Original article

Study of host and virological factors of patients with chronic HCV infection and associated laboratory or clinical autoimmune manifestations

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ABSTRACT

Objective. *Chronic hepatitis C virus (HCV) infection is associated with an array of autoimmune laboratory and clinical manifestations. The goals of our study were to identify host and/or virological factors that are implicated in the pathogenesis of these manifestations.*

Methods. *We performed a detailed prospective study of various demographic, virological, biochemical, immunological (including lymphocyte subsets, Fcγ-receptor and HLA class-II genotyping), histological and host genetic parameters in 3 well defined subgroups of HCV patients (n = 40): patients with liver disease only (group I, n = 11) or with laboratory (group II, n = 20) and clinical (group III, n = 9) autoimmune manifestations.*

Results. *Group III patients, mainly with features of mixed cryoglobulinemia, were older, with higher levels of rheumatoid factor and circulating cryoglobulins while they tended to have a longer estimated disease duration compared to the other two groups of patients. We did not identify any specific immunological features that could differentiate symptomatic versus asymptomatic patients, except from the elevated soluble interleukin-2 receptor levels. An increased frequency of the R/R131 FcRγIIIA and the NA1/NA1 FcγRIIIB genotypes was observed in our total HCV population, regardless of autoimmune manifestations, compared to historical controls. No statistically significant differences in HLA class II allele frequencies was detected between patient subgroups or in comparison to healthy controls.*

Conclusions. *Chronically infected HCV patients with symptomatic mixed cryoglobulinemia display a number of unique characteristics that differentiate them from asymptomatic patients with chronic hepatitis C.*

Introduction

Chronic hepatitis C virus (HCV) infection is uniquely associated not only with chronic liver disease and its complications (cirrhosis, hepatocellular carcinoma) but also to a number of autoimmune phenomena (1, 2). The role of the host immune response in the development of liver disease is crucial (3, 4). HCV by itself is not cytopathic and chronic liver damage is thought to be mediated primarily through host immune responses (5). On the other hand, the distinctive propensity of HCV to establish chronic infection in the majority of exposed individuals (50-85%) (6), indicates the ability of the virus to circumvent the host defense mechanisms, at least during the initial phases of HCV infection. Once chronic HCV infection has been established, a subset of patients (~20%) develops chronic liver necroinflammation that eventually leads to cirrhosis (7). A number of host, viral and external factors have been identified so far that confer an increased risk for progressive liver disease (7).

Between 10 to 55% of patients with chronic HCV infection demonstrate a number of circulating autoantibodies including rheumatoid factor (RF), cryoglobulins, antinuclear antibodies (ANA), antithyroid (antimicrosomal or antithyroglobulin) antibodies, smooth muscle antibodies (SMA) and antimitochondrial antibodies (AMA) (1, 8). Although these autoantibodies are frequently detected in the circulation of infected patients, only a small percent-

age of these patients develop clinical autoimmune syndromes (8, 9). Among them the syndrome of HCV-associated mixed cryoglobulinemia is the best characterized and studied (9, 10). Other autoimmune manifestations include autoimmune thyroiditis, sialadenitis (Sjögren-like) and arthritis (8). In regards to the pathogenesis of these clinical extrahepatic autoimmune manifestations, data are limited. It is currently unclear what are the major determinants, either host or virus specific, that differentially regulate the development of laboratory and/or clinical autoimmune manifestations in a subset of chronically infected HCV patients.

The goals of our study were to better characterize and compare a number of host and virological factors between well-defined subgroups of chronically infected HCV patients. Specifically, we included patients with evidence of liver disease only (Group I), asymptomatic patients with circulating autoantibodies (Group II) and patients with symptomatic autoimmune manifestations (mainly mixed cryoglobulinemia, Group III). An extensive array of clinical, laboratory (RF, cryoglobulin levels, autoantibody titers), virological (HCV RNA levels, viral genotype, liver histology), immunological (lymphocyte subpopulations, serum cytokine levels such as interleukin-2, -4, -8, -10, interferon-β, tumor necrosis factor-α, serum cytokine receptor levels such as soluble interleukin -2 receptor) and genetic (Fcγ Receptor genotypes, HLA class II typing) parameters were evaluated and compared between these groups.

Materials and methods

Study design - Patients

This was a prospective study of patients with chronic HCV infection presenting to the Departments of Gastroenterology and/or Rheumatic and Immunologic Diseases at the Cleveland Clinic Foundation, Cleveland, Ohio. Patients belonging to Groups I and II were consecutive patients seen at the Department of Gastroenterology by one of the authors of the study (Z.M.Y.) while patients from Group III were patients seen at the Department of Rheumatic and Immunological Diseases during the study period. All patients had chronic HCV infection documented by the presence of anti-HCV antibodies and HCV RNA in the serum. Patients with co-existent liver diseases (including co-infection with hepatitis B virus, alcoholic liver disease, drug-induced liver diseases, Wilson's disease, alpha-1 anti-trypsin deficiency, hemochromatosis, malignancies, autoimmune liver diseases), human immunodeficiency virus infection, history of organ transplantation, pregnancy and those who were receiving treatment with antivirals and/or immunomodulatory agents (< 4 weeks prior to the recruitment in the study) were excluded from the study. The one month cut-off period was set arbitrarily in order to avoid interference with the laboratory and immunological assays. The study was approved by the Institutional Review Board of the Cleveland Clinic and patients signed an informed consent prior to their enrolment in the study.

For each patient a detailed clinical history and physical examination was performed. According to their initial laboratory and clinical data, patients were classified into three groups:

- Group I included patients with chronic HCV infection only, without laboratory or clinical manifestations of autoimmunity.
- Group II included patients with chronic HCV infection and circulating autoantibodies such as RF, cryoglobulins, ANA, antithyroid (antimicrosomal or antithyroglobulin) antibodies, SMA or AMA, without any associated clinical manifestations.
- Group III included patients with chronic HCV infection, circulating autoantibodies and associated clinical autoimmune manifestations including symptoms of mixed cryoglobulinemia (glomerulonephritis, arthritis, neuropathy or skin manifestations such as palpable purpura, ulcers or digital gangrene), sialadenitis (Sjögren's like-syndrome) or thyroiditis.

Laboratory assays

Each patient has blood drawn once at the initial visit and a number of tests were performed while serum was also kept in -70ºC for subsequent assays. Each specimen was examined for the presence of anti-HCV antibodies by a commercial enzyme immunoassay (Cobas Core Anti-HCV EIA, Roche) and a quantitative HCV RNA assay (Cobas Amplicor HCV, Roche Diagnostics). In the majority of patients, the genotype of the infecting HCV strain was determined using a commercially available line probe assay (Inno-Lipa HCVII, Innogenetics).

Specimens which were positive for both assays (anti-HCVand HCVRNA) were further tested with a number of standard assays which included: a complete blood cell count and differential, cryoglobulin quantification (measured in μg/ml, positive > 50 μg/ml), RF (positive > 20 IU/ml), ANA, AMA, SMA, antithyroid antibodies, anti-HIV antibodies and hepatitis surface antigen (HBsAg).

Flow cytometry analysis

Fresh whole blood specimens were stained with a panel of monoclonal antibodies labeled with FITC, PE, PerCP or APC and subsequently analyzed on a utilizing four-color flow cytometric analysis in a FACScan flow cytometer (Becton Dickinson/BD, CA). The monoclonal antibodies that were utilized in this study included: CD3 - APC, CD4 - FITC, CD8 - PerCP, CD25 - PE, HLA DR - PE, CD19 - FITC, CD45 - APC, CD13- PE. All labeled antibodies and their respective controls were from BD. After gating in the lymphocyte population, 5000 events were acquired and the percentages of the different lymphocyte subpopulations were estimated in the gated population.

Serum cytokine and cytokine-receptor assays

All serum specimens were stored initially at -70ºC until the specific assays were performed. Serum soluble interleukin-2 receptor (sIL2R) levels were measured using a commercially available assay (normal range < 600 Units/ml). The serum levels of interferon-γ (IFN-γ), IL-2, IL-4, IL-8, IL-10 and

tumor necrosis factor-α (TNF-α) were determined by commercially available sandwich ELISAs (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Each specimen was assayed in duplicates and all results were expressed as pg/ml. The sensitivity of each assay was for: IFN-γ = 3 pg/ml, IL-2 = 7 pg/ml, IL-4= 10 pg/ml, IL-8 = 10 pg/ml, IL-10 = 2 pg/ml, TNF-α = 4.4 pg/ml. Specimens with undetectable levels were reported as 0 pg/ml for statistical purposes.

HLA class II typing

A blood sample from each patient was assayed for HLA class II typing by polymerase chain reaction - sequence specific oligonucleotide probing (PCR-SSOP), (Allogen Laboratories, The Cleveland Clinic Foundation, Cleveland, OH). The results of HLA class II typing for the whole HCV population was compared to a control group of cadaveric donors (n= 191) from the same institution.

Fcγ-receptor (FcγR) genotyping

The FcγR-IIA, FcγR-IIIA and FcγR-IIIB genotypes were determined at the University of Alabama at Birmingham, Birmingham, AL (Dr. Robert P. Kimberly), as previously described (11). Specifically, for each FcγR the following genotypes were determined: for FcγR-IIA = H/H131, H/R131 and R/R131; for FcγR-IIIA = F/F, F/V and V/V176 and for FcγR-IIIB = NA1/NA1, NA1/NA2, NA2/NA2. Furthermore, the allelic frequency of H or R/131 (FcγR-IIA), NA1 or NA2 (FcγR-IIIA) and F176 or V176 (FcγR-IIIB) was estimated in each group and for the whole group of HCV infected individuals.

Liver biopsy data

Thirty-one patients (77%) underwent liver biopsy as part of their evaluation for their chronic liver disease. Each liver biopsy was graded for necroinflammatory activity by the modified Histologic Activity Index (HAI, score 0-18) and for the stage of fibrosis (score 0-6), as previously described (12). The HAI and fibrosis score was calculated for each group of patients and compared between groups.

Table I. Demographic characteristics of the 3 patient subgroups with chronic HCV infection. Statistical significant differences between groups are indicated with asterisks.

Parameter	Group I (n=11)	Group II (n=20)	Group III (n=9)
Age (years) (mean ± S.D.)	43.1 ± 5.2	47.1 ± 4.8*	54.2 ± 14**
Sex (M/F)	8/3	12/8	7/2
Estimated disease duration (mean ± S.D.) (years)	16.6 ± 8.6	17.6 ± 8.8	22.4 ± 4.3
Mode of transmission			
Transfusion	1/11 (9%)	3/20 (15%)	4/9 (44%)
IVDU	6/11 (54%)	6/20 (30%)	3/9 (33%)
Unknown	4/11 (37%)	11/20 (55%)	2/9 (22%)
ALT(U/L (mean ± S.D.) (normal < 45 U/L)	93.6 ± 48.5	89.9 ± 61	73.7 ± 51.1
HCVRNA(copies/ml, median)	1.3 x 10^6	1.4 x 10^6	0.5 x 10^6
Genotype 1 (%)	5/9 (56%)	17/18 (94%)***	6/6 (100%)
Liver biopsy			
HAI score	5 ± 2.7	5.5 ± 1.7	5.4 ± 2.3
Fibrosis score	2.4 ± 1.5 (n=9)	2.8 ± 2 (n=17)	2.4 ± 1.8 (n=5)

* p = 0.05 (Group I versus II), ** p = 0.05 (Group I versus III), *** p = 0.03 (group I versus II)
IVDU: intravenous drug use; HAI: Histologic Activity Index.

Statistical analysis

For comparison of non-parametric values the Wilcoxon rank sum test or two-tailed student's t-test were used. Comparison of percentages between groups was performed with the $\chi 2$ or Fisher's exact test. Statistical significance was set at p-value of 0.05. For the HLA analysis, the Fisher's exact test was utilized with the Bonferroni correction for multiple tests (P value x the number of alleles examined). Correlation between nonparametric values was performed using the Spearman rank correlation method and a correlation coefficient was estimated for each comparison.

Results

Patient characteristics

Forty patients (27 males, 13 females) with chronic HCV infection were prospectively recruited for the study. Chronically infected HCV patients were categorized into three groups: Group I (n = 11) consisted of patients without circulating autoantibodies or clinical manifestations of autoimmunity; Group II (n = 20) included patients with circulating autoantibodies only while in group III (n = 9) patients with clinical autoimmune manifestations were recruited.

The demographic characteristics of the 3 groups of patients are presented in Table I. Patients in groups II (mean age = 47 years) and III (mean age = 54 years) were older compared to patients in groups I (mean age= 43 years, p value = 0.05 for both comparisons). There was no significant difference between groups in terms of sex, estimated disease duration (EDD), mode of HCV transmission, ALT or HCV RNA levels. Most of the patients had elevated aminotransferase levels (73% in group I, 75% in group II and 62% in group III) but their levels did not differ significantly between groups (Table I). Similarly, the HCV RNA titers were comparable between the 3 groups. Specifically, in group I patients the HCV RNArange was between 1.7 x 10^3 to 7.3 x 10^6 copies/ml (mean = 2.2 x 10^6 copies/ml), in group II 23 x 103 to 2.9 x 10^6 copies/ml (mean = 1.3 x 10^6 copies/ ml) and in group III 262 x 10^3 to 3.8 x 10^6 copies/ml [mean = 1.2 x 10^6 copies/ ml, median = 0.5 x 10^6 copies/ml)]. Serum HCV RNAlevels tended to be lower in cryoglobulin positive (mean = 1.38 x 10^6 copies/ml, median= 0.94 x 10^6 copies/ml, n=18) compared to cryoglobulin negative (mean = 1.7 x 10^6 copies/ml, median = 1.33 x 10^6 copies/ml, n = 21)

Table II. Autoantibodies in patients with laboratory or clinical autoimmune manifestations. The frequency of various autoantibodies in patients with laboratory (Group II) or clinical (Group III) autoimmune manifestations are presented. Comparisons between groups were performed with the Fisher's exact test and the Wilcoxon rank sum test (see Patients and Methods) and the respective p-values are also shown.

Autoantibody	Group II (n=20)	Group III (n=9)	p-value
Anti-thyroid	1/20 (5%)	2/9 (22%)	NS
ANA	2/20 (10%)	4/9 (44%)	NS
AMA	0/20 (0%)	1/9 (11%)	NS
SMA	8/20 (40%)	4/9 (44%)	NS
RF	15/20 (75%)	8/9 (89%)	NS
Median (IU/ml)	33	275	0.002
Cryoglobulins	10/20 (50%)	9/9 (100%)	0.01
Median (μg/ml)	55	520	> 0.001

patients, but the difference did not reach statistical significance. The majority of patients were genotype 1 (28/ 33, 85%). Liver biopsy was performed in the majority of studied patients (31/40, 77%). The necroinflammatory activity (assessed by the modified HAI score) and the fibrosis stage (assessed by the fibrosis score) were similar across all subgroups of HCV patients (Table I). The incidence of cirrhosis as evidenced by an advanced fibrosis stage (score 5-6) was 11% (1/9), 29% (5/17) and 20% (1/5) of patients in groups I, II and III, respectively (p=NS).

Autoantibodies

Patients belonging to groups II and III displayed a number of circulating autoantibodies (Table II). The percentage of patients with anti-thyroid, AMA, ANA or SMA was similar between group II and III patients (Table II). Group III patients with mixed cryoglobulinemia were more commonly positive for serum cryoglobulins (100%) compared to patients with circulating autoantibodies only (50%, p = 0.01). Although the number of patients with positive RF did not differ between groups (p=0.635), the levels of serum RF were much higher in patients with clinical autoimmune features (median =275 IU/ml) compared to asymptomatic RF positive patients (median= 33 IU/ml), p = 0.002. Similarly, the concentration of circulating cryoglobulins was much higher in symptomatic (median = 520 μg/ml) vs. asymptomatic patients (median = 55 μg/ml), p < 0.0001.

The correlation between the RF and cryoglobulin levels was estimated for the whole group of HCV patients, using the Spearman rank correlation method. A statistically significant correlation between RF and cryoglobulin levels was detected (correlation coefficient = 0.672, p = 0.0001, n=40).

Patients with clinical autoimmune manifestations (Group III)

The demographic, clinical and laboratory findings of the nine patients with clinical autoimmune manifestations are presented in Table III. The majority of patients were males (7/9, 78%) with long disease duration (22±4 years). All patients had circulating cryoglobulins (100%, median concentration = 520 μg/ml, range 203-10165 μg/ml) while all except one patient were RF positive

Table III. The clinical and laboratory findings of patients with clinical autoimmune manifestations (group III).

Patient	Sex/Age (years)	Mode of transmission	EDD (years)	Clinical manifestations	RF (IU/ml)	Cryos (_g/ml)	Other autoantibodies
1	M/72	Transfusion	20	LCV	341	2260	
2	M/49	IVDU	30	Arthritis/MPGN/ LCV/Sialadenitis	187	4890	
3	M/40	IVDU	20	Neuropathy /skin nodules	Neg	401	ANA(1:80)
4	F/52	Unknown	Unknown	Retinal vasculitis/ arthritis	36	520	ANA(1:640) AMA(1:320) SMA(1:80)
5	M/43	IVDU	20	Arthritis	76	309	ANA(1:320) SMA(1:80) Anti-thyroid
6	F/62	Transfusion	19	LCV/MM/ GN/thyroiditis	76	309	Anti-thyroid
7	M/46	Transfusion	27	Digital necrosis/ GN	161	367	
8	M/44	Transfusion	21	Digital necrosis	61	663	ANA(1:320) SMA(1:80)
9	M/80	Unknown	Unknown	LCV/neuropathy	1370	10165	

IVDU: Intravenous drug use; EDD: estimated disease duration; LCV: leukocytoclastic vasculitis; MPGN: membranoproliferative glomerulonephritis; MM: mononeuritis multiplex; GN: glomerulonephritis; RF: rheumatoid factor; cryos: cryoglobulins; ANA: antinuclear antibodies; AMA: antimitochondrial antibodies; SMA: smooth muscle antibodies.

Table IV. Lymphocyte subsets in the three groups of HCV patients. The absolute number and percentage of different lymphocyte subpopulations are presented. All values are expressed as mean ± 1 S.D. (standard deviation).

Parameter	Group I (n=11)	Group II (n=18)	Group III (n=9)
Lymphocytes (/μl)	2281.8 ±658.5	2179.4 ±618.3	1681.1 ±750.4
CD4 (%)	48.7 ± 9.4	48.9 ± 7.9	41.9 ± 10.6
CD8 (%)	25.7 ± 9	24.8 ± 8.2	18.9 ± 6.5
CD19 (%)	12.2 ± 5.6	12.1 ± 6.1*	25.4 ± 16.4**
CD4CD25 (%)	20.5 ± 4.4	18.3 ± 6.7	18.4 ± 5.8
CD8CD25 (%)	4.6 ± 2.2	4.5 ± 4.2	2.6 ± 2.4
CD4DR+ (%)	6.9 ± 2.6	8.2 ± 3.2	10.9 ± 4.7
CD8DR+ (%)	9 ± 8	9.2 ± 3.3	8.4 ± 3.3

(8/9, 88%). Their clinical manifestations were typical of symptomatic mixed cryoglobulinemia and consisted of vasculitis (5/9, 55%), arthritis (3/9, 33%), neuropathy (3/9, 33%), skin manifestations like digital necrosis or nodules (3/9, 33%) and glomerulonephritis (2/9, 22%). Four patients (44%) were ANA positive (titer range 1:80 – 1:640), three were SMA positive (33%), two had circulating anti-thyroid antibodies (22%) and one patient was AMA positive (11%). Despite the presence of these autoantibodies, none of the patients had other clinical or laboratory features indicative of a specific autoimmune disease such as autoimmune hepatitis, primary biliary cirrhosis or systemic lupus erythematosus. Of the two patients with anti-thyroid antibodies, one (patient #6) had thyroiditis. None of these patients were on antiviral or immunomodulatory treatment at the time of their evaluation. Three patients had previously received therapy: patient #3 combination antiviral therapy with IFNα/ribavirin and low dose prednisone; patient #4 IFNa alone and patient #6 IFNα and low dose prednisone. All patients had completed their therapy 7, 13 and 2 months, respectively prior to their enrollment to the study.

Lymphocyte subpopulations

The number of total lymphocytes did nor differ between the 3 groups of studied patients (Table IV). Although there was a mild decrease in the absolute number of total lymphocytes in patients with clinical autoimmune manifestations (1681 ± 750/μl), the difference compared to group I (2281 ± 658/μl) and group II (2179 ± 618/μl) patients did not reach statistical significance.

Similarly, there was no difference in the subpopulations of CD4 and CD8 T cells (CD3+) between the 3 groups (Table IV). When the subsets of activated CD4 or CD8 T cells (CD25 or DR+) were compared between patients with different laboratory or clinical autoimmune manifestations, no statistically significant difference was demonstrated (Table IV).

The population of B cells (CD19+) was expanded in patients with clinical manifestations (25.4 ± 16.4%) compared to group I (12.2 ± 5.6%, p = 0.02) and group II (12.2 ± 6.1%, p = 0.01) patients. The absolute number of B cells though did not differ between the 3 groups, most likely due to the decreased absolute number of lymphocytes in group III patients (data not shown). Similarly, there was no difference in the number of CD5+ cells among the total lymphocyte population or in the B cell subpopulation (data not shown).

Serum cytokines and soluble cytokine receptors

A number of different Th1 and Th2 cytokines were measured in the serum of patients from each group. Serum Th1 cytokines such as IL-2 and IFN-γ were undetectable by the ELISA assay that was utilized in our study (R&D Systems, MN, USA, data not shown). IL-4 was detected in very low levels in the whole group of HCV patients (mean = 0.7 ± 1.71 pg/ml, n=35), without significant differences between the 3 groups (data not shown). Another prototype Th2 cytokine, the IL-10 was detected similarly in relatively low levels (mean = 2.16 ± 1.78 pg/ml, n=34). Despite its frequent presence in the serum of HCV infected patients there was no statistically significant difference among the 3 groups (Fig.1).

TNF-α is a pleiotropic cytokine that plays a significant role in tissue inflammation including hepatitis and vasculitis. Serum levels of TNF-α were measured in the serum of 35 HCV patients utilizing a sensitive ELISA assay (sensitivity 4.4 pg/ml, see Materials and Methods). The levels of TNF-α were detected in relatively low levels (1.74 ± 2.3 pg/ml, n = 35). Patients from group II displayed higher levels (2.56 ± 2.57 pg/ml) compared to group I (0.56± 1.32pg/ml, p = 0.03) and group III (0.82 ±1.19, p=0.1) patients.

Serum IL-8 was found in high levels in the majority of patients with chronic HCV infection (180.6 ± 356.9 pg/ml, n = 36). Despite the presence of increased IL-8 levels, we did not detect any differences between patients belonging to the different groups (Fig. 1).

sIL2R levels were elevated in the majority of HCV patients (37/40, 92%). The levels were particularly elevated in patients with symptomatic mixed cryoglobulinemia (1405 ± 552 U/ml, normal < 600 U/ml) compared to patients with laboratory autoimmune phenomena only (826 ± 349 U/ml, p = 0.01) or patients without laboratory and clinical autoimmune manifestations (916 ± 252 U/ml, p=0.07).

FcγR-genotyping

Genetic polymorphisms of the FcγR-IIA, FcγR-IIIA and FcγR-IIIB have been linked to an increased risk of specific autoimmune diseases and certain of their clinical manifestations (13). The FcγR genotypes were determined in our HCV population and their frequency was compared between the 3 patient groups.

FcγRIIA is expressed mainly in monocytes/macrophages and neutrophils. A gene polymorphism at position 131 creates two different allotypes; one contains histidine (H131) and the other arginine (R131) at this position. Cells

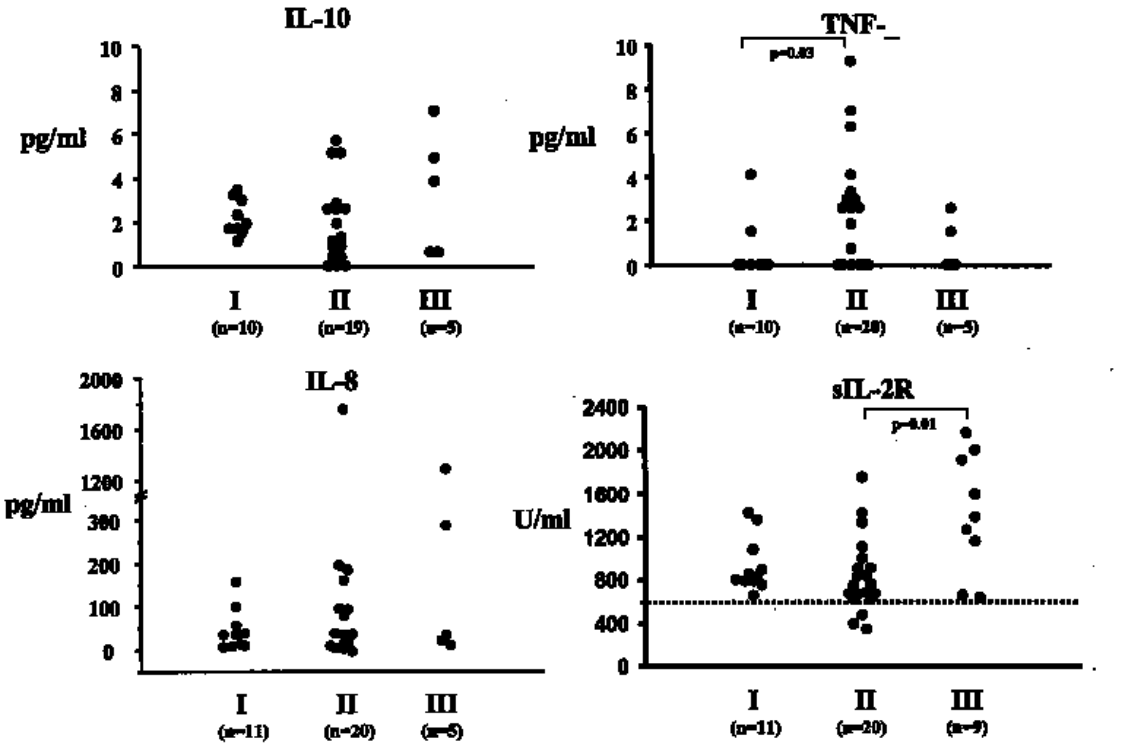

Fig. 1. The serum levels of interleukin-10 (IL-10), IL-8, TNF-α and sIL2R in the 3 groups of patients are displayed.

Table V. Distribution of FcγRIIA, FcγRIIIAand FcγRIIIB genotypes among the 3 different subgroups of HCV patients. The frequency of the FcγR genotypes and alleles in the different HCVpatient subgroups are shown. The number of patients with the particular genotypes as well as the percentage of its genotype(in parentheses) in each group are presented.

	FcγRIIA				FcγRIIIA				FcγRIIIB		
Genotype frequency Patient groups	R/R131	H/R131	H/H131	Patient groups	F/F176	F/V176	V/V176	Patient groups	NA1/NA1	NA1/NA2	NA2/NA2
I (n=11)	2 (18.2%)	6 (54.5%)	3 (27.3%)	I (n=11)	5 (45.5%)	6 (54.5%)	0 (0%)	I (n=10)	2 (20%)	4 (40%)	4 (40%)
II (n=17)	4 (23.5%)	11 (64.7%)	2 (11.8%)	II (n=18)	9 (50%)	4 (22.2%)	5 27.8%)	II (n=17)	6 (35.3%)	5 (29.4%)	6 (35.3%)
III (n=7)	2 (28.6%)	2 (28.6%)	3 (42.9%)	III (n=7)	3 (42.9%)	4 (57.1%)	0 (0%)	III (n=7)	2 (28.6%)	1 (14.3%)	4 (57.1%)
Total (n=35)	8 (22.9%)	19 (54.3%)	8 (22.9%)	Total (n=36)	17 (47.2%)	14 (38.9%)	5 (13.9%)	Total (n=34)	10 (29.4%)	10 (29.4%)	14 (41.2%)
Allelic frequency Patient groups	R131	H131		Patient groups	F176	V176		Patient groups	NA1	NA2	
I (n=11)	0.45	0.54		I (n=11)	0.73	0.27		I (n=10)	0.4	0.6	
II (n=17)	0.56	0.44		II (n=18)	0.61	0.39		II (n=17)	0.5	0.5	
III (n=7)	0.43	0.57		III (n=7)	0.71	0.29		III (n=7)	0.36	0.64	
Total (n=35)	0.5	0.5		Total (n=36)	0.67	0.33		Total (n=34)	0.44	0.56	

expressing the R131 allele or the R/R genotype demonstrate a lower affinity for IgG binding. The overall frequency of the R/R, H/R and H/H genotypes was 23%, 54% and 23% respectively in our HCV population. The overall frequency of the H131 and R131 allele was 0.5 for both genes. No statistically significant difference between patient groups was noted.

FcγRIIIA is expressed mainly in natural killer (NK) cells and mononuclear phagocytes (14). It has been shown to play a critical role in IgG1/IgG3 binding and hence, in immune-complex handling. A polymorphism at nucleotide (nt) 559 (T→G) creates a substitution of phenylalanine (F) to valine (V) at amino acid position 176 of the extracellular domain (EC2) of the receptor. V/V homozygotes have been found to bind IgG more efficiently (especially IgG1 and IgG3 subclasses) (14).

In our HCV population (n=36), the frequency of the low-binding genotype (F/F176) was 47% and of the F176 allele 0.67. The frequency of the low-binding gene or allele was independent of the presence or absence of laboratory and/or clinical autoimmune manifestations (Table V).

The FcγRIIIB is the FcγR expressed exclusively in human neutrophils and has been shown to play a significant role in neutrophil activation induced by immunoglobulins and/or immune complexes (14). A polymorphism of the responsible gene is associated with the differential biallelic expression of the neutrophil-specific antigen NA1 or NA2. Neutrophils from individuals homozygous for the NA1 allele (NA1/NA1) demonstrate a greater ability to bind to IgG1/IgG3 as well as to phagocytose IgG-opsonized targets (14).

The frequency of the NA1/NA1 homozygotes in patients with HCV infection (n = 34) was 29% (Table V) with an overall frequency of the NA1 gene of 0.44. No significant variation in gene or allele frequency between the 3 patient groups was noted.

HLA class II typing

HLA class II genotype was determined by the PCR-SSOP method in the majority of studied patients (38/40, 95%). A control group of cadaveric donors (n=191) from the same institution was also included in the study. The ethnic background of our HCV population was similar to that of the control population (33/40 or 82% Caucasians versus 135/191 or 71%, p=NS).

The allele frequency in the different patient subgroups as well as of the total HCV population is shown in Table VI. No statistically significant differences were observed between the different groups. There was a tendency for decreased expression of DRB1*07 (DR7) in patients with clinical manifestations of mixed cryoglobulinemia (0% in group III) compared to patients without clinical manifestations (45% and 30% in groups I and II, respectively) or healthy controls (25%). We also observed an increased frequency of DRB1*15 in patients from group III (57%) compared to groups I and II (18% and 15%, respectively). In both cases though, the difference did not reach statistical significance.

Discussion

Although the association of chronic HCVinfection with autoimmune manifestations is well established, its pathogenesis remains unclear. Furthermore, it is so far unknown if the patients with laboratory and/or clinical autoimmune manifestations, represent distinct subsets of chronically infected HCV patients with special genetic, immunological or virological features compared to patients with liver involvement alone. In our study a number of host and virological factors were determined in chronically infected HCV patients categorized according to the presence or absence of autoimmune features.

Previous studies have compared the characteristics of HCV patients with or

Table VI. HLA class II allele frequency in HCV patients and healthy controls. The allelic frequency of HLAclass II (as a percentage) in the 3 subgroups of HCVpatients is shown.

		Group I	Group II	Group III	Total HCV	Healthy controls
n		11	20	7	38	191
Allele frequency (%)						
DRB1						
	*01	9	15	43	18	12
	*03	45	20	29	29	19
	*04	9	15	14	13	29
	*07	45	30	0	29	25
	*11	9	30	29	24	17
	*13	18	25	14	21	24
	*15	18	15	57	24	30
DRB3						
	*01	18	25	29	24	22
	*02	27	45	43	39	38
	*03	9	5	0	5	12
DRB4						
	*01	45	45	14	39	50
DRB5						
	*01	18	20	57	26	29
	*02	0	20	0	3	4
DQB1						
	*02	82	35	29	47	37
	*03	18	45	43	37	55
	*04	18	10	0	11	7
	*05	9	40	43	32	28
	*06	27	40	57	39	45
DPB1						
	*01	18	20	14	18	18
	*02	27	40	29	34	31
	*03	18	20	0	16	14
	*04	63	65	71	66	66

without circulating cryoglobulins (15, 15-17), but not of those with symptomatic HCV-associated mixed cryoglobulinemia. Furthermore, our study was unique as far as we examined an array of virological, immunological, histological and genetic features of these patients that could be implicated in the pathogenesis of their autoimmune manifestations. The patients that were included in the study have either never received antiviral and/or immunomodulatory therapy or have completed their treatment at least 2 months prior to their enrollment in the study.

Patients with symptomatic autoimmune diseases (mainly with mixed cryoglobulinemia) were older and tended to have a longer EDD compared to patients from the other 2 groups. The majority of published studies in the literature have also indicated an older age and prolonged EDD in HCV patients with cryoglobulinemia (18).

We did not observe any correlation between autoimmune manifestations (either clinical or laboratory) and markers of the underlying liver disease, assessed by ALT levels and liver biopsy findings. There was no evidence of increased incidence of cirrhosis in patients with autoimmune characteristics. In a recent metaanalysis of published studies, Kayali *et al*., reported a 40% incidence of cirrhosis in HCV patients with cryoglobulinemia compared to 17% in patients without detectable cryoglobulins (18). The association remained significant after adjusting for variables such as age, gender and EDD (18). In our study, we did not find such an increased incidence of cirrhosis in patients with cryoglobulinemia (data not shown).

Limited data are available in the literature on quantitative HCV RNA levels in HCV patients with or without autoimmune phenomena (16, 19-21). Utilizing a sensitive quantitative assay for serum HCV RNA measurement, we were able to detect similar levels of viremia between the 3 groups of patients. Although there was a trend for lower HCV RNA levels in symptomatic patients and patients with cryoglobulinemia in general, the difference did not reach statistical significance. These results are in contrast with recent findings by Schmidt *et al*. who detected lower plasma HCV RNA levels in cryoglobulin positive patients (16). As the authors suggested though, plasma HCV RNA levels may underestimate HCV virions present in immune complexes in secondary lymphoid tissues or in the cryoprecipitate (16).

A number of autoantibodies were detected in patients belonging to groups II and III including antithyroid, ANA, SMA and rarely AMA. Symptomatic patients were more often positive for antithyroid, ANAand AMA but the difference did not reach statistical significance. Patients with symptomatic mixed cryoglobulinemia (group III) though, displayed statistically significant higher levels of cryoglobulins compared to asymptomatic patients. A similar correlation between elevated cryocrit levels and cryoglobulinemic symptoms has been recently reported by Trejo *et al*., in a large group of patients with cryoglobulinemia (n = 443, 73% HCV+) (17). Although the exact mechanism by which HCV induces the production of cryoglobulins is unknown, similar findings of elevated cryoglobulin levels have been observed in HCV negative patients with symptomatic cryoglobulinemia in a recent study by Rieu *et al*. (22)..

Another characteristic laboratory finding in patients with symptomatic disease was the frequent presence of RF (8/9, 89%). RF was detected in much higher levels in this group of patients compared to asymptomatic patients with autoimmune characteristics. This finding is unique to patients with HCV-associated cryoglobulinemia, since RF is detected less frequent in HCV-negative cryoglobulinemic patients (17, 22). A strong correlation between RF and cryoglobulin levels was observed in our study ($p = 0.0001$). A similar correlation between cryocrit percentage and RF levels was also found in HCV patients with cryoglobulinemia in a recent study by Schmidt *et al*. (16).

Cellular immune responses are critical in the pathogenesis of HCV-induced liver damage as well as in tissue injury in HCV-associated mixed cryoglobulinemia (23-25). In our study a detailed analysis of lymphocyte subpopulations did not reveal any significant differences between the 3 patient groups. An expanded population of B cells was noted in patients with symptomatic autoimmune diseases compared to patients with liver disease or laboratory autoimmune findings. Although the percentage of B cells (CD19+) was higher in symptomatic patients, the absolute number of B cells did not differ between groups. Cacoub *et al*. also did not detect any statistically significant differences in lymphocyte subsets in HCV patients with and without cryoglobulinemia (15), although there was no distinction between symptomatic and asymptomatic cryoglobulinemic HCV positive patients.

The Th1-Th2 type of immune response has been studied extensively in patients with past or chronic HCV infection (3,4). Patients who clear HCV demonstrate a strong Th1 response while chronically infected patients display a mixed pattern (Th1/Th2) in the periphery and a predominant Th1 response in the liver (26,27). We measured a number of Th1 and Th2 cytokines in the serum of HCV patients. In general, the levels of circulating cytokines were either undetectable (IL-2, IFN-γ, IL-4) or present in small quantities (IL-10, TNF-α). The levels of IL-8, a chemokine with known chemoattractant activity for polymorphonuclear cells and T lymphocytes (28) were detected in high levels in the majority of HCV patients. Despite the elevated levels of serum IL-8 in our patients, we did not identify a different pattern of expression in patients with autoimmune features (laboratory and/or clinical).

sIL2R is released from the surface of activated lymphocytes (T and B) and mononuclear cells and thus represents a marker of disease activity in various autoimmune and viral diseases (29). A number of studies in chronic hepatitis C have demonstrated elevated levels of sIL2R (30-32) while a gradual decrease of its levels after IFNα treatment is predictive of sustained virological response (33). In agreement to these previous studies, we detected elevated levels in the majority of patients with chronic hepatitis C. This was particu-

larly evident in patients with symptomatic mixed cryoglobulinemia (group III) who displayed much higher levels than patients with liver disease only or with circulating antibodies. No correlation between the serum levels of sIL2R and liver necroinflammatory activity or fibrosis score was found (data not shown), that could explain these findings.

Recently, Lamprecht *et al.* reported elevated levels of sIL2R in eight patients with HCV-associated cryoglobulinemic vasculitis that correlated with disease activity (34). Successful antiviral treatment with IFNα and low dose prednisone was accompanied by significant decrease in sIL2R levels. Since measurement of sIL2R is routine in many clinical laboratories, its study as a potential marker of disease activity and/or as a prognostic factor to antiviral or immunomodulatory therapy in these patients should be further explored.

Genetic polymorphisms of the Fc-receptors for the IgG immunoglobulins (FcγRs) have been associated with variable risk for the appearance, progression or response to immunomodulatory treatment of different autoimmune diseases (14, 35). There have not been any studies of FcγRs polymorphisms in HCV-associated autoimmune manifestations and especially in mixed cryoglobulinemia. We studied the genetic polymorphisms of FcγRIIA, FcγRIIIAand FcγRIIIB in the majority of our HCV patients.

Polymorphisms of the FcγRIIA, a receptor present on the surface of monocytes/macrophages and polymorphonuclear cells, are associated with variable degree of IgG binding and phagocytosis of IgG-coated cells (14). A recent meta-analysis has shown that the low-binding R/R131 genotype is enriched in the SLE population (especially in patients without nephritis) compared to control populations (36). The frequency of the different FcγRIIA genotypes in our HCV population did not differ from the reported frequencies in healthy populations in the literature. Specifically, we found a total frequency of 23%, 54% and 23% of the R/R, R/H and H/H genotypes compared to a reported frequency of 22-30%, 42-54% and 19-33%, respectively in healthy populations (mainly Caucasians) (14, 37). Similarly, the overall frequency of the R and H alleles was 0.5 compared to a reported frequency of 0.45 – 0.55 in healthy controls (14, 37). Furthermore, no significant variation between the different patient subgroups was noted.

The role of FcγRIIIA in immune complex handling appears to be important (14, 35). There is a strong association between the low-binding phenotype (F/F176) and the development of SLE (with associated nephritis) (11, 35) and an increased relapse rate in patients with Wegener's granulomatosis (38). In our study an increased frequency of the low-binding genotype (F/F176) and allele (F) was noted (47% and 0.67 respectively). Interestingly none of our patients with symptomatic mixed cryoglobulinemia (group III) possessed the high-binding genotype (V/V176). Wu *et al.* have found the low-binding genotype (F/F176) in 26% of healthy controls (n=113) with a 0.56 frequency of the F176 allele (11). The results of our study indicate a higher frequency of the low-binding genotype and its allele in patients with chronic HCV infection compared to historical controls. Our results are similar to those obtained in an SLE population in the same study by Wu *et al.* (11). Although the small number of patients included in our study and the absence of a control group, limits the generalization of our data, further studies are clearly needed in order to confirm these preliminary findings.

FcγRIIIB is expressed only on neutrophils and its NA1/NA1 genotype has been associated with enhanced phagocytic activity (14). Homozygotes for the high binding NA1 allele are enriched in patients with Wegener's granulomatosis and renal involvement (39). We observed a high frequency of the high binding NA1/NA1 genotype and NA1 allele in HCV patients (29% and 0.44 respectively). The frequency of the NA1/NA1 genotype in healthy populations ranges between 10-16% and of the NA1 allele between 0.35–0.38 (14, 37). Similarly, to the other two FcγR polymorphisms, we did not observe any significant differences between the 3 different patient subgroups.

Among the host genetic factors that determine the susceptibility and outcome of chronic viral infections, the HLA genotype appears to be of major importance. This has been documented in chronic viral infections due to HIV (40) and hepatitis viruses (B and C) (41). A number of studies have examined the HLA class I and II genotypes in patients with chronic HCV infection and autoimmune characteristics (including cryoglobulinemia), with conflicting results (42-49).

We determined the HLAclass II alleles in our HCV patients and compared their frequency to a control group of cadaveric donors with similar ethnic background. Although no statistically significant differences were identified, a few points can be made regarding our observations. HLA DRB1*11 was found more often in our HCV patients (24%) compared to healthy controls (17%) but the difference was not statistically significant. Furthermore, patients with laboratory or clinical autoimmune features displayed more often this allele (approximately 30%) compared to HCV patients with liver disease only (9%) (p=NS). Cacoub *et al.* reported recently an increased frequency of this phenotype in HCV patients with cryoglobulinemia (35% regardless of vasculitic symptoms) in comparison to patients without cryoglobulins (17%) and healthy individuals (23%) (43).

Interestingly, we found a decreased frequency of DR7 (DRB1*07) in symptomatic patients with mixed cryoglobulinemia (0%) compared to patients with liver disease only (45%), laboratory autoimmune features (30%) or healthy controls (25%). Similar results have been reported by Cacoub *et al.* (43) and Ossi *et al.* (49).

In conclusion, we report here the findings of a detailed study of the demographic, laboratory, histological, immunological and host genetic characteristics of patients with chronic HCV infection and autoimmune features (either laboratory or clinical). The statistically significant differences between the 3 groups are shown in Table

Table VII. A summary table showing the statistically significant differences in various host and virological parameters between the study groups.

Parameter	Group I (n=11)	Group II (n=20)	Group III (n=9)	p values
Age (years, mean ± S.D.)	43.1 ± 5.2	47.1 ± 4.8*	54.2 ± 14**	* 0.05 (I vs II) / ** 0.05 (I vs III)
Genotype 1 (%)	5/9 (56%)	17/18 (94%)*	6/6 (100%)	* 0.03 (I vs II)
RF titer (IU/ml, median)	NA	33	275 *	* 0.002 (II vs III)
Cryoglobulins				
% positive	NA	50%	100% *	* 0.01 (II vs III)
Titer (μg/ml, median)	NA	55	520 *	* 0.001 (II vs III)
B cells% (CD19 positive)	12.2 ± 5.6	12.1 ± 6.1*	25.4 ± 16.4**	* 0.01 (II vs III) / ** 0.02 (I vs III)
sIL2R levels (U/ml, mean ± S.D.)	916.6 ± 252.3	826.3 ± 349.2	1405.7 ± 552.4*	* 0.01 (II vs III)

sIL2R = Soluble interleukin 2 receptor

VII. Multicenter studies including large number of patients and respective healthy and disease controls from different ethnic and genetic backgrounds are clearly needed in order to confirm our findings. The application of the new genomic and proteomic technology will certainly assist in that direction.

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