Serum samples from patients were studied blindly at three time-points (entry, end of treatment and end of followup). For the detection of autoantibodies we used indirect immunofluorescence, commercial and in-house ELISAs.

RESULTS: Sustained biochemical response was associated with ANA-negativity at the entry or end of follow up. Sustained virological response was associated with the absence of PCA at the entry. Combined virological and biochemical sustained response (CVBSR) was associated with the absence of antinuclear antibodies (ANA) at the end of follow up and PCA-negativity at the entry. Sustained virological and CVBSR were associated with a reduction of ANA and SMA titers during therapy.

CONCLUSION: Although PCA and/or NOSA seropositivity should not affect the decision to treat HCV patients, the presence of some of them such as ANA, PCA and SMA before treatment or their increase during therapy with IFN- α may predict a worse response, indicating the need for a closer monitoring during treatment of HCV patients positive for these autoantibodies.

Key words: HCV infection; Autoantibodies; Interferon-alpha

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INTRODUCTION

Hepatitis C virus (HCV) infection has been associated with several immune-mediated phenomena including autoimmune thyroiditis[1], Sjogren's-like syndrome[2], essential mixed cryoglobulinemia[3], and autoantibody production[4-16].

Administration of interferon-alpha (IFN-α) in combination with ribavirin has already been proved to be the most promising therapeutic approach for the treatment of HCV infection[17-19]. Various side-effects have been reported in patients treated with this cytokine, including the appearance or exacerbation of underlying autoimmune diseases and the development of a variety of organ and non-organ specific autoantibodies (NOSA)[4-16,20-22]. However, very few studies in adults have been strictly designed to address: whether the prevalence and the titre of organ and NOSA in serial samples of HCV-treated patients were affected by IFN-α therapy, and the impact of these autoantibodies on the treatment outcome of HCV patients[6,23-28].

We conducted a retrospective serological study in order to evaluate at three time-points (entry, end of treatment, end of follow-up) whether parietal cell autoantibodies (PCA) and NOSA in HCV-treated patients were affected by the IFN-α treatment and furthermore, to address which was, if any, their impact on the treatment outcome. We report here our first results.

MATERIALS AND METHODS

Patients

Sera from 57 selected HCV patients, previously treated with a course of antiretroviral therapy, were studied. According to the aim of the study, serum samples were collected at three time-points: baseline, end of treatment and end of follow-up (6-12 mo after suspension of therapy). In order to address our hypothesis, these samples were selected from our records in an attempt to make three groups of HCV patients matched for age, sex, HCV-genotype and HCV viral load. The first group was consisted of 19 patients who achieved combined sustained virological and

biochemical response (responders), the second of 16 who had relapsed after an initial response at the end of treatment (relapsers) and the third of 22 who had no response (non-responders). At the end of treatment, biochemical response was achieved in 46 patients (81%), virological response in 37 (65%) and combined biochemical and virological response in 35 (61%). At the end of follow up, sustained biochemical response was observed in 33 patients (58%), sustained virological response in 20 (35%) and combined sustained biochemical and virological response in 19 (33%).

Diagnosis of HCV infection was based on clinical, laboratory and histological evaluations as we described previously[9,11,29]. All patients had no clinical laboratory or histological signs of autoimmune hepatitis (revised score for the diagnosis of this disease was less than 10 in all of them)[30]. Twenty-seven patients were treated with IFN-α (3 MU three times weekly) and ribavirin (19 patients infected with genotype 1 or 4 and two patients with undetermined genotype received 1 000-1 200 mg ribavirin daily, while 6 patients with genotype 2 or 3 received 800 mg/d) for 48 wk. The remaining 30 patients received IFN-α only (3 MU three times weekly) for 24-48 wk. Patients were followed up at least for 18 mo from the beginning of treatment.

Patients were regularly seen in the outpatient clinic for physical examination, blood tests and virological assays. Serum samples of the patients were investigated at the three time-points for PCA and NOSA. A complete medical history, routine liver function tests, virological tests and histological evaluation were also available.

The end-of-treatment and end-of-follow-up responses were defined as the normalization of serum alanine aminotransferase level (ALT) at the end of treatment and follow-up period (biochemical response) and as the clearance of serum HCV-RNA by polymerase chain reaction (PCR) at the same time-points (virological response).

Histology

Liver biopsy specimens were available in 35 cases before the initiation of therapy. Nineteen HCV-infected patients with genotype 2 or 3 were excluded from biopsy, because of the favorable response to treatment and 3 patients were denied. The histological evaluation was assessed using the Knodell histologic/activity index score[31]. The inflammation score was obtained by combining the scores for the first three components of the Knodell index: portal, periportal and lobular inflammation (range 0-18). The Knodell fibrosis scores were: 0 (no fibrosis), 1 (portal fibrosis), 2 (portal fibrosis with few septa), 3 (bridging fibrosis) and 4 (cirrhosis)[31]. According to the previous publications of our group[29,32], patients were divided in to two groups according to inflammation: minimal/mild (0-8) and moderate/severe (9-18) and fibrosis none/mild/moderate (0-2) and severe fibrosis/cirrhosis (3-4).

Virologic tests

Serologic evidence of HCV infection was determined by the detection of antibodies to HCV (anti-HCV) using a third generation enzyme immunoassay (Murex Diagnostics, Temple Hill, Datford, UK). HCV-RNA levels were available in all patients using a commercially quantitative PCR (Cobas Amplicor HCV Monitor, Roche). HCV genotypes were determined by a reverse-hybridization method (InnoLipa HCV II, Innogenetics). Classification of genotypes was done according to Simmonds *et al*[33]. None of the patients studied was positive for hepatitis B surface antigen and antibodies to human immunodeficiency virus.

Detection of autoantibodies

Serum samples were tested for PCA and NOSA by two independent observers (N.G and G.D) blindly to the clinical status and the treatment outcome of the patients (double blind study).

Antinuclear antibodies (ANA) were detected by indirect immunofluorescence (IIF) on HEp-2 cells (INOVA Diagnostics) following standard protocols (positive titre $\geqslant$1:80), while smooth muscle antibodies (SMA), liver-kidney microsomal autoantibodies (anti-LKM), PCA, anti-mitochondrial antibodies (AMA) and antibodies against liver cytosol (anti-LC) were detected by IIF on rat liver, kidney and stomach sections as we described elsewhere[7,11,34]. Significant titers were considered $\geqslant$1:80 for SMA and $\geqslant$1:40 for anti-LKM, PCA and AMA. Anti-neutrophil cytoplasmic antibodies (ANCA) were detected by IIF on ethanol-fixed granulocytes (INOVA Diagnostics; positive titre $\geqslant$1:20). All ANCA-reactive samples by IIF were further investigated for the presence of IgG antibodies against proteinase 3 (anti-PR3, INOVA Diagnostics) and myeloperoxidase (anti-MPO, INOVA Diagnostics).

All samples were investigated for the presence of IgG antibodies against double stranded DNA (anti-dsDNA) and IgG anticardiolipin antibodies (anti-CL) using in-house ELISAs following published protocols by us[4,9,10,29,35]. The specificity, reproducibility and optimal conditions of these assays were determined in extensive preliminary experiments as described[4,9,10,29,35]. In each assay, the between-day variation of the optical density (A) values was eliminated by running serial dilutions of a positive control (standard curve) on each plate. Briefly, a standard curve was constructed by assaying repeatedly control sera positive for IgG anti-dsDNA and IgG anti-CL in serial dilutions (1:50 to 1:6 400). The A value of the 1:6 400 dilution was arbitrarily chosen as 1 binding unit (BU). The BU values for the test samples were calculated according to this curve. This was accomplished by dividing the A of each sample by the A value, which corresponded to 1BU for that plate. Finally, the results were expressed as binding index (BI) calculated by dividing the BU of every sample by the mean BU of the healthy controls plus 4SD, multiplied by 100. According to this formula, a BI of 100 was defined as the cut-off point of the assays. The adoption of this stringent cut-off point precluded the possibility of false-positive results[4,9,10,29,35].

All subjects consented to participate in the study at the time of interview. The Ethis Committee of Larissa University Hospital approved the study protocol.

Statistical analysis

Associations between the presence or absence of autoantibodies and different variables were assessed using the univariate unadjusted χ^2 statistic. Statistical comparisons between means were calculated by one-way analysis of variance.

RESULTS

Demographic, epidemiologic, clinical, virologic and histologic characteristics of the patients at baseline are shown in Table 1. The duration of HCV disease was considered the period from the first time of iv drug abuse in iv drug abusers, while in the remaining HCV patients the duration was calculated from the first time of anti-HCV detection. No significant correlation was found between treatment outcome and sex, risk factors for contracting HCV and HCV-viral load. Patients below 45 years old had significantly increased virological (P = 0.006) and combined virological and biochemical response rates at the end of follow up (P = 0.01), while the presence of genotypes other than 1b or 4 was correlated with a significantly higher virological response rate at the end of treatment (P<0.03). In addition, none of the patients with severe fibrosis or cirrhosis achieved sustained biochemical and virological responses (P<0.05).

The prevalence of PCA and NOSA in HCV patients studied

at the three time-points is shown in Table 2. There was no significant difference between the mean titers and autoantibodies detected at the three different time-points (Table 2). The prevalence of autoantibodies was not associated with the treatment schedule, which was administered to the patients (IFN-α with or without ribavirin; data not shown). Before treatment, the most frequent autoantibody detected was SMA (51/57; 89.5%), while ANA were found in 54.4%. Age over 45 years was significantly associated with ANA positivity at the end of treatment (22/26 patients over 45 years old *vs* 18/31 patients under 45 years old, $P<0.05$).

ANCA were detected commonly in HCV-infected patients (80.7-82.5%). In most cases a diffuse cytoplasmic pattern (cANCA) was recorded. However, due to the absence of anti-PR3 and anti-MPO by ELISAs in all ANCA-positive samples, we considered them as ANCA-negative and they were excluded from further statistical analysis.

In overall, anti-LKM were detected in 4 out of 57 patients (7%) (Tables 2, 3). The only anti-LKM-positive patient who had a sustained biochemical and virological response was that with the highest titer at the entry, which then decreased (end of treatment) and disappeared at the end of follow up (Table 3).

The statistically significant correlations between the presence of PCA and/or NOSA and the response to treatment are shown in Tables 4A-C. The presence of ANA at the entry and at the end of follow up was significantly associated with the reduced rates of sustained biochemical response (Table 4). A combined sustained biochemical and virological response was achieved in a significantly higher proportion of ANA negative patients at the end of follow-up compared to those with ANA reactivity at the same chronic period (Table 5). None of the PCA-positive patients at the entry achieved a sustained virological or sustained biochemical and virological response (Tables 5 and 6).

The treatment outcome was associated with alterations of the titres in some NOSA during the three time-points of investigation (Tables 7,8 and 9). The decrease of ANA titers was associated with a significantly higher proportion of patients with sustained virological ($P = 0.02$) and combined sustained responses ($P = 0.02$) (Tables 7 and 8). The decrease of SMA titers was correlated with a significantly increased rate of sustained biochemical ($P = 0.005$), sustained virological ($P = 0.003$) and combined biochemical and virological responses ($P = 0.001$) (Tables 7, 8 and 9).

Table 1 Characteristics of HCV patients studied

	Non-responders (n = 22)	Relapsers (n = 16)	Sustained responders (n = 19)
Sex (M/F)	15/7	10/6	13/6
Age (Mean/range)	48±15	50±10	37±13
Disease duration (yr)	2.4±2.1	2.9±3	2.5±2.4
Source of HCV Infection			
Transfusion before 1990	8	4	4
Iv drug abuse	3	3	6
Multiple hospitalizations	3	0	0
Multiple sexual partners	0	1	2
Unknown	8	8	7
Genotype			
1a/1b	11	8	8
2a/c	2	2	0
3a	4	4	7
4	4	1	2
Undefined	1	1	2
Viral load			
>2.10^6 copies/mL	9	10	10
⩽2.10^6 copies/mL	13	6	9
Histologic data (Yes/No)	14/8	11/5	10/9
Minimal/mild inflammation	12	6	9
Moderate/severe inflammation	2	5	1
None/mild/moderate fibrosis	10	5	10
Severe fibrosis or cirrhosis	4	6	0
HBV and/or HIV co-infection	0	0	0

HBV = hepatitis B virus, HIV = human immunodeficiency virus, M = male, F = female, n = the number of individuals

Table 2 Prevalence (%) and mean titre of PCA and non-organ specific autoantibodies in HCV patients at three time-points

	Before treatment (n = 57)		*End of treatment* (n = 57)		*End of follow up* (n = 57)		P value
	Pos (%)	Mean titre	Pos (%)	Mean titre	Pos (%)	Mean titre	
ANA	54.4	1/188	70.2	1/194	71.9	1/171	NS
SMA	89.5	1/127	89.5	1/135	84.2	1/118	NS
Undefined cytoplasmic staining[1]	12.3	1/108	19.31	1/76	14	1/248	NS
AMA	0		0		0		
Anti-LKM	5.3	1/133	7	1/100	5.3	1/67	NS
PCA	15.8	1/76	17.5	1/108	15.8	1/231	NS
Anti-LC	1.8	1/40	3.5	1/60	0		NS
ANCA	80.7	1/31	80.7	1/31	82.5	1/31	NS
CANCA	78.9	1/28	77.2	1/30	77.2	1/28	NS
PANCA	3.5	1/90	5.3	1/53	8.8	1/64	NS
Anti-PR-3	0		0		0		
Anti-MPO	0		0		0		
Anti-dsDNA	22.8	129 BI	24.6	135 BI	21.1	134 BI	NS
Anti-CL	19.3	148 BI	29.8	138 BI	17.5	129 BI	NS

Abbreviations are same as in the text. pANCA = ANCA with perinuclear pattern, NS = not statistically significant, n = the number of individuals studied in each group. P values were calculated by one-way analysis of variance for the mean titres and by total χ^2 for autoantibodies positivity. [1]Means an AMA-like pattern by indirect immunofluorescense on HEp-2 cells, which did not give an AMA pattern on frozen liver, renal and stomach sections and which did not react in specific ELISA for AMA and immunoblots on human liver.

Table 3 Alterations of anti-LKM reactivity in association with the response to therapy in the four anti-HCV positive/anti-LKM-positive patients

Gender	Genotype	*Anti-LKM titre*			*Response*	
		1st	2nd	3rd	End of treatment	End of follow-up
M	1b	1/320	1/40	0	Responder	Sustained responder
F	1b	1/40	1/160	1/80	Responder	Relapser
F	2a/c	1/40	1/40	1/40	Non-responder	Non-responder
M	4	0	1/160	1/80	Non-responder	Non-responder

Abbreviations are same as in the text. M = male, F = female, 1st, 2nd and 3rd refers to the sample obtained at the entry, at the end of treatment and at the end of follow-up, respectively.

studied in each group. Data are given as mean±SD.

Table 4 Impact of ANA detection on sustained biochemical response of the patients

	Biochemical response at the end of follow up [*n* (%)]			
	n	Yes	No	*P*
ANA entry				
Pos	31	13 (41.9)	18 (58.1)	0.017
Neg	26	20 (76.9)	6 (23.1)	
ANA end of follow up				
Pos	41	20 (48.8)	21 (51.2)	0.037
Neg	16	13 (81.3)	3 (18.7)	

Abbreviations are same as in the text. *n* = the number of individuals in each group, *P* values were calculated by Fischer's exact test or χ^2 where applicable.

Table 5 Impact of PCA and ANA on sustained biochemical and virological response of the patients

	Combined sustained response at the end of follow up [*n* (%)]			
	n	Yes	No	*P*
PCA entry				
Pos	9	0 (0)	9 (100)	0.022
Neg	48	19 (39.6)	29 (60.4)	
ANA end of follow up				
Pos	41	10 (24.4)	31 (75.6)	0.048
Neg	16	9 (56.3)	7 (43.7)	

Abbreviations are same as in the text. *n* = the number of individuals in each group, *P* values were calculated by Fischer's exact test or χ^2 where applicable.

Table 6 Impact of PCA on sustained virological response of the patients

	Virological response at the end of follow up [*n* (%)]			
	n	Yes	No	*P*
PCA entry				
Pos	9	0 (0)	9 (100)	0.02
Neg	48	20 (41.7)	28 (58.3)	

Abbreviations are same as in the text. *n* = the number of individuals in each group, *P* values were calculated by Fischer's exact test or χ^2 where applicable.

Table 7 Impact of ANA and SMA alterations at the three time-points of investigation on the sustained virological response of the patients

	Virological response at the end of follow up [*n* (%)]			
	n	Yes	No	*P*
Alteration of ANA titres				
Increase	20	8 (40)	12 (60)	0.02
Decrease	13	8 (61.5)	5 (38.5)	
Constant	24	4 (16.7)	20 (83.3)	
Alteration of SMA titres				
Increase	14	3 (21.4)	11 (78.6)	0.003
Decrease	18	12 (66.7)	6 (33.3)	
Constant	25	5 (20)	20 (80)	

Abbreviations are same as in the text. *n* = the number of individuals in each group of patients. Alterations of autoantibodies titres are referred to the period from entry to end of follow-up. *P* values were calculated by total χ^2.

Table 8 Impact of ANA and SMA alterations at the three time-points of investigation on the sustained combined biochemical and virological response of the patients

	Combined sustained response at the end of follow up [*n* (%)]			
	n	Yes	No	*P*
Alteration of ANA titers				
Increase	20	7 (35)	13 (35)	0.02
Decrease	13	8 (61.5)	5 (38.5)	
Constant	24	4 (16.7)	20 (83.3)	
Alteration of SMA titers				
Increase	14	2 (14.3)	12 (85.7)	0.001
Decrease	18	12 (66.7)	6 (33.3)	
Constant	25	5 (20)	20 (80)	

Abbreviations are same as in the text. *n* = the number of individuals in each group of patients. Alterations of autoantibodies titres are referred to the period from entry to end of follow up. *P* values were calculated by total χ^2.

Table 9 Impact of SMA alterations at the tree time-points of investigation on the sustained biochemical response of the patients

	Biochemical response at the end of follow up [*n* (%)]			
	n	Yes	No	*P*
Alteration of SMA titers				
Increase	14	7 (50)	7 (50)	0.005
Decrease	18	16 (88.8)	2 (11.2)	
Constant	25	10 (40)	15 (60)	

Abbreviations are same as in the text. *n* = the number of individuals in each group of patients. Alterations of autoantibodies titres are referred to the period from entry to end of follow-up. *P* values were calculated by total χ^2.

DISCUSSION

We demonstrated that the majority of HCV patients had circulating NOSA while a significant proportion had also PCA. Several organ and NOSA has already been associated with HCV infection both in adults and children[4-16,22-28]. So far however, very few studies have been strictly designed to investigate the alterations of these autoantibodies and their impact on the treatment outcome after a course of IFN-α[6,23-28]. In addition, some of the previous data regarding the variation and influence of NOSA on the response to IFN-α were referred to the childhood[6,25] and not to the adult population.

Our study confirmed that PCA and NOSA were frequently detected in HCV patients. Similarly to previous reports by Gregorio *et al*[6,36], these autoantibodies appeared to be a part of the natural course of chronic hepatitis C, since their prevalence and titre were unaffected by IFN-α. In contrast, Agarwal *et al*[26] showed a disappearance of PCA and NOSA during treatment with IFN-α, but in this study a relatively small number of patients were followed up (7 patients only).

Interestingly however, we found that the presence and the titre alterations in some of these autoantibodies during the follow-up period had an effect on the treatment response. In particular, the presence of ANA at the entry and the end of

follow up, as well as the detection of PCA at the entry were correlated with a worse response. Additionally, the decrease of ANA and SMA titres from the entry to the end of follow up was associated with a better outcome after IFN-α therapy. Our findings are in accordance with only two very recent studies by Muratori *et al*[25] and Wasmuth *et al*[28], which investigated the impact of NOSA on IFN-α with or without combination with ribavirin treatment in HCV-infected children and adults. However, other studies have shown no relation of organ and NOSA with response to treatment[6,23,24,27,37,38].

Apart from treatment outcome, the clinical significance of PCA and NOSA in our series of patients remains obscure. Among several autoantibodies investigated, only ANA were associated with older ages, which is in agreement with the findings of Squadrito *et al*[14], but in contrast with other studies[22,39]. In addition, similarly with a recent study by Stroffolini *et al*[39], we were not able to show any correlation between the positivity of autoantibodies and liver damage. In contrast, other reports supported that continuous hepatocellular damage due to HCV might be an essential step for the production of autoantibodies[8,13,38]. Under this context and in view of the growing evidence for molecular mimicry as a mechanism of autoimmunity, a recent elegant study by Gregorio *et al*[40] has shown that ANA and SMA in chronic HCV-infection may arise as a consequence of cross-reactive immune responses to HCV and host smooth muscle/nuclear antigens. The latter speculation could support, at least in part, our findings where a favorable biochemical and virological response was associated with a decline of ANA and SMA titres (the lower the viral load, the lower the cross-reactive immune response with host antigens).

We were not able to show any significant alterations of PCA during therapy, which is in contrast with recent observations by Fabbri *et al*[15]. These researchers demonstrated a statistically significant increase of patients positive for PCA during IFN-α treatment, which was associated with the development of autoimmune gastritis and autoimmune thyroiditis[15]. ANCA were considered to be negative because we did not find reactivity against MPO or PR-3 by specific ELISAs. This finding is similar with that reported previously by our group[41] and others[42]. However, a recent study in HCV patients by Wu *et al*[12] reported a high prevalence of cANCA by identifying PR-3 as their major target autoantigen.

The prevalence of anti-LKM in our patients is comparable with previous works from our group and others[5,6,8,11,24,25]. Only one of our HCV+/anti-LKM+ patients succeeded a sustained response. Interestingly, this patient had the highest titre at the entry among the anti-LKM positive patients and was infected with genotype 1b. Nevertheless, the progressive decline of anti-LKM titre up to disappearance at the end of follow up was associated with a combined sustained biochemical and virological response. The disappearance of this antibody in combination with the disappearance of the virus further supports the concept that anti-LKM production in HCV infection may be at least in part, the result of a cross-reactive immune response between the virus and cytochrome P4502D6 (CYP2D6)[7,43]. Additional supports to this hypothesis emerged from recent studies by Kerkar *et al*[44] and Bogdanos *et al*[45]. In the first study the authors were able to show cross-reactivity between the immunodominant epitope 193-212 of CYP2D6 and homologies of two unrelated viruses (HCV 2977-2996 and CMV 121-140)[44]. In the second study the investigators for the first time gave experimental support to the notion that molecular similarities between CYP2D6, HCV and herpes simplex virus could result in anti-LKM production via a cross-reactive response[45].

Although occasionally IFN-α might unmask or provoke autoimmune hepatic reactions and even "true" autoimmune hepatitis in HCV-treated patients[7,24,30,37], the response rate to IFN-α was in general the same both in anti-LKM-positive and anti-LKM-negative HCV patients. This was also the case in our patients.

In conclusion, PCA and NOSA, including anti-LKM, are commonly found in adult HCV patients. Although seropositivity for these autoantibodies should not affect the decision to treat HCV patients, the presence of some of them such as ANA, PCA and SMA before treatment or their increase during IFN-α therapy may predict a worse response, suggesting the need for a more intensive follow up during treatment of HCV patients positive for these autoantibodies. Two very recent studies, one in children and the other in adult population with hepatitis C, could enhance our findings since both of them have demonstrated a lower benefit from IFN-α therapy among NOSA-positive/HCV-positive subjects[25,28]. Future prospective studies are needed to uncover the full spectrum of these associations and to provide new insights into their operating mechanisms.

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