RANTES, TNF-α, Oxidative Stress, and Hematological Abnormalities in Hepatitis C Virus Infection

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Background: Chronic infection with hepatitis C virus (HCV) is associated with failures of T-cell–mediated immune clearance and with abnormal B-cell growth and activation. Hepatitis C virus infection is characterized by a systemic oxidative stress that is most likely caused by a combination of chronic inflammation, iron overload, liver damage, and proteins encoded by HCV. After a viral infection, multiple proinflammatory mediators contribute to recruitment of immune cells to the liver and to the generation of an antiviral immune response. Recent publications mark chemokines and their receptors as key players in leukocyte recirculation through the inflamed liver.

Materials and Methods: The present study involved 75 male subjects, divided into 2 groups: group 1 (n = 30), control group; group 2 (n = 45), patients with chronic HCV. For all subjects, the following investigations were performed: estimation of the levels of bilirubin, albumin, prothrombin concentration, glycosylated hemoglobin, creatinine, α-fetoprotein, HCV RNA, and activities of alanine and aspartate transaminases as well as alkaline phosphatase. In addition, regulated on activation normal T cell expressed and secreted (RANTES), tumor necrosis factor alpha, malondialdehyde (MDA) and nitric oxide (NO) were assessed. Plasma HCV-RNA concentration (viral load) was determined by real-time polymerase chain reaction (PCR) StepOne system using Applied Biosystem. Complete blood picture was assayed using Abbott Cell-Dyn 3700 hematology analyzer.

Results: There were significant increases of the levels of RANTES, tumor necrosis factor alpha, MDA, and NO in HCV-infected patients compared with the control group ($P < 0.05$); and in these patients, these levels showed significant positive correlation with the HCV RNA viral load. Also, mild leukopenia, thrombocytopenia, neutropenia, and lymphocytosis, with consequent significant increase in the lymphocytes/neutrophils ratio, were detected in these patients.

Conclusion: The data support the concept of chemokines (RANTES) as mediators of liver cell injury in HCV infection. In addition, MDA and NO levels might be used as monitoring markers for oxidative stress in hepatitis C infection.

Key Words: chronic liver disease, RANTES, TNF-α, nitric oxide, MDA, real-time PCR, complete blood picture

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The World Health Organization (WHO)[1] estimates reported that approximately 3% of the world's population has been infected with hepatitis C virus (HCV),

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Hepatitis C virus is a major human pathogen, infecting more than 170 million individuals; approximately 70% of those infected become chronic carriers and are at severe risk for developing liver fibrosis and cirrhosis.[2]

In most persons with HCV infection, the infection progresses to chronic infection, which can lead to hepatic fibrosis and the subsequent occurrence of cirrhosis, liver failure, and hepatocellular carcinoma.[3]

During many chronic infections, the virus spreads rapidly from the site of initial infection to distal tissues. T cells must first become activated in the lymph nodes and spleen and then gain the ability to migrate to infected organs. Chemokines orchestrate all stages of that T-cell response from recruitment of naive T cells to inflamed lymphoid tissue, migration of T cells within lymphoid organs, movement of activated T cells from lymphoid tissues to effector sites, and the movement of effector T cells within nonlymphoid tissues.[4]

Regulated on activation normal T cell expressed and secreted (RANTES) is a chemokine that plays a role in immune responses to viral infections. It was originally considered a T-cell–specific chemokine, but now, it is known to be expressed by other cell types including epithelial cells and platelets.[5] It is a member of the chemokine family with potent chemotactic properties for T lymphocytes, natural killer, memory T cells, eosinophil, dendritic cells, and monocytes.[5–9]

Production of chemokines in the liver is likely to play a role in HCV infection, as infiltration of lymphocytes has been observed in concomitance with chronic disease.[10]

Increased levels of circulating tumor necrosis factor alpha (TNF-α) and TNF receptors have been reported in patients with hepatitis C, although the exact role of that cytokine in the pathogenesis of HCV infection is still unclear.[11]

Oxidative stress occurs owing to discordance in balance between pro-oxidants and antioxidants. Normally, a rise in oxidative stress concomitantly enhances the antioxidative activity to protect the cell from damage. Chronic exposure to increased levels of oxidative stress may result in excess of reactive oxygen species within the hepatocytes.[12]

Hepatitis C virus up-regulates hepatic inducible nitric oxide synthase (iNOS) gene expression leading to liver inflammation, hepatocellular damage, and fibrosis.[13] Liver tissue from patients with HCV infection was shown to express elevated levels of iNOS transcripts compared with patients without HCV infection.[14]

Recently, Streiff et al.[15] have reported abnormal peripheral blood count in patients with HCV infection.

The present study was aimed to assess the serum levels of RANTES, TNF-α, complete blood count, and oxidative stress markers in patients with hepatitis C and to find out if they have a relation to the virus load in these patients.

MATERIALS AND METHODS

The present study included 45 male patients with HCV. They were recruited from the internal medicine department

of October 6 University Hospital. In addition, 30 healthy male subjects, matched for age and body mass index, were recruited as a control group. The control subjects were among the attendants to the outpatient clinic for minor surgery, that is, hernia, pilonidal sinus, etc. All patients underwent a thorough clinical examination including history taking, complete medical and laboratory evaluation including a liver ultrasound scan.

Hepatitis C virus infection was diagnosed by clinical as well as biochemical data and by detection of anti-HCV antibodies by Abbot AxSYM system (Abbott Laboratories, Abbott Park, IL) and was confirmed by estimation of plasma HCV-RNA concentration (viral load) that was determined by real-time polymerase chain reaction (PCR) using StepOne TaqMan (Applied Biosystems, Foster City, CA) with detection limits greater than 50 IU/mL of plasma.

RNA Extraction

For the isolation of HCV RNA from plasma, the QIAamp miniElute virus spin kit (QIAGEN, Hilden, Germany) was used following manufacturer's instructions. RNA was extracted from 200 μL plasma with 1 μL of the internal control (1X internal positive control RNA; Applied Biosystems) and eluted in 50 μL buffer.

Real-Time PCR Assay (Quantitative PCR)

Amplification was performed in a 25-μL reaction mixture containing the following: 1X TaqMan Universal PCR Master Mix, 16.5 μmol/L of each primer and probe, and 8.5 μL of extracted RNA. As an internal control, we used the TaqMan exogenous internal positive control reagents kit with 1X internal positive mix (primers and TaqMan labeled with fluorescein amidite and VIC). All reagents were obtained from Applied Biosystems.

Absolute quantification of HCV RNA was performed with StepOne real-time PCR (Applied Biosystems). Amplification started with an incubation at 45°C for 10 minutes to inactivate possible contaminating amplicons, followed by 10 minutes at 95°C that activated AmpliTaq. Polymerase chain reaction cycling program consisted of 40 two-step cycles of 15 seconds at 95°C and 45 seconds at 60 °C.

Inclusion criteria included patients with positive HCV infection. Exclusion criteria included patients with positive hepatitis B surface antigen, HIV concomitant infection, a history of schistosomiasis (rectal snip), prior anti-HCV treatment, recent use of steatogenic or antiviral agents: hepatic decompensation (ascites, jaundice, bleeding varicocele, or hepatic encephalopathy); patients with a history of alcohol intake or malaria were also excluded.

None showed clinical or biochemical signs of advanced liver or kidney diseases (their plasma prothrombin time, serum albumin, and creatinine were within reference ranges).

Twelve milliliters of blood were withdrawn from each patient and control subject after 10 hours of fasting. Blood was withdrawn in sterile syringe; 1.8 mL from the blood sample was taken in 200-μL sodium citrate and centrifuged, and plasma was separated and used for estimation of prothrombin concentration by using the clotting method on Stago STA compact analyzer, France. Two milliliters of the blood sample was placed in a vacuum tube containing K_2 EDTA; 1 ml was used as such for estimation of glycosylated hemoglobin,[16] using automated Hitachi 911, and for complete blood counts, which were performed in duplicate within 20 minutes of collection by Abbott Cell-Dyn 3700 hematology analyzer (Abbott Laboratories). The remaining milliliters of the K_2 EDTA sample was centrifuged, and the separated plasma was used for estimation of the viral load of HCV in patients using real-time PCR (Fig. 1). The remaining part of the blood sample was placed in sterile plain tubes and centrifuged, and the serum was stored at −70°C until used for estimation of the levels of total bilirubin, albumin, creatinine, NO, malondialdehyde (MDA), and the activities of serum alanine transaminase (ALT), aspartate transaminase, and alkaline phosphatase using automated Hitachi 911 multichannel analyzer (Boehringer Manheim Diagnostics, Indianapolis, IN).

Hepatitis markers (hepatitis B surface antigen[17] anti-hepatitis C antibody-Ab by third-generation enzyme-linked immunosorbent assay [ELISA])[18] were searched in the same serum sample, which was also used for assay of α-fetoprotein by microparticle enzyme immunoassay method on Abbott AxSYM (Abbott Laboratories), and RANTES as well as TNF-α, which were measured by sandwich ELISA technique using RANTES and TNF-α antigen ELISA kits purchased from Raytech ELISA kits (Helena Laboratories, Beaumont, TX).[19,20]

Statistical Analysis

Statistical analysis was done using SPSS (Statistical Package for Social Science) program version 17. The Kolmogorov-Smirnov test was used to verify the normal or nonnormal distribution of values. The quantitative data were presented as mean ± SD. Unpaired Student *t* test was used for comparison of 2 groups. The qualitative data were presented in the form of number and percentage. The Pearson correlation for nonparametric data was used for correlations of viral load and the biochemical data. Significance was considered at $P < 0.05$; insignificance was considered when $P > 0.05$.

RESULTS

The present study showed a significant increase of serum levels of RANTES, TNF-α, MDA, and NO in the patients with chronic HCV infection compared with the control group (Table 1; Fig. 2). Furthermore, these parameters showed significant positive correlations with the HCV load and with the activities of ALT (Table 2). In addition, total leukocyte, platelet, and neutrophil mean counts were significantly lower, whereas the lymphocyte mean count was significantly higher in the patients with HCV compared to the controls (Table 1).

DISCUSSION

During HCV infection, chronic inflammation, regeneration, and fibrosis are the key elements leading to liver dysfunction.

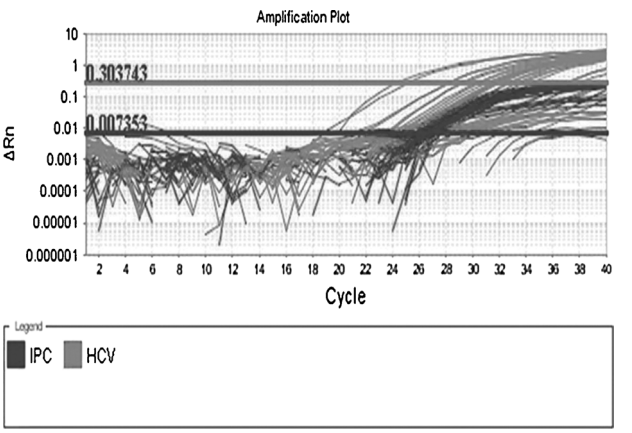

FIGURE 1. Amplification plot of HCV by real-time PCR.

TABLE 1. Clinical Data, Liver Function Tests, RANTES, TNF-α, MDA, NO, and Blood Picture in Patients With HCV Versus Control Group (Mean ± SD)

	Control n = 30	Chronic HCV n = 45	*P*
Age, yrs	41.43 ± 3.2	42.8 ± 5.8	>0.05
Body mass index, kg/m^2	24.27 ± 0.97	24.35 ± 1.30	>0.7
Total bilirubin, mg/dL	0.84 ± 0.08	1.09 ± 0.16	<0.001*
AST, IU/L	21.13 ± 3.40	50.0 ± 13.6	<0.001*
ALT, IU/L	22.13 ± 3.06	63.3 ± 18.2	<0.001*
Alkaline phosphatase, U/L	90.73 ± 11.9	95.76 ± 17.7	>0.1
Albumin, g/dL	4.16 ± 0.19	4.03 ± 0.305	<0.05*
Prothrombin concentration, %	84.7 ± 4.9	74.4 ± 6.29	<0.001*
α-Fetoprotein, ng/mL	3.96 ± 0.54	4.35 ± 0.9	<0.05*
HbA_{1c}, %	4.73 ± 0.47	4.88 ± 0.55	>0.2
Hb, g/dL	13.77 ± 0.56	13.45 ± 0.81	>0.05
Red blood corpuscles, 1×10^6cells/mm^3	4.7 ± 0.49	4.6 ± 0.99	>0.05
TLC, 1×10^3cells/mm^3	5.97 ± 1.16	5.21 ± 1.07	<0.01*
Neutrophils, %	55.30 ± 8.85	51.13 ± 8.53	<0.05*
Lymphocytes, %	32.77 ± 5.41	40.78 ± 7.51	<0.001*
L/N ratio	0.591 ± 0.016	0.795 ± 0.054	<0.001*
Monocytes , %	5.1 ± 1.33	5.18 ± 2.40	>0.05
Eosinophil, %	2.92 ± 2.3	2.94 ± 2.03	>0.05
Basophils, %	0.57 ± 0.31	0.59 ± 0.41	>0.05
Platelets, 10^3cells/mm^3	242.33 ± 48.83	169.67 ± 28.11	<0.001*
Creatinine, mg/dL	0.82 ± 0.11	0.86 ± 0.14	>0.1
RANTES, ng/mL	24.97 ± 12.17	77.27 ± 25.45	<0.001*
TNF-α, pg/mL	17.17 ± 7.01	49.84 ± 13.07	<0.001*
MDA, nmol/mL	4.81 ± 0.87	13.63 ± 1.53	<0.001*
NO, μmol/L	17.29 ± 2.63	20.49 ± 3.98	<0.01*
Anti-HCV Ab (signal-to-cutoff ratios)	Undetected	611.27 ± 374.85	<0.001*
HCV-RNA, IU/mL	Undetected	1212860 ± 373662.8	<0.001*

*$P < 0.05$ is considered significant.

Cytokines and chemokines are major regulators of these processes. Therefore, the outcome of HCV infection depends in part on a complex network of cytokine and chemokine interactions that orchestrate innate and adaptive immune responses to HCV infection.[21]

The role of chemokines in HCV infection is poorly understood.[22] Li et al.[23] found that Toll-like receptor-3 senses HCV infection in cultured hepatoma cells, leading to nuclear factor kappa B activation and the production of numerous chemokines and inflammatory cytokines, such as RANTES, macrophage inflammatory proteins 1α and β, inducible protein-10 (IP-10), and IL-6.

Although the precise role of TNF-α in the immunopathogenesis of HCV infection is far from clear, it represents a key mediator in determining HCV clearance and hepatitis progression.[24]

In our present study, we observed considerable increase of serum levels of RANTES and TNF-α in patients with chronic HCV infection compared with control group ($P < 0.05$).

Elsammak et al.[25] have demonstrated increased levels of serum TNF-α in 27 Egyptian patients with HCV infection, which showed significant positive correlation with the HCV RNA viral load. In addition, Talaat[26] has reported elevation of TNF-α levels in 82 patients with HCV infection (20 patients with mild cirrhosis, 20 patients with moderate cirrhosis, 20 patients with severe cirrhosis, and 22 patients with hepatocellular carcinoma) at different stages of disease. A significant positive correlation between serum levels of TNF-α and grade of disease was recorded.

Hung et al.[27] have shown that HCV infection interferes with the insulin signaling pathway in hepatocytes, increasing the inflammatory response with production of cytokines such as TNF-α and IL-6, and increasing oxidative stress. In addition, Parvaiz et al.[28] have reported that HCV infection up-regulates the inflammatory cytokine TNF-α.

Yoneda et al.[29] have reported significantly higher levels of serum RANTES levels in 79 Japanese patients with chronic HCV before therapy compared to controls.

It was postulated that RANTES was transcriptionally induced in human hepatoma cells by treatment with TNF-α via activation of nuclear factor kappa B and p38 mitogen-activated protein kinase, presumably suggesting that TNF-α–induced expression of RANTES plays important roles in cell-mediated liver injury in liver diseases.[30] Hepatitis C virus was shown to be capable of RANTES gene expression in both nonhepatic and hepatic cell lines.[9]

Hepatitis C virus infection was reported to be associated by increased markers of oxidative stress.[31] Under conditions of oxidative stress, as seen in certain chronic inflammatory disorders including hepatitis C, reactive NO species, such as peroxynitrite and nitrogen oxides, are currently considered as the main mediators of the deleterious effects to the host of NO, including cytotoxicity and DNA damage.[32]

Malondialdehyde is the end product of lipid peroxidation and forms by degradation of the polyunsaturated lipids by reactive oxygen species.

In the current study, patients with HCV infection showed significantly increased serum levels of stress markers (MDA and NO), and these levels showed significant positive correlation with the HCV RNA viral load.

Ali et al.[33] have shown that serum MDA and NO levels and myeloperoxidase activity were significantly higher, and the activities of paraoxonase and arylesterase were significantly lower in 23 Egyptian patients with chronic HCV hepatitis compared

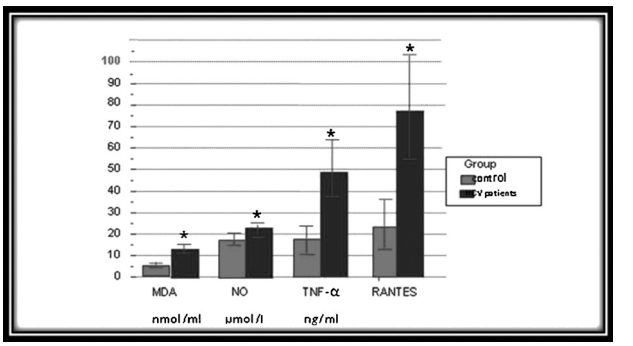

FIGURE 2. Mean serum levels of RANTES, TNF-α, NO, and MDA in patients with chronic HCV compared with control group.

TABLE 2. Correlation Between Biochemical Parameters and HCV RNA Viral Load Among Patients With HCV Infection

	RANTES (ng/mL)		TNF-α (pg/mL)		MDA (nmol/mL)		NO (μmol/L)		ALT (IU/mL)	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
HCV RNA	0.608	<0.001*	0.728	<0.001*	0.570	<0.001*	0.625	<0.001*	0.563	<0.001*
ALT	0.786	<0.001*	0.720	<0.001*	0.666	<0.001*	0.791	<0.001*		

to 21 healthy subjects. Nakhjavani et al.[34] have reported that oxidative stress and lipid peroxidation play a major role in liver injury in chronic HCV infection and that viral load correlated with the serum level of oxidized LDL.

Moreover, Ozenirler et al.[35] have reported that oxidative stress increases in patients with chronic hepatitis manifested by increases in MDA.

Furthermore, in the current study, viral load showed significant linear relation with ALT serum activity (a marker of liver injury). Recently, Ijaz et al.[36] had reported a similar but weak correlation between viral load and ALT activities in Turkish patients with HCV infection.

In the present study, the mean counts of platelets, total leukocytes, and neutrophils were observed to be lower and that of lymphocytes was found higher, whereas those of hemoglobin and erythrocytes were not significantly different in the patients with HCV infection compared with the controls. Streiff et al.[15] have reported significant neutropenia and thrombocytopenia (TCP) but with no significant difference in the means of hemoglobin and erythrocytes between patients with HCV infection and controls. Louie et al.[37] have reported that the prevalence of TCP ranged from 0.16% to 45.4%, whereas more than half of the studies reported a TCP prevalence of 24% or more. In the current study, considering TCP as a platelet count of $150 \times 10^3/mm^3$ or less,[38] 13 (28.9%) of the 45 Egyptian patients had TCP. Such TCP could be attributed to multiple mechanisms including platelet splenic sequestration, bone marrow suppression, and reductions of the level or activity of the hematopoietic growth factor thrombopoietin.[39] Recently, Martinez-Camacho et al.[40] have calculated the lymphocyte/neutrophil (L/N) ratio and found that the ratio was significantly higher in HCV infection (0.86) than that in HBV infection (0.56) and of African American controls. Lymphocytosis was defined as an L/N ratio greater than 0.6. Lymphocyte/neutrophil ratios were calculated to avoid the impact of hypersplenism and constitutional leukopenia seen in African Americans.

In the present study, the calculated L/N ratio was significantly higher in the patients with HCV infections (0.795) than the controls (0.591); $P < 0.001$ among Egyptians.

Hemoglobin A_{1c} levels were estimated in the present study to asses the glycemic state of the controls and the patients with chronic HCV because it is now widely recognized that chronic hepatitis C is a metabolic disease strongly associated with type 2 diabetes mellitus and insulin resistance[29]

CONCLUSION

High linear correlation of RANTES, MDA, NO, and TNF-α with HCV RNA viral load has been demonstrated, and this makes the measurement of these peptides a reliable marker. Oxidative stress was documented in HCV cases manifested by high levels of MDA and NO. Hepatitis C virus testing should be considered for persons with unexplained neutropenia and thrombocytopenia. The L/N ratio was significantly higher in HCV infection, a finding that requires to be confirmed by repeating the assay on a large number of cases, and to be performed during and after therapy to ascertain its ability to serve as an inexpensive pretreatment tool to predict poor virological response to HCV therapy.

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