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Biochemical Study on Lymphocyte Cell Surface Antigen in Hepatitis C Infected Patients

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ABSTRACT

Aim of work:
The current study aimed at assessment of peripheral blood lymphocyte cells in hepatitis C patients compared to Healthy controls and evaluating the potential diagnostic role and relation to disease severity and complications.

Patients and Methods: The present study included 27 hepatitis C patients and 27 healthy controls. Full history and clinical assessment of hepatitis C were performed for patients. Blood samples were collected from patients and controls for estimation of laboratory parameters (AST, ALT, T. Bilirubin, D. Bilirubin, PCR, CBC (WBCs, PLT, and HB), and T-cell activation marker (CD69). The flow- cytometer was used to measure CD69 %.

Results: The results of this study revealed significant increase in AST, ALT. D. Bilirubin, and CD69%. In hepatitis C patients comparing with controls. In addition, hepatitis C patients had statistically significant decrease in Albumin and PLT count less than controls. No statistically significant decrease was detected in HB and WBCs count in hepatitis C patients comparing with healthy control. and no statistically significant increase in T. Bilirubin in hepatitis C group more than control was detected.

Conclusion: The present study could suggest that CD69 cells are important determinants of immune status and prognosis in hepatitis C patients.

INTRODUCTION

HCV is hepatotropic, and in many countries chronic hepatitis C is a leading cause of liver disease including fibrosis, cirrhosis and hepatocellular carcinoma. In primary exposure, serum HCV RNA cannot be detected before a window of 1-3 wk. Symptoms are mild and non-specific, so patients often do not seek medical assistance. Elevated ALT levels indicating the first signs of liver injury can be detected 4-12 wk. after infection, and wide fluctuations are common. Severe liver inflammation is uncommon, and fulminant hepatitis is rare. Seroconversion may occur between 4 and 10 wk. after exposure (Santantonio T *et al*., 2008). HCV persists in 50–85% of infected patients, and once chronic infection is established, spontaneous clearance is rare.

It is now well established that HCV is a global health challenge, with an estimated 2%-3% of the global population having chronic HCV infection (WHO. (2014)). Estimates over the last 15 years show HCV affection to have increased to 2.8%, which means > 185 million infections worldwide (Mohd Hanafiah K *et al*., 2013), to control non-cytopathic viral infections, the activation of the adaptive immune system, especially the cellular immune response, is necessary.

Naïve specific CD4+ and CD8+ T cells are primed by dendritic cells in the lymph nodes. Once these cells become activated, they change their phenotype into effector cells and migrate to the infected tissue attracted by the chemokines produced by the parenchymal cells. Primed specific CD4+ cells are essential to allow the adequate activation of specific cytotoxic T cells by secretion of T helper (Th)-1 cytokines (Larrubia *et al*., 2009) subsequently, these specific cytotoxic T lymphocytes (CTL) play a major role in resolution of spontaneous infection because they are able to recognize the infected cells and destroy them by cytolytic mechanisms. On the other hand, they also produce type-1 cytokines that eliminate the virus without tissue damage.

Both CD4+ and CD8+ cell activation depends on the engagement between T cell receptor and the Major Histocompatibility Complex (MHC)/epitope complex as well as the interaction between co-stimulatory molecules with their ligands and the adequate cytokine milieu (Choudhuri *et al*., 2005).

When these cells have finished their effector task, they express negative co-stimulatory molecules and pro-apoptotic factors to switch-off their activity, and a subsequent constriction in the specific T cell population is produced. After this event, a memory T cell population is maintained for years to come to respond faster to a new infection and in certain cases to keep under control occult viral infection (Appay *et al*., 2008).

CD69 is a lymphoid activation antigen whose rapid expression (< or = 2 h post activation) makes it amenable for the early detection of T-cell activation and for subset activation analyses.

CD69 is the first activation-induced protein that can be detected on the surface of lymphocytes (Reddy *et al*., 2004; Testi *et al*., 1989). Already at 2 h after the stimulation, this receptor can be found on the surface of human lymphocytes, but its expression is transient as it peaks 18–24 h after stimulation and decreases then (Testi *et al*., 1994).

One early study on human peripheral blood mononuclear cells (PBMCs) demonstrated that such a rapid induction of CD69 is due to the presence of this molecule in the cytoplasm of resting lymphocytes as its induction was independent of RNA and protein synthesis (Risso *et al*., 1991). This is why CD69 is widely used in studies for the identification of recently activated leukocytes, especially lymphocytes and NK cells, but the role of CD69 in regulating immune processes has not been intensively studied.

MATERIALS AND METHODS

The present study is cross sectional study performed on 27 hepatitis C patients and 27 healthy controls during the period from August 2014 to October 2015 in Gastroenterology and Hepatology Clinic, Faculty of Medicine, Fayoum University.

Inclusion criteria: Any Patient presented to the Gastroenterology and Hepatology Clinic, Faculty of Medicine, Fayoum University with hepatitis C (HCVAb positive) from 18-60 years old is recruited to participate in the study after obtaining an informed consent. Full history was obtained and Exclusion Criteria included patients having co-infection with hepatitis B or HIV.

Informed consent: Objective of the study and steps were explained clearly to every patient.

Examination:

Blood collection: Five ml blood samples were collected for analysis of serum (AST, ALT, T. Bilirubin, D. Bilirubin, PCR, CBC (WBCs, PLT, and HB), and peripheral whole blood used to measure T-cell activation marker (CD69).

Data collected were statistically analyzed to estimate the relationship between hepatitis C patients and controls regarding clinical data, and laboratory parameters (AST, ALT, T. Bilirubin, D. Bilirubin, PCR, CBC (WBCs, PLT, and HB), and CD69%.

Immunophenotyping of peripheral blood leucocytes by flow-cytometry. The primary outcome biomarker was the immunophenotypic analysis of circulating leucocytes performed by flow cytometric immunofluorescence procedure as recommended by the manufacturer (Beckman coulter Epics XL-MCL) (Dacie, 2011). Molecular analysis Quantitative determination of Serum HCV titer using real time PCR, using Abbott Molecular Inc. Des Plaines, IL 60018 USA (Germer *et al*,2005, Saldanha *et al*., 2005). Measurement of BC (PLT count - WBCs count and hemoglobin) are automatically measured by SYSMEX-XS instrument. Determination of serum (ALT) activity and serum (AST) using Human Gesellschaft für Biochemica und Diagnostica mbH (Germany) laboratories diagnostic kits (Schumann, 2003; Schumann *et al*., 2002). Colorimetric method for determination of serum total and direct bilirubin level was applied using Human Gesellschaft für Biochemica und Diagnostica mbH (Germany) laboratories diagnostic kits (Van den Bergh, 1916).

The Statistical analysis was performed using SPSS software version 16 in windows 7. The data obtained in the study were expressed as means± SD.

In-depended student T-test was used to compare quantitative data of independent groups. The level $p < 0.05$ was considered the cut-off value for significance. ROC curve was done by using SPSS software version 16 which was constructed by sensitivity percent (true positive fraction) and 100-specificity percent (false positive fraction) of the marker at several cutoff points.

RESULTS

The present study included 27 patients with hepatitis C and 27 healthy individuals as controls group. The patients group included 16 females (59.3%) and 11 males (40.7%), their ages ranged from 20 to 60 years. The healthy control group included 13 females (48.1%) and 14 males 51.9%), their ages ranged from 18 to 60 years. There was no statistically significant difference between the two groups (p. value =0.06).

Our results showed that The Cluster of differentiation (CD69) % had a high significant increase in the HCV infected group when compared to control group (P value=0.004) as shown in Table (1) and Figs. (1and 2).

Table 1: HCV infected vs control group

Groups / Parameters	Control n= 27	HCV infected n= 27	P value
CD69 (%)			
Range	0.01 – 0.07	0.01 -6.93	0.004
Mean ± S.D	0.04 ± 0.02	0.57± 1.35	

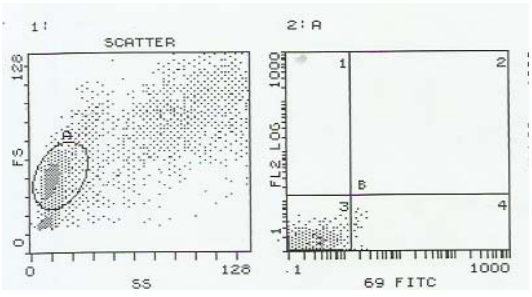

Fig. 1:CD69 % gate in hepatitis C cases

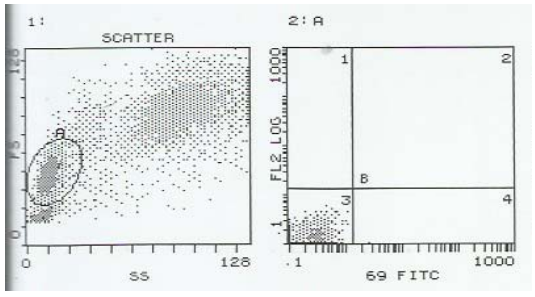

Fig. 2: CD69 % gate in healthy persons (control)

When using the ROC curve for the HCV infected group compared with control group, to improve the specificity and sensitivity of CD69 percent, the cutoff value was 0.08 %, Area Under the Curve (AUC) was equal to 0.91 and yields a sensitivity and

specificity of 77.8% and 100%, respectively (best cutoff).

Our results also proved that there was significant increase in serum AST, ALT, D. Bilirubin and PCR levels in hepatitis C patients more than healthy control. There was non-significant increase in T. Bilirubin. There was significantly decreased serum albumin in hepatitis C group less than control. Moreover, there was significant decrease in PLT count in hepatitis C less than control. And non-significant decrease in HB and WBCs count in hepatitis C less than control.

DISCUSSION

HCV is a small single stranded ribonucleic acid (RNA) of positive polarity, and is an enveloped virus belonging to the Hepacivirus genus within the Flaviviridae family (Preciado *et al*., 2014). It consists of approximately 9600 nucleotides in length, which encode three structural proteins (core, E1, and E2), the ion channel protein p7, and six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Tang, Grisé, 2009). Because each protein is involved in HCV entry, infection, replication, or maturation, they are potential antiviral targets. Hepatitis C virus replication takes place entirely within the cytoplasm; therefore, it does not establish latency making it easier to cure (Lambers *et al*., 2011).

The definitive barrier to control HCV infection is the adaptive immunity. This response has two arms to fight against pathogens; humoral and cellular immune response. Humoral immune response that means neutralizing and non-neutralizing antibodies (Non-nAbs), can endorse antiviral activity (Guidotti, Chisari, 2006).

The activation of the adaptive immune system, especially the cellular immune response, occurs in hepatitis C patients to control non-cytopathic viral infections, Naïve specific CD4+ and CD8+ T cells are primed by dendritic cells in the lymph nodes. Once these cells become activated, they change their phenotype into effector cells and migrate to the infected tissue attracted by the chemokines produced by the parenchymal cells. Primed specific CD4+ cells are essential to allow the adequate activation of specific cytotoxic T cells by secretion of T helper (Th)-1 cytokines Subsequently, these specific cytotoxic T lymphocytes (CTL) play a major role in resolution of spontaneous infection because they are able to recognize the infected cells and destroy them by cytolytic mechanisms. On the other hand, they also produce type-1 cytokines that eliminate the virus without tissue damage. (Jovana *et al*., 2013).

We chose the CD69 antigen as a marker for short-term activation as it is one of the earliest markers to appear on the surface of activated T cells, generally parallels the ensuing proliferative response in vitro, and gives a good estimate of T cell functionality in the clinic.

Moreover, CD69 is a well-established activation marker that is virtually undetectable on the plasma membrane of resting T cells but is rapidly and transiently upregulated upon cell stimulation; these facts make it highly suitable for the direct measurement of T cell responses to various experimental stimuli. (Jovana *et al*., 2013).

Various cellular approaches such as immunohistochemical staining or flow cytometry have already been used to analyze some phenotypic and function characteristics of Intrahepatic lymphocytes (IHL) during chronic hepatitis C (CHC), (Deignan *et al*., 2002) however, these procedures are difficult to apply, and lymphocyte isolation can introduce some bias because of cell loss or changes in surface marker expression. (Curry *et al*., 2000).

The aim of this study was to assess the role of activation T-cells in the progress and chemical pictures in case of hepatitis C patients.

The study included 27 hepatitis C patients and 27 healthy controls. There was no statistically significant difference between them.

Patients were clinically evaluated with thorough revision of clinical progress and

radiological findings. They all had positive Serum Hepatitis C antibodies (anti-HCV).

All the healthy controls had negative serum Hepatitis C antibodies (anti-HCV).

Laboratory investigations were performed to the hepatitis C patients and healthy controls (HCs) including routine hematological investigations like (WBCs count, PLT count and HB), liver enzymes (AST, ALT), T. Bilirubin, D. Bilirubin and albumin, also immunophenotypic analysis of lymphocyte subpopulations in the form of CD69.

The results were summarized in tables and were analyzed for possible differences and statistical relationship with radiological findings.

AST and ALT levels were significantly higher in hepatitis C patients than the healthy controls (P value <0.0001), a finding in agreement with many authors who reported abnormal increase in both AST and ALT levels in hepatitis C patients which might be explained at least partially by the underlying chronic inflammatory disorder. (Yasser *et al*., 2013).

Serum Albumin level was significantly lower in hepatitis C patients than the healthy controls (P value = 0.009), a finding in agreement with many authors who reported subnormal serum albumin level in hepatitis C patients which might be explained at least partially by the underlying chronic inflammatory disorder. (Mostafa *et al*., 2010).

In this study, there was anon significant increase in serum T. Bilirubin in hepatitis C patients than the healthy controls (P value = 0.06), a finding is not in agreement with many authors who reported a significant abnormal increase in serum T. Bilirubin level in hepatitis C patients. (Tarek *et al*., 2013).

This difference likely resulted from the relatively lower severity of infection in the HCV-infected group in the present study.

In contrast, in this study, there was significant increase in serum D. Bilirubin in hepatitis C patients than the healthy controls (P value = 0.03), a finding is in agreement with Mostafa *et al*., 2010 (Mostafa *et al*., 2010).

Who reported abnormal increase in serum D. Bilirubin level in hepatitis C patients.

Hemoglobin level, and WBCs count were non-significantly lower in hepatitis C patients than the healthy controls (P. value = 0.11 and 0.25 respectively), a finding is not in agreement with Mei-Hua Tsai *et al*., 2015(Mei-Hua *et al*., 2015). Who reported a significant subnormal HB and WBCs count level in hepatitis C Patients This difference likely resulted from the relatively lower severity of infection in the HCV-infected group in the present study.

Mei-Hua Tsai *et al*., 2015(Mei-Hua *et al*., 2015) also stated that there was a significant lower in PLT count and this was in agreement with our study This difference likely resulted from the relatively lower severity of infection in the HCV-infected group in the present study.

In our study, the mean percentage of CD69 cells (the T- lymphocytes) in hepatitis C patients (0.57± 1.35) was significantly higher compared to controls (0.04 ± 0.02) with P. value = 0.004.

Our results agreed with the results of Albert Tran *et al*., 1997(Albert *et al*., 2008) in a research utilized flow cytometry analysis to examine the phenotype of intrahepatic (IHL) and peripheral blood lymphocytes (PBL) in 36 patients with chronic hepatitis C. Data were compared with findings of six control patients without chronic hepatitis.

On the basis of both forward and side scatter on a FACScan cytofluorometer (Becton Dickinson), they proved that CD69 (activation inducer molecule (AIM)) on IHL in hepatitis C is higher than IHL in healthy control this indicate that cellular immune responses do take place in HCV-infected livers. These T lymphocytes become activated in the liver and could play an important role in mediating cellular injury.

C.M.O.de Almeida *et al*., 2011(C.M.O.de Almeida *et al*., 2011) also proved that HCV patients displayed an enhanced frequency of activated

lymphocytes (CD69+) than the healthy control, in a research worked by Immunophenotyping of peripheral blood leucocytes by flow cytometry.

These results were in agreement with our results. There is increasing evidence that the T cell response to HCV plays an important role in the course and the pathogenesis of the disease and that CD4+ and CD8+ T cell specificity and functional significance correlate with disease activity.

Moreover, our results coincided with Hanan *et al*., 2016 (Hanan *et al*., 2016) that showed significant elevation of Tregs specific genes (CD69) in both groups of HCVs whether naïve or after treatment in comparison to healthy controls during a study conducted in Biochemistry and Molecular Biology Unit, Cairo University, Faculty of Medicine.by Real-time quantitative PCR using SYBR Green Step One plus real-time PCR system.

The study proved that there is significant increase in CD69 in the hepatitis C patient comparing with control. Sreetha Sidhar than *et al*., 2014 (Sreetha *et al*., 2014) additionally proved that PBMC expression of CD69 was greater in the HCV monoinfected cohort than healthy volunteers where CD69 is a receptor that is induced upon antigen-associated activation of T cells and transmits signals to other lymphocytes in a research using DNA Microarray Analysis and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction where Total RNA isolated from PBMCs was reverse-transcribed using random primers with the High Capacity cDNA Reverse Transcriptase Kit (Lif Technologies).

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