Original Article

Cryoglobulinaemia and autoimmune markers in Hepatitis C virus infected patients on renal replacement therapy

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Abstract

Objective: To investigate the association of cryoglobulinaemia and autoimmune markers with hepatitis C virus (HCV) infection in patients on maintenance haemodialysis (HD) and post renal transplantation.

Methods: Serum samples of 103 HCV-antibody (anti-HCV) positive and 105 anti-HCV negative patients were investigated for cryoglobulins. These comprised 136 patients on HD and 72 renal transplant recipients. Serum creatinine and liver function tests were obtained on all patients. Rheumatoid factor (RF), anti nuclear antibodies (ANA), anti smooth muscle antibodies (ASMA), liver kidney microsomal antibodies (LKM), immunoglobulins (Igs) and complement levels were performed on all cryoglobulin positive (cryopositive) samples. HCV RNA and genotyping detection tests were done for cryopositive patients.

Results: The prevalence of cryoglobulins in patients on HD or after renal transplantation was found to be higher (57.6%) among anti-HCV positive patients compared to the anti-HCV negative patients (42.4%) (P=0.000). RF, ANA and ASMA were also higher in cryopositive HCV infected patients. HCV RNA was present in 84.2% of anti-HCV positive patients. Cryoprecipitable RF activity was found in a higher number of symptomatic patients with HCV genotype 1compared to HCV genotype 3.

Conclusion: There is an association of cryoglobulinaemia and autoimmune markers in HCV infected patients on HD, and in HCV positive renal transplant recipients. Also HCV genotype 1 is associated with symptomatic mixed cryoglobulinaemia (JPMA 57:225;2007).

Introduction

Cryoglobulins are immunoglobulins (Igs) that reversibly precipitate in serum at temperatures below 37°C. Three types of cryoglobulins are recognized: Type I with monoclonal Igs, type II with both mixed monoclonal and polyclonal Igs and type III with polyclonal Igs. Type I cryoglobulins have been shown to be associated with neoplastic conditions, whereas type II and III are linked to infections and autoimmune diseases as well as malignancies.[1-3] Clinically, cryoglobulinaemic syndrome presents with small and medium vessel vasculitis resulting in palpable purpura, arthralgia/arthritis and weakness. Cryoglobulinaemia can result in glomerulonephritis, neuropathies, pulmonary vasculitis and carditis.[4,5]

Hepatitis C virus (HCV) has been reported as the major cause of mixed cryoglobulinaemia (MC) with 60-100% HCV antibody (anti-HCV) positivity in cryoglobulin positive patients with or without rheumatoid factor (RF) activity.[1,5,6] HCV genotype I is linked to type II MC and genotype 3 has been shown to be associated with type III MC.[6,7] Also, cryoglobulinaemia has been reported more frequently in anti-HCV positive compared to anti-HCV negative patients on HD or after renal transplantation.[8,9]

In Pakistan, the prevalence of HCV infection is reported to be around 6%.[10] HCV infection is a major concern in patients on HD, with a prevalence rate of up to 68%.[11-13] Many of these patients are candidates for renal transplantation and the presence of cryoglobulins may complicate patient management both pre or post transplantation. Also, detection of autoimmune markers in the workup of such patients may be confusing. The occurrence of cryoglobulinaemia or autoimmune markers in HD patients or renal transplant recipients has not been reported from Pakistan. Since the prevalence of HCV infection in patients on HD in Pakistan is reported to be very high[11-13], the incidence of MC is also expected to be high in this patient population. Many of these patients undergo renal transplantation and receive immunosuppressive therapy that may influence cryoglobulin formation. This study was undertaken to determine the association of cryoglobulinaemia and autoimmune markers with anti-HCV status in chronic renal failure patients on HD or Renal transplant recipients. The association of HCV genotypes with cryoglobulin formation was also investigated.

Patients and Methods

A case control study was conducted on 208 chronic renal failure patients on renal replacement therapy at Sindh Institute of Urology and Transplantation (SIUT) Karachi from July to November 2004. Study subjects were selected on the basis of anti-HCV status. Informed consent was obtained prior to patient enrolment in the study. The ethical Committee of Sindh Institute of Urology and Transplantation (SIUT) approved this study. There were 103 anti-HCV positive patients and 105 were anti-HCV negative. One-hundred and thirty six patients were selected from HD unit and during the same period, 72 renal transplant recipients were enrolled from the post-transplant follow up clinic. Patients who were positive for hepatitis B surface antigen were excluded from the study.

Venous blood samples (10-12 ml) were collected in pre-warmed syringes and allowed to clot at 37°C. Serum was separated and stored with sodium azide (0.1gm/L) at 4°C for 7 days and checked daily for cryoprecipitation. Samples positive for cryoprecipitates were warmed at 37°C to check for resolubility. Cryoprecipitates were washed three times with cold saline at 4°C, diluted in 0.01 M NaOH and protein concentration determined at 280 nm.[14] Characterization of cryoprecipitates was done by immunofixation electrophoresis (IFE) method (Paragon electrophoresis system, Beckman Coulter USA). Sample application and electrophoresis were carried out at 37°C.[14]

Serum creatinine, total and direct bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and glutamyl transferase (GT) were measured by Hitachi 911 auto analyzer.

Immunological markers, IgG, IgA, IgM and complement components (C3, C4 and C1q) were determined by radial immunodiffusion (The Binding Site Birmingham, UK) at 37°C. Detection of anti-nuclear antibody (ANA) was done on fixed Hep2 cells, and of anti-smooth muscle antibody (ASMA) and liver kidney microsomal (LKM) antibody on fixed rat tissue sections by indirect immunofluorescence (DiaSorin USA). RF activity was determined in the serum, cryoprecipitate and supernatant of positive samples by latex particle agglutination (Randox UK) and nephlometery (The Binding Site Birmingham UK).

The presence of HCV RNA in serum samples was detected by RT-PCR (reverse transcription-polymerase chain reaction). RNA was extracted using High Pure Viral RNA Kit (Roche Germany). Conserved sequences in the 5'-untranslated region were amplified by an in-house assay.[15] Versant HCV Genotype Assay (LiPA) (Bayer, USA) was used for HCV genotype determination.

Data was analysed using SPSS system version 11. Results were expressed as prevalence rates and mean ± standard deviation for qualitative and quantitative measurements respectively. Chi-square test was used for qualitative results; independent Student's t-test was for quantitative measurements and Mc Nemar test to calculate the significance of paired qualitative variables. All P values calculated were two-tailed and P = 0.05 was considered statistically significant.

Results

Out of 208 patients, there were 139 males and 69 females. From 103 anti-HCV positive patients, 77 were on maintenance HD and 26 were renal transplant recipients. Among 105 anti-HCV negative patients, 59 were receiving maintenance HD and 46 had received a renal transplant. Mean duration on HD and post transplant period was 20.94 ± 29.5 and 36.5 ± 42.1 months respectively. In the HD group, 25 patients had a previous history of renal allograft rejection.

In the 208 patients studied, cryoglobulins were detected in 66 (31.7%). Of these 38 (57.6%) were anti-HCV positive and 28 (42.4%) were anti-HCV negative (Mc Nemar test: P=0.000). Cryoglobulin positivity was found in 30.9% and 33.3% of HD patients and renal transplant recipients respectively (Table 1).

A significantly higher incidence of RF, ANA and ASMA were seen in anti-HCV positive patients (table 2). In HD patients positive for anti-HCV and cryoglobulins, mean RF activity was found in 8 (28.6%), ANA in 9 (32%) and ASMA in 11 (39.3%) samples. In anti-HCV negative-cryopositive HD patients, RF activity was found in 2 (14.3%), ANA in 3 (21.4%) and ASMA in 4 (28.6%). RF and ANA were found only in 1 (10%), while ASMA was positive in 4 (40%) HCV infected cryopositive renal transplant recipients. In anti-HCV negative-cryopositive group, RF, ANA and ASMA were positive in 2 (14.3%), 1 (7.1%) and 4 (28.6%) patients respectively. RF activity in the cryoprecipitates was found in 16 (59%) symptomatic patients (mean concentration: 100.4 ± 102 IU/ml). In nonsymptomatic patients cryoprecipitable RF (mean concentration: 179.7 ± 444 IU/ml) was present in 17 (43.5%) samples.

HCV RNA was detected in 32/38 (84.2%) of anti-HCV positive samples (table 2). The dominant HCV genotype identified was type 3, present in 18 (56%) patients. Genotype 1 was detected in ten (31%), while 4 (13 %) patients were coinfected with types 1and 3. Eight patients with HCV genotype 3 had RF activity in cryoprecipitates (mean RF activity: 295.9 ± 627.9 IU/ml) and three (37.5%) were symptomatic. In patients with genotype 1, six had RF activity in cryoprecipitates (mean RF activity: 113 ± 111.6 IU/ml), five (83%) of whom were symptomatic. Three patients with mixed genotypes had cryoprecipitable RF with mean RF activity of 285.9 ± 329.6IU/ml and 2 (66.6%) of these were symptomatic. In symptomatic patients with HCV genotypes 3 and 1, mean cryoprecipitable RF was 106.9 ± 119 and 128.3 ± 108 IU/ml respectively. Mean RF activity in the cryoprecipitates of symptomatic patients with mixed genotypes was 193.7 ± 76.4.

Immunochemical characterization of 39 cryoprecipitates (1.0-22.9 mg/ml) by IFE revealed type III cryoglobulins in 21 (53.8%), and type II in a single sample. In the type II cryoglobulin sample, monoclonal component detected was IgMk. It was difficult to interpret Ig heavy and light chain bands of 17 cryoprecipitates due to low protein concentrations (<1.5 mg/ml). The characterization of 25 cryoprecipitates with protein concentrations <1mg/ml was not performed.

There was no significant difference in the clinical features observed between the two groups, but the classical features of purpura and arthritis/arthralgia associated with cryoglobulinaemic syndrome were more common in anti-HCV positive patients (table 3). The incidence of transplant rejection was also higher in anti-HCV positive seen in eight patients (table 3). These patients had a previous history of graft rejection and had reverted on to dialysis.

Table 1. Patient characteristics and biochemical markers according to cryoglobulin positivity and HCV status.

	CG (+)-HCV (+) n (%)	CG (+)-HCV (-) n (%)	CG (-)-HCV (+) n (%)	CG (-)-HCV (-) n (%)	P-value
n =208	38 (57.6)	28 (42.4)	65 (45.8)	77 (54.2)	0.000†*
HD n =136	28 (36.4)	14 (23.7)	49 (63.6)	45 (76.3)	0.000†*
Renal transplant recipients: n=72	10 (38.5)	14 (30.4)	16 (61.5)	32 (69.6)	0.856†
Biochemical Markers					
T bil (mg/dl): mean ± std.dev	0.60 ± 0.3	0.517 ± 0.18	0.62 ± 0.27	0.66 ± 0.33	N.S#
D.bil (mg/dl): mean ± std.dev	0.2 ± 0.16	0.16 ± 0.09	0.19 ± 0.11	0.16 ± 0.10	N.S#
ALT (U/L): mean ± std.dev	23 ± 14.68	18.4 ± 18.59	23 ± 24.35	22.09 ± 28.23	N.S#
AST (U/L): mean ± std.dev	38.5 ± 23.93	28.04 ± 15.79	36.81 ± 28.29	38.73 ± 38.32	N.S#
ALP (U/L): mean ± std.dev	222.77 ± 319.79	115.44 ± 63.74	253.5 ± 298.05	139.24 ± 106.6	N.S#
?GT (U/L): mean ± std.dev	59.61 ± 73.41	30.25 ± 27.56	53.4 ± 69.86	44.96 ± 45.47	N.S#
Age (years): mean ± std.dev	29.98 ± 9.63	27.4 ± 9.12	27.41 ± 9.12	30.19 ± 10.48	N.S#
Sex: M/F	20/18	20/8	44/21	55/22	N.S

CG (+) = cryoglobulin positive, HCV (+) = hepatitis C virus antibody positive, HCV (-) = hepatitis C virus antibody negative,
CG (-) = cryoglobulin negative, HD = haemodialysis, Renal transplant recipients = renal transplant,
T.Bil = total bilirubin, D.Bil = direct bilirubin, ALT = alanine aminotransferase,
AST = aspartate aminotransferase, ALP = alkaline phosphatase, ?GT = gamma glutamyl transferase,
M = male; F = female, N.S = not significant
† = Mc Nemar test applied, *= significant P-value = 0.05, # = Student's t t-test applied,

Table 2. Immunological features and HCV RNA status of cryoglobulin positive patients.

	HCV (-) n = 28	HCV (+) n = 38	P-value
CG conc. (mg/ml)	0.704 ± 0.434 (0.25-2.2)	1.735 ± 3.63 (0.3-22.9)	0.065#
IgG (g/l)	16.22 ± 6.14 (6.8-36.7)	17.54 ± 5.82 (5.4-34)	#
IgM (g/l)	2.23 ± 0.96 (1.15-4.71)	1.86 ± 0.69 (0.65-3.4)	N.S#
IgA (g/l)	2.57 ± 1.26 (0.35-4.75)	2.78 ± 2.01 (0.45-12.56)	N.S#
C3 (g/l)	1.08 ± 0.36 (0.43-1.98)	1.12 ± 0.33 (0.4-1.89)	N.S#
C4 (g/l)	0.27 ± 0.15 (0.01-0.62)	0.35 ± 0.35 (0.05-1.9)	N.S#
C1q (g/l)	0.36 ± 0.43 (0.02-1.8)	0.33 ± 0.36 (0.09-1.8)	N.S#
Autoimmune Markers			
ANA % +ve	14	26	0.000†*
ASMA % +ve	32	40	0.020†*
RF-SR % +ve	14	24	0.000†*
RF-SN %+ve	7	16	0.000†*
RF-CP (IU/ml)	117.8 ± 346.64 (0.00-1843)	166.96 + 344.98 (0.00-2040)	0.57#
LKM: %+ve	0.0	0.0	N.A
HCV RNA %+ve	32	84	0.000†

HCV (+) = hepatitis C virus antibody positive,
HCV (-) = hepatitis C virus antibody negative,
conc = concentration,
Ig = immunoglobulins,
ANA = antinuclear antibodies,
+ve = positive,
ASMA = anti smooth muscle antibodies,
RF = rheumatoid factor;
CP = cryoprecipitate,
SR = serum,
SN = supernatant,
LKM = liver kidney microsomal antibodies;
N.S = not significant;
NA= not applicable.
*= significant
P-value = 0.05,
= Student's t-test applied,
† = Mc Nemar test applied

Table 3. Clinical features of symptomatic cryoglobulin positive patients.

	HCV (-) n = 10	HCV (+) n= 17	p-value
Purpura (%)	1 (10)	7 (41)	0.068
Arthritis (%)	3 (30)	9 (53)	0.177
Rash (%)	1 (10)	2 (12)	0.744
Swelling (%)	4 (40)	5 (29)	0.895
Mouth ulcers (%)	0	1 (6)	0.387
Splenomegaly (%)	1(10)	1 (6)	0.826
Transplant rejection (%)	2 (20)	8 (47)	0.119

HCV (-) = hepatitis C antibody negative,
HCV (+) = Hepatitis virus C antibody positive

Discussion

The association between chronic HCV infection and MC is now well recognized. The interaction between HCV antigen E2 and CD 81 on B-cells leads to polyclonal B-cell proliferation. This may result in the formation of cryoglobulins and/or autoimmune antibodies, which may be pathogenic.[1] The reported frequencies of cryoglobulins in anti-HCV positive patients range from 19% to 59%.[16,17] Among cryopositive patients, the prevalence of anti-HCV positivity as high as 92% has been reported.[18]

Cryoglobulins have been reported in up to 32% HCV infected patients on HD, and 38% anti-HCV positive renal transplant recipients.[8,9,19] We found similar prevalence rates in our anti-HCV positive patient group, i.e. 36.4% in HD patients, and 38.5% in renal transplant recipients. Although the number of anti-HCV positive renal transplant recipients in this study was less compared to anti-HCV positive dialysis (25% vs. 75%) patients and a larger sample size may alter the association of HCV infection and cryoglobulinaemia in this patient population. But the significant association seen between HCV infection and cryoglobulin formation is unlikely to change. The cryopositivity rate of 23.7% in anti-HCV negative HD patients was relatively high compared to previously reported figures of 6-11%.[8,9] The fact that HCV RNA was positive in 32% of these patients may partly explain these findings, and adds to the increasing evidence for a role of HCV infection in cryoglobulin pathogenesis. Also, patients on HD and renal transplant recipients are immunocompromised, with increased risk of acquiring other viral and non-viral infections that may induce cryoglobulinaemia. It is likely that at least in some of our patients cryoglobulins were present transiently. We cannot confirm this possibility, as these patients were not followed for repeat testing.

We also did not find significant differences in the cryoglobulin concentrations of both anti-HCV positive and negative patients as previously reported.[5] However, two anti-HCV positive patients had very high cryoglobulin levels at 22 mg/ml and 7 mg/ml and were considered as outliers in the statistical analysis. Both were on HD with classical symptoms of palpable purpura and arthralgia. One died later of hepatic encephalopathy, and the other was ruled out for renal transplantation because of persistent anaemia and thrombocytopenia.

The relationship between HCV genotypes and cryoglobulinaemia is not clear. Lenzi et al[7] showed association of type1 with cryoglobulinaemia, while others reported no relationship with a particular genotype.[5,16] We also did not find a significant genotype association, although type I was found in majority of symptomatic patents with cryoprecipitable RF.

Association of autoimmune markers with cryopositivity in relation to HCV infection is vague. We found RF activity (29%), ANA (32%) and ASMA (39%) positivity in HD cryopositive patients comparable to previously reported prevalence of these markers.[8,19] However, Ozdemir et al[20] with similar results of autoimmune markers found only 16% cryopositivity in their study population. Conversely, Wu et al[9] found a higher prevalence of RF activity (43.8%) in HD patients. Similarity, differences were also found in the prevalence of these markers in renal transplant recipients. We found only 10% RF positivity in HCV infected cryopositive patients, compared to 25% reported by Wu et al[9] and 55.4% reported by Rostaing et al irrespective of cryopositivity.[21] Interestingly cryoglobulins were present in only 2.7% of these patients. These differences may reflect variable patient selection criteria, besides genetic and environmental factors. The anti-HCV and cryopositive patients in this study did not have clinical evidence of any other autoimmune or connective tissue disorder. Therefore ANA positivity seen could be an autoimmune manifestation of HCV infection.

Information regarding HCV associated MC in renal allograft rejection is lacking. It has been hypothesized that HLA expression in liver in the presence of increased HCV antigens result in cryoglobulin formation post transplantation.[22] In the present study, although a higher incidence of renal allograft rejection was found in HCV infected cryopositive patients, the number of these patients was small. Since these patients had reverted back to HD at the time of cryoglobulin detection, other causes of rejection cannot be ruled out. Thus it is difficult to establish a relationship between HCV infection associated MC and renal allograft rejection.

In conclusion there is a significant association of MC and autoimmune markers with HCV infection in patients on renal replacement therapy particularly in HD patients. Immunosuppression seems to have a favourable effect, evident by low RF and ANA positivity in transplant recipients. HCV genotype 1 may be an important contributing factor in the pathogenesis of cryoglobulinaemic syndrome. Thus chronic renal failure patients presenting with symptoms suggestive of MC should be screened for cryoglobulins.

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