CELLULAR IMMUNE RESPONSE TO INFECTION BY DIFFERENT GENOTYPES OF HEPATITIS C VIRUS

Mohamed El-Shahat Ebeid and Kadry Abdel-Kader El-Bakry*

Molecular Biology Department ,Genetic Engineering and Biotechnology Research Institute (GEBRI), Minufiya University, Sadat City, Egypt.and * Zoology Department, Faculty of Science (Damietta), Mansoura University, Egypt.

ABSTRACT

The present study aimed to estimate the cellular immune response to infection by different HCV genotypes in some Egyptian patients with chronic liver disease. HCV-RNA and HCV genotypes were conducted using type-specific primers to amplify the core region of HCV genome. The levels of IFN-gamma and TNF-alpha, PIIINP, SOD enzyme were also determined. The results showed that genotype 4 was the prevalent one (88.57%). Genotype 3 was found mixed with genotype 2 and 4 (11.42% and 34.28% respectively). Genotype 1 was absent from patients under study. No significant differences were observed in the level of IFN-gamma interferon, SOD enzyme and ALT enzyme between those genotypes. On the other hand the level of TNF-alpha was greatly increased in patients infected with mixed HCV genotypes 3, 4 compared with patients with genotype 4 and those infected with mixed genotypes 2, 3. Otherwise PIIINP was decreased significantly of the same group compared with other groups.

KEY WORDS

Cellular Immune response, HCV genotypes, IFN-gamma Interferon.

INTRODUCTION

Hepatitis C virus (HCV) is the leading cause of chronic liver disease and is associated with hepatocellular carcinoma. Egypt has the highest countrywide prevalence of hepatitis C virus (HCV) in the world, with an estimated 8–10 million among a population of 68 million having been exposed to the virus and 5–7 million active infections (1) and the prevalence of antibodies to HCV is approximately 10-fold greater than in the United States and Europe (2). However, there have been few studies on the distribution and genetic diversity of HCV isolates in non-developed countries (3).

Accumulated data show that there are two main patterns for

the distribution of HCV genotypes in the Middle East; in the first pattern, genotype 4 is prevalent in most of the Arab countries and in the second pattern, genotype 1a or 1b predominates in the non-Arab countries (4).

The cellular immune response to viral infection involves nonspecific mechanisms, such as interferon (IFN) release and natural killer NK cell activity, and antigen specific mechanisms including cytotoxic T-lymphocytes and inflammatory cytokine release (5). Both direct cytolysis and secreted antiviral factors, tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) can be implicated in host defense. Type I interferons (IFN), both alpha and beta, are the only agents proved to be effective in the treatment of HCV infection (6). Cytokine pattern secreted by T cells at the site of viral replication may influence the final outcome of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections (7).

Liver injury is reportedly associated with a chronic inflammatory response involving tumor necrosis factor- α (TNF- α) and interleukin-1b (IL-1b). The former plays a central role in liver injury, triggering the production of other cytokines that in turn recruit inflammatory cells, promote fibrogenesis and further activate oxidative burst (8). It is worth stressing that both TNF-

Address for Correspondence :

Dr. Mohamed El-Shahat Ebeid,
Molecular Biology Department,
Genetic Engineering and Biotechnology Research Institute
(GEBRI), Minufiya University, Sadat City,
Egypt, P.O 22857-79
Tel: +2-048-2601265(work), +2-048-2603874 (Home)
E-mail:moelshahat69@yahoo.com.

alpha and IL-1b are involved in controlling the balance between cytoproliferation and apoptosis, thus taking part in determining the liver cell's fate and progression to liver cancer (9). The aim of the present study was to clarify the levels of gamma interferon and tumor necrosis factor- alpha in patients infected with different HCV genotypes. Along with these cytokines, the level of superoxide dismutase enzyme and procollagen III N-terminal peptide were also studied.

MATERIALS AND METHODS

In the present study, HCV genotyping was done for HCV-RNA positive patients with chronic hepatitis C collected from the outpatients of Gastroenterology Surgical Center, Mansoura University, Mansoura, Egypt to estimate the different genotypes of HCV. Furthermore, immunological study was done to determine the levels of the proinflammatory cytokines in those samples to explain the immune status in each genotype. Biochemical study also was done to determine the levels PIIINP (as a fibrotic marker) and activity of superoxide dismutase enzyme in each genotype.

I- Molecular Biology Study

Polymerase Chain Reaction (PCR): All chemicals used in this study were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). The HCV RNA was extracted by the silica method (10). PCR was performed as described (11). Briefly, for RT step, 10 µl of the extracted RNA solution was added to 10 pM of downstream primer 5' CCA TGG CGT TAG TAT GAG TG-3' and subjected to cDNA synthesis for 45 minutes at 39°C in a mixture containing 10mM tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 20 unit of RNase inhibitor, 200 µM of each dNTP and 10 µl of reverse transcriptase (Bethesda research laboratories, Gaithersburg, MD, USA) in a final volume of 20 µl. Nested - PCR was carried out using 10 µl of the cDNA that was added to reaction mixture containing 200 µM of each dNTP, 50mM of KCl, 1.5 mM of MgCl₂ and 0.01% of gelatin, the outer primers [5'- TGC TCA TGG TGC ACGGTC TA-3' and 5'- CCA TGG CGT TAG TAT GAG TG-3'] in a concentration of 10 pM each and Taq polymerase (2.5 units) in a final volume of 50 µl reaction mixture. Amplification was performed by incubation in a programmable thermal cycler (PTC-100, MJ research Inc., Water town, MA, USA) at 95°C for 5 minutes and 35 cycles (95°C for one minute, 55°C for 90 seconds and 72°C for 2 minutes) followed by 10 minutes at 72°C. After completion of the first round PCR, 2 µl of the product were transferred to the second round reaction mixture containing 10 pM of each inner primer [5'-AGA GCC ATAGTG GTC GG-

3' and 5'-CTT TCG CGA CCC AAC ACT AC -3' and the reaction mixture as the same as described in the first round. The reaction was incubated in the thermal cycler for 5 minutes at 95°C and 30 cycles (95°C for one minute, 58°C for one minute and 72°C for 90 seconds) followed by 10 minutes at 72°C for one cycle. After completion of the second round PCR 10 µl was tested on 2% agarose gel electrophoresis after staining with ethidium bromide.

HCV Genotyping: HCV genotyping technique was done for sera of patients HCV positive using PCR technique. The method was done as described (12, 13).

II- Immunological and biochemical studies: For the genotyped samples of the present study the following immunological and biochemical parameters were studied.

Determination of gamma interferon (IFN -γ) and tumor necrosis factor alpha (TNF-α): The method of determination based on the sandwich-type of enzyme immunoassay (sandwich ELISA). Briefly, the wells of the microtiter plate were coated with anti-human IFN-γ or TNF-α monoclonal antibodies and incubated overnight at room temperature. Serum samples (of HCV positive patients) were then added to the microtiter wells. Subsequently, a biotinylated polyclonal antibody to human IFN-γ or TNF-α were added to bind to the IFN-γ or TNF-α immune complex respectively, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin. After washing, a substrate solution was added to the wells. The absorbance of each well (Standard wells, samples wells, and reagent well) was measured using ELISA reader (Bio- TEK Instrument, VT., USA) at 450 nm. The absorbance of each standard well was plotted against the concentration of the same well in semi logarithmic paper to obtain the standard curve. From the standard curve of IFN-γ and TNF-α their concentrations can be determined in each serum sample (CLB Co., Amsterdam, Netherlands).

Determination of procollagen IIIN-aminoterminal peptide (PIIINP): PIIINP levels were determined by using competitive radio-immunoassay. Briefly, a serum sample containing an unknown amount of PIIINP was mixed with a standard amount of radioactively labeled derivative of PIIINP. The labeled and unlabelled antigens were then allowed to compete for the limited number of high affinity binding sites of the antibody. The amount of radioactive antigen in the antibody – antigen complex was reversely proportional to the amount of unlabelled antigen in the sample. After separating the free antigen from the antibody– antigen complex, the residual radioactivity was counted and the actual concentration is calculated with the

aid of a standard curve (Orion Diagnostica, Finland).

Determination of superoxide dismutase enzyme activity:

This assay based on the ability of the enzyme to inhibit the phenazine methosulphate – mediated reduction of nitroblue tetrazolium dye (14).

Statistical analysis: Student's 't' test was performed using the statistical program package, instate software, version 2.03 (Graphpad, USA) and an IBM PC/AT compatible computer. The degrees of significance, $P < 0.05$ significant, $P < 0.01$ highly significant and $P < 0.001$ extremely significant were used.

RESULTS

HCV genotyping: The percent of single infection with HCV genotype 4 (G4) was 54.28%, ($n=19$). Genotype 3 was mixed with the genotype 2 and genotype 4 with a percent of 11.42%, ($n=4$) and 34.28%, ($n=12$) respectively (Table 1). According to the results of HCV genotyping Patients were consequently divided into; patients with genotype 4 (G4; $n=19$), patients with mixed genotypes 3, 4 (G3&4; $n=12$) and patients with mixed genotypes 2, 3 (G 2& 3; $n=4$).

Gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α): Fig 1 showed the level was decreased none significantly in patients infected with mixed genotypes 3, 4

Fig 1: Serum levels of gamma interferon (IFN-gamma) in patients with different HCV genotypes

HCV genotypes were detected accordingly, patients were grouped into three groups, infected with single genotype 4 (G4), infected with genotypes 2&3 (G2&3) and genotypes 3&4 (G3 and G4). The level of IFN- gamma was determined in comparison with healthy control group using Sandwich ELISA and the value represented in Pg/ml.

compared with mixed genotypes 2, 3 as well as single genotype 4. Collectively, the level of IFN- γ in HCV infected patients was decreased non-significantly compared with the healthy control group. Fig 2 showed that the level of TNF- α was elevated in patients with mixed genotypes 3&4 compared with the single genotype 4 and mixed genotypes 2, 3 ($P < 0.001$ and $P < 0.05$ respectively). The level was increased significantly in Patients with different HCV genotypes compared with healthy control group.

Fig 2: Serum levels of tumor necrosis factor-alpha (TNF-alpha) in patients with different HCV genotypes

The level of TNF-alpha was determined in all groups using Sandwich ELISA and the value represented in Pg/ml. *, Significant; **, highly significant; *** Extremely significant; P1 compared with normal control, P2, compared with G4, P3, compared with G2,3.

Serum level of procollagen IIIN-aminoterminal peptide (PIINP):

The level of PIINP in sera of patients infected with mixed HCV genotypes 3, 4 was reduced significantly in ($P < 0.05$) compared with the level in sera of patients infected with HCV genotype 4 and those with mixed genotypes 2,3 (Fig.3). No significant difference was observed between patients with single genotype 4 and mixed genotypes 2, 3. The level was increased in patients of genotype 4 and genotype 2&3, but the level was decreased nonsignificantly in patients with mixed genotypes 3&4 compared with the healthy control group (Fig. 3).

Serum levels of superoxide dismutase (SOD) and ALT enzymes: From Fig. 4 and Fig. 5, there is no significant

Fig 3: Serum level of PIINP in patients with different HCV genotypes

The level of PIINP was determined in all groups using competitive radioimmunoassay and the value represented in mg/l; *, Significant; *** Extremely significant; P1 compared with normal control, P2, compared with G4, P3, compared with G2,3.

difference in serum activity of SOD and ALT between the different HCV genotypes. However a significant difference was observed between patients with different HCV genotypes and healthy control group (Fig. 4 and Fig. 5).

DISCUSSION

Phylogenetic analyses have grouped HCV variants into six major genotypes, which have a star-like distribution and several minor subtypes (15). In the United States and in European countries, the majority of patients are infected with

Fig 4: Serum level of SOD activity in patients with different HCV genotypes

*** Extremely significant; P1 compared with normal control.

Fig 5: Serum level of ALT enzyme in patients with different HCV genotypes

*** Extremely significant; P1 compared with normal control

genotype 1, 2 or 3 (16). A high prevalence of HCV genotype IV was found in Middle Eastern countries, including Egypt, Saudi Arabia and Yemen and genotype 1a or 1b predominates in the non-Arab countries of the Middle East (4,16,17,18). The present study showed that, genotype 4 was detected in 88.57% in the tested HCV patients. Not only that but also genotypes 2 and 3 were found in mixed infection either with each other or with genotype 4. The present study agrees with that mentioned in (4) which genotype 1a or 1b predominates in the non-Arab countries of the Middle East.

In Egypt, it was found that the genotype IV is the most prevalent genotype and represents 95% among serum samples analyzed. In addition they detected genotypes and subtypes 1b and 2b equally in 2.5% of patients (19). Also, in Egypt, HCV genotype 4a is highly prevalent, where it contributed 85% of the tested samples in comparison to 10, 2.5, and 2.5% for subtypes 1b, 2a, and 3a, respectively (20). Others found that 84.3% of HCV infected patients were infected with a single genotype, including 1a in 12.4%, 1b in 2.2% and 2a in 11.2% (21). However the present study found that 54.28% of

Table 1: Number and percent of HCV genotypes in patients infected with HCV

HCV genotypes	No of cases	% of cases
G1	0	0
G4	19	54.28%
G,2,3	4	11.42%
G,3,4	12	34.28%
Total	35	100%

patients were infected with single genotype 4 only and a high percent of mixed genotypes were present (45.72%) in which, 34.285% of patients were infected genotypes 3, 4 and 11.42% were infected with mixed genotypes 2, 3.

The present study aimed to analyze the level of the pro-inflammatory cytokines (IFN-gamma and TNF-alpha) in patients with different HCV genotypes. Mixed infection with genotype 3, 4 greatly reduces the level of Procollagen III-N terminal peptide (PIIINP) and non-significantly reduces the level of IFN-gamma compared with other genotypes 4 and 3&4. On the other hand elevates the level of TNF-alpha. Otherwise no significant difference was observed in the levels of IFN-gamma, TNF-alpha, PIIINP, SOD activity and ALT activity. Different mechanisms including immunological liver damage, direct cytotoxicity mediated by different viral product and inductions of oxidative stress have been suggested as playing a pathogenic role in this infection (22). The initiation of a number of intracellular signal pathways involving apoptotic and/or anti-apoptotic signals should also be included amongst the effects of TNF- α and HCV infection is indeed associated with an increase in TNF- α production, and the expression of viral proteins apparently results in more severe liver injury and hepatocyte death (23). Others determined the DNA oxidative damage that correlated with TNF-alpha (a cytokine that can produce oxidative stress by stimulating the generation of reactive oxygen species), over-expression in chronic HCV-mediated liver damage and reported that evolution to cirrhosis was characterized by an increased oxidative DNA damage, c-myc expression and IL-1b release. When disease activity was severe, it was paralleled by an increased expression of IL-1b and c-myc associated with genotype 1b infection (9). They suggested that chronic HCV-mediated oxidative DNA damage in the liver may have an impact not only on hepatocyte proliferation rate through c-myc activation but also on cell proliferation and apoptosis through TNF- α activation (9). The present study agrees with the suggestion of Farinati et al (9) in which, the production of TNF-alpha, in patients with HCV infection (all patients with different HCV genotypes) was accompanied with a reduction in the activity of antioxidant enzyme, superoxide dismutase (SOD) and increase of production of PIIINP (except those patients infected with mixed genotypes 3&4) compared with healthy control group. Cell defense against ROS includes over expression of Mn-superoxide dismutase (SOD), an inducible mitochondrial enzyme. (24). In HCV infection an induction of Mn-SOD was present in PBMC but absent in the liver, suggesting that this organ could be less protected against oxidative damage and the oxidative stress could participate in the pathogenesis of HCV infection (24).

The levels of SOD and catalase were decreased both in group of children with chronic hepatitis C, and B in comparison to the healthy children (25). Loginov et al studied antioxidant system in adults with chronic active hepatitis, and demonstrated that, SOD decrease correlating with severity of inflammatory process (26). In another study was found that SOD activity was significantly low in chronic active hepatitis C patients (27). On the contrary, some author has detected high SOD levels in CHC patients (22, 25). The reduction in the amount of SOD, and GSH-Px reflects both a decrease in the synthesise capacity of liver, and the antioxidant defense power of the patients with CHC. It can be argued that increased lipid peroxidation is caused by the inflammation related to viral infection and decreased the antioxidant levels may be an early marker of the oxidative stress. Lipid peroxides formed can be chemotactic for the neutrophils causing increased inflammation, which further drives oxidant-mediated injury in the liver (28) and may be due to the inflammatory cytokine, TNF-alpha as in the present study.

Madwar et al (29) determined the prevalence and clinical significance of HCV genotypes, to evaluate clinical relevance of percentage of CD4+ and CD8+ T cells in blood, and to find out predictors of response to interferon (IFN) therapy in Egyptian chronic hepatitis C (CHC) patients. Results of Madwar et al revealed that genotype 4a is the prevalent HCV-genotype (85%) in Egypt. Only 21% of cases responded to IFN (29). Zekri et al reported that, liver disease progression from chronic liver disease to hepatocellular carcinoma due to HCV genotype-4 infection is associated with an imbalance between Th1 and Th2 cytokines (21).

In the viral hepatitis, virus also infects the peripheral lymphocytes. The infected lymphocytes produce interferon to stimulate healthy cells against viruses (30). Although interferon and ribavirin are not antioxidants, their antiviral capacity might reduce viral load and inflammation and perhaps through this mechanism might reduce virus-induced oxidative stress. Gorenek et al (31) similarly showed that SOD was significantly decreased in CHC patients before treatment when compared with healthy subjects ($p < 0.001$). In conclusion, genotype 4 was prevalent and genotype 1 was absent in patients under study in Dakahlia governorate, Egypt. The mixed genotypes (2&3 and 3, &4) were also present in these patients. No difference was observed in cellular immune response and antioxidant activity between patients with single infection with genotype 4 and patients with mixed infection with genotypes 2&3 and 3&4.

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