ISSN: 0067-2904

The Role of Toll Like Receptor -2 in Hepatitis B Infection

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Abstract

Hepatitis B virus (HBV) infection is a global public health problem. It is estimated that there are 240 million HBV carriers in the world, of whom roughly 600,000 die annually from HBV-related liver disease. A total of 150 individuals were included in this study, 130 individuals of them had hepatitis B infection (patients group); HBs-Ag was detected in their sera by enzyme linked immunosorbent assay (ELISA) technique and was confirmed by real time PCR analysis to detect the viral genetic material, the others were control. Most of HBV patients in this study were located within 20-40 years group with a percentage of 47.7% and within the 40-60 years group with a percentage of 38.5%. Acute infection was confirmed by detection of anti-HBc IgM antibodies, they were significantly higher ($P<0.05$) in acute hepatitis B patients than other groups in this study. Although there were no significant differences in biochemical tests, TSB, AST, ALT and ALP concentrations between study groups, the concentration levels of TSB, AST and ALT were higher than normal value in acute and chronic without treatment hepatitis B infected groups.

Toll like receptor-2(TLR-2) serum levels were upregulated in hepatitis B patients, it was significantly higher ($p\leq 0.001$) in Hepatitis B infected patients than control. The highest level was in chronic hepatitis B patients without treatment, chronic with treatment, acute, then carrier groups. Indicating that, TLR2 might participate in the pathogenesis of HBV infection, probably through altering the innate immune responses during infection.

Keywords: Hepatitis B virus, Toll-like receptor, anti-HBc IgM antibodies, biochemical tests.

دور TLR-2 في خمج التهاب الكبد الفايروسي نوع B

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الخلاصه

يعد خمج التهاب الكبد الفايروسي من المشاكل الصحيه العامه على الصعيد العالمي حيث يقدر عدد الحاملين لهذا المرض بالعالم ب 240 مليون منهم ما يقارب 600 الف مريض يموتون سنويا بامراض الكبد التي لها علاقه بالتهاب الكبد الفايروسي نوع B. شملت الدراسة على 150 فرد منهم 130مرضى مصابون بالتهاب الكبد الفايروسي نوع B. اغلب المرضى اعمارهم تتراوح مابين20 – 40 سنة وبنسبة 47,7 % و مابين 40 – 60 سنة بنسبة 38,5 %. تم تشخيصهم عن طريق كشف HBs– Ag في مصل المرضى بواسطة استخدام تقنية التحري المناعي الممتز المرتبط بالأنزيم وتأكيد الاصابة بالكشف عن المادة الوراثية للفايرس بتقنية التفاعل عديد البلمره. التهاب الكبد الفايروسي B الحاد تم تاكيده من خلال الكشف عن Anti– HBc IgM في مصول المرضى وكان مرتفع معنويا ($p < 0.05$) لديهم مقارنة بالمجاميع الاخرى. بالرغم

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من عدم وجود اختلافات معنوية بتراكيز الفحوصات الكيمياويه (TSB و ALT و AST و ALP) بين المجاميع المدروسه الا ان مستوياتها اعلى من المستويات الطبيعية في مرضى التهاب الكبد الفايرسي B الحاد والمزمن بدون علاج. كما ان مستويات TLR−2 في مصول مرضى التهاب الكبد الفايروسي B كانت غير منتظمه و مرتفعه معنويا ($p < 0.001$) مقارنة بعينات السيطرة. أعلى مستوى لل TLR−2 كان في مرضى التهاب الكبد الفايروسي نوع B المزمن بدون العلاج ، وبعدها مرضى التهاب الكبد الفايروسي نوع B المزمن اللذين يتعاطون العلاج، ومن ثم المرضى من النوع الحاد واخيرا الحاملين للمرض. مما يشيرالى احتماليه اشتراك TLR−2 في امراضيه التهاب الكبد الفايروسي نوع B من خلال تغيير الاستجابه المناعيه الطبيعيه اثناء الخمج.

Introduction

Viral hepatitis refers to infections that affect the liver and are caused by viruses. It is a major public health issue in the worldwide. Not only does viral hepatitis carry a high morbidity, but it also stresses medical resources and can have severe economic consequences. Seven viruses, hepatitis viruses A through G, are responsible for most cases of viral hepatitis. About a third of the world population has been infected with hepatitis B virus at one point in their lives, including 240 million to 350 million who have chronic infections. Over 750,000 people die of hepatitis B each year. The majority of all viral hepatitis cases are preventable [1].

Although the prevalence of chronic HBV infection was Low (<2%) in Iraq [2], still there are remaining threats to the successful eradication of HBV worldwide [3]. Hepatitis B belongs to the hepadnaviridae class of viruses. HBV is a small, partially double-stranded DNA genome (3.2 kb) encoding four genes-HBsAg (surface envelope glycoprotein), HBcAg (viral capsid protein), HBV Pol/RT (polymerase reverse transcriptase), and X gene (transcriptional activator). It is transmitted by direct percutaneous or permucosal exposure to infected blood. The hepatitis B infection occurs in adolescents and adults and can lead to acute hepatitis, subclinical infection, or the development of chronic infection [4].

The innate and adaptive immune responses of the HBV-infected host contribute to the development and pathogenesis of chronic HBV infection, and often affect the efficacy of anti-HBV drugs. Innate immunity is responsible for recognition of viral nucleic acids, viral proteins, and host tissue damage. It induces an antiviral state against infected cells by producing interferons (IFNs) [5]. IFNs are divided into three types: type-I mainly represented by IFN-α and -β, type-II by IFN-γ, and type III by IFN-lambda (IFNL) family. Each IFN family member mediates important anti-viral activity via engagement with their respective IFN receptor [6]. IFNL are directly induced through sensing of viral infection via pattern recognition receptors such as Toll-like receptors (TLRs). In TLR2-mediated antiviral responses, hepatocytes play an active role during hepadnaviral infection. The mutual inhibition of HBV replication and TLR2 signaling represents an important aspect of HBV infection that should be considered in the new therapeutic concept against chronic HBV infection [7]. This study aimed to investigate the role of TLR-2 in the immune response of Iraqi patients with hepatitis B viral infection.

Materials and Methods

1. Study groups

A total of 150 individuals were included in this study, 130 individuals of them had hepatitis B infection (patients group); HBs Ag was detected in their sera by ELISA technique and confirmed by real time PCR analysis to detect the viral genetic material. These investigations with the diagnosis was made by the consultant medical staff in the Gastroenterology and Hepatology Teaching Hospital in Baghdad. The patients were 87 males and 43 females with age range 9-70 years, while the other 20 individuals were the control group, 11 males and 9 females their age range matched with patient group.

Seventy four Hepatitis B patients (20 acute, 20 chronic with treatment, 20 chronic without treatment and 14 carriers) beside fourteen controls from pervious 150 individuals were included in the investigation by TLR-2 ELISA kits.

2. Specimens collection

Specimens were collected by venipuncture, 5 ml of blood was drawn using disposable syringes. The blood was placed in plastic disposable tubes, then left to stand at room temperature (18-25°C) to

clot. Sera were separated by centrifugation for 5 minutes at 3000 round per minute (rpm). The separated serum was distributed into 3 aliquots in Eppendorf tube and kept at -20ºC until assayed.

3. Biochemical Tests

- **Total Serum Bilirubin (TSB) determination**

In order to determine TSB, BILTS Total Bilirubin Special Kit, for quantitative TSB in human serum and plasma of adults were used by cobas^{TM} c 111 chemistry analyzer and according to the manufacture instructions [8].

- **Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) determination**

Reflotron® GPT (ALT), GOT (AST) and ALP reagent strips were used for Quantitative determination of ALT, AST and ALP, respectively in blood, serum and plasma with Reflotron® Plus System by Roche Company and according to the manufacture instructions [9; 10; 11].

4. Detection of HBc IgM Antibodies

A solid phase, two-step incubation, antibody capture ELISA kit for qualitative determination of IgM-class antibodies to hepatitis B virus core antigen in human serum or plasma was used and according to the manufacture instructions [12].

5. Assessment of TLR-2 serum levels

Sera of patients and controls were assessed for the level of TLR-2 using commercially available kits (Boster, Austria) by ELISA system and the procedure was done according to the kit instructions [13].

6. Statistical analysis

The results were analyzed statistically and the values were expressed as mean ± SD. The differences between means were assessed by ANOVA (Analysis of Variance) and correlation by correlations coefficient (r) using Microsoft office Excel 2007 software. All Values were deemed significantly different when $P< 0.05$[14].

Results and Discussion

The distribution of HBV patients according to age in this study were shown in Table-1. It was found that the age of HBV patients ranged between 9-70 years. The mean ± SD of acute, chronic and carrier patients were 40.6 ± 2.6, 37.8 ± 15.0 and 35.8 ± 9.5 years, respectively. Most of HBV patients in this study were located within 20-40 years group with a percentage of 47.7% and within the 40-60 years group with a percentage of 38.5%; whereas 8.5% and 5.3% were located within more than 60 years and less than 20 years groups, respectively.

These results agreed with Tarky *et al*., [15] results who found that the prevalence of HBsAg was lowest in the first decade of life (0.9%), and increase with age to reach a maximum prevalence rate of 2.4% in the fifth decade of life. The risk of testing positive for HBs antigen is almost doubled in the third decade of life compared to first decade of life. Being in the fifth decade of life significantly increased the risk of having positive HBs antigen by 2.6 times compared to first decade of life. Also the results of this study coincide with several studies done in Iraq like Ahmed, [16] who found that most of chronic hepatitis B patients were located within third and forth decade 21-40 year with a percentage of 51.3% and it was found that the seventh decade 61-70 year constitutes the least percentages 5% and with Abid *et al*., [17] who reported that the infection with chronic hepatitis B virus was distributed in patients with age ranged (30-50) years. Similar result was found in the United States, The highest incidence of acute hepatitis B had been seen among persons 25–44 years of age and the lowest among children less than 15 years of age. Over time, the incidence decreased in all age groups with the greatest proportional decline occurring among age groups <15 years (95% decline) and 15–24 years (87% decline) [18]. In 2013, incidence of acute hepatitis B were the highest for persons aged 30–39 years; the lowest rates were among children and adolescents aged <19 years [19].

Table 1-The distribution of HBV patients according to age

Age groups (years)	No. of patients	%
Less than 20	7	5.3
20-40	62	47.7
40-60	50	38.5
More than 60	11	8.5
Total	130	100%

Anti-HBc IgM antibodies mean ± SD were significantly higher (P<0.05) in acute hepatitis B patients 3.7 ± 6.02 than chronic with and without treatment (0.35 ±0.24 and 0.41 ± 0.31), carrier patients (0.28 ± 0.23) and control (0.14 ± 0.12), as shown in Table-2. The frequency of positive anti-HBc IgM in hepatitis B infected patients included in this study was found in 7.1% of them. This result was conducted with Lavarini *et al*., [20] study who found that in Italy 85% of patients with positive IgM anti-HBc test result confirmed that hepatitis was due to primary infection with hepatitis B virus while patient's absence of the IgM marker indicated that they were previously unrecognised long term carriers of HBsAg.

The frequency of isolated anti-HBc relates directly to the prevalence of HBV infection, 3% of HBsAg-positive patients were also positive for anti-HBc IgM in study conducted from Mashhad/Iran Shakeri *et al*., [21] which disagreed with the results of this study that found 7.1% of HBs Ag positive patients were also positive for anti-IgM HBc Ab. IgM anti-HBc appears in persons with acute disease about the time of illness onset and indicates recent infection with HBV. IgM anti-HBc is the best serologic marker of acute HBV infection. A negative test for IgM-anti-HBc together with a positive test for HBsAg in a single blood sample identifies a chronic HBV infection [22].

Table 2-Anti-HBc IgM antibody index unit in Hepatitis B patients

Specimen groups	**Anti HBc IgM antibodies index unit Mean ± SD**
Acute hepatitis B patients	3.7 ± 6.55
Chronic hepatitis B patients with treatment	0.35 ±0.24
Chronic hepatitis B patients without treatment	0.41 ± 0.31
Carrier	0.28 ± 0.23
Control	0.14 ± 0.12
P value	0.00094

Although there were no significant differences in biochemical tests (TSB, AST, ALT and ALP) among study groups as shown in Table-3, the concentrations levels of TSB, AST and ALT were higher than normal value in acute and chronic without treatment hepatitis B infected groups. TSB and ALT concentrations were higher in acute hepatitis B patients (1.99 ± 2.6 and 61.8± 67.0, respectively) than control (0.55± 0.15 and 22.92±11.4, respectively). Also there was higher concentration of ALT in acute group (61.8 ± 67.0) than treated chronic patients (26.47± 15.0) and carrier group (24.0±13.0).

The results agreed with WHO report that mentioned hallmark of acute viral hepatitis was the striking elevation in serum transaminase (aminotransferase) activity. The increase in aminotransferase, especially ALT, during acute hepatitis B varies from a mild/moderate increase of 3- to 10-fold to a striking increase of >100-fold [4] and with Zainal, *et al.*, [23] who found that patients with HBV in Baghdad had a significant higher, AST, ALT, ALP and TSB ($p < 0.00$) compared to control. Also with Al-Shaikle, [24] who reported that TSB and ALT were increased among HB infected patients, while it was normal among control group. Liver enzymes are located in liver cells that can leak out into the bloodstream when liver cells are injured.

Table 3-Biochemical tests in hepatitis B patients and controls.

Specimen groups	Biochemical tests concentrations Mean ± SD			
	TSB mg/dl	AST U/L	ALT U/L	ALP U/L
Acute hepatitis B patients	1.99 ± 2.6	58.9±114.6	61.8± 67.0	52.1± 18.6
Chronic hepatitis B patients with treatment	0.75± 0.58	21.46±6.22	26.47± 15.0	61.18±21.43
Chronic hepatitis B patients without treatment	1.32±2.75	55.63±137.1	64.1± 179.3	52.1±34.27
Carrier	0.68±0.27	19.62±8.8	24.0±13.0	53±20.2
Control	0.55± 0.15	15.0±4.9	22.92±11.4	43.65±22.0
P Value	0.129 NS	0.36NS	0.44 NS	0.38NS

Optimal values: TSB up to 1.4 mg/dl, ALT up to 41 U/L, AST up to 40 U/L and ALP 40-129 U/L [25].

Toll like receptor-2 levels in hepatitis B infected patients was significantly higher ($p \leq 0.001$) than control. The highest level was in chronic hepatitis B patients without treatment, chronic with treatment, acute, then carrier groups. TLR2 serum levels were upregulated in hepatitis B patients, as shown in Table-4.

Mechanisms by which hepatitis B virus (HBV) establishes persistent infection remain unclear, although viral inhibition of host defenses is likely to be important. Most studies of the immunological aspects of persistent HBV infection have focused on adaptive immunity, with little research on the innate immune response to HBV [26]. One of the key components of the innate immune response is the family of TLRs, evolutionarily conserved pattern recognition receptors (PRR) [27]. Activation of TLRs by various motifs common to microorganisms, known as pathogen-associated molecular patterns (PAMP), triggers the release of inflammatory mediators such as tumor necrosis factor alpha (TNF-α) [28].

Table 4- TLR-2 serum levels in study groups

Specimen groups	TLR-2 levels pg/ml
Acute hepatitis B patients	3895 ±1032
Chronic hepatitis B patients with treatment	4168 ±1128
Chronic hepatitis B patients without treatment	4947±1306
Carrier	3467±1077
Control	162.6±51.99
P value	0.001 S

Previous studies had shown that some viruses suppress TLR-mediated immune mechanisms, thereby disabling an important aspect of the host's antiviral defenses [29-31]. TLR-2 plays an important role in the immunopathogenesis of HBV infection. The relationship between TLR2 expression and clinical outcome of chronic HBV infection is not yet elucidated in details so far. Huang *et al*., [32] demonstrated that TLR2 was mainly expressed in monocytes and its ligand stimulation resulted in TNF-α, IL-6 and IL-10 production. Serum soluble TLR2 (sTLR2) levels were negatively correlated with TLR2 mRNA in peripheral blood monocyte cells (PBMC). As compared with immunotolerant carriers and inactive carriers, chronic HB patients showed an elevated TLR2 expression and TNF-α, IL-6 induction in PBMC, but had a decreased level of sTLR2 in serum. However, TLR2 expression and TNF-α induction in monocytes of chronic HB patients was remained lower than healthy controls. Furthermore, higher TLR2 expression in PBMCs and lower level of sTLR2 in serum at baseline was predictive of a complete response to 52weeks of telbivudine (LdT) therapy. Temporal dynamic analysis showed that TLR2 expression was restored with viral suppression and ALT normalization from week 12 to 24. However, peg-IFN-α-2a therapy induced a slightly decline in TLR2 expression. This study concluded that TLR2 expression and function in monocytes were impaired by chronic HBV infection. Higher TLR2 levels in PBMC and lower sTLR2 in serum at baseline were associated with a complete response to LdT therapy, and dynamic TLR2 expression was differently regulated by LdT and peg-IFN-α-2a therapy.

Visvanathan *et al*., [33] reported that in the absence of HBeAg, HBV replication was associated with up-regulation of the TLR2 pathway leading to increased TNF- production. Riordan *et al*., [34] found that CD14-positive peripheral blood monocyte expression of TLR2 was significantly reduced in HBeAg-positive patients compared to both controls and HBeAg-negative patients, with values ranging from 80% to as low as 5% of normal. TLR2 expression in HBeAg-negative patients was not significantly different from that found in controls.

It is widely accepted that chronic hepatitis B virus (HBV) infection is the result of an ineffective antiviral immune response against HBV infection. Wang *et al*., [35] found that the hepatitis B surface Ag (HBsAg) was related to decreased cytokine production induced by the TLR2 ligand (Pam3csk4) in PBMCs from chronic hepatitis B patients, and demonstrated that HBsAg selectively inhibits Pam3csk4- stimulated IL-12 production in monocytes/macrophages by blocking the JNK-MAPK pathway and provide a mechanism by which HBV evades immunity and maintains its persistence.

Recognition mechanisms of innate immune response help to improve immunotherapeutic strategies in HBeAg-negative chronic hepatitis B. TLR-2 is an important component of innate immunity. Moradzadeh *et al*., [36] reported that dominance of G1896A pre-core mutation of HBV variants was

correlated with serum TLR2. Moreover TLR2 is critical for induction of inflammatory cytokines and therefore ALT elevation. Few studies suggested that the peripheral blood levels of TLR2 and TLR4 in HBV groups were up-regulated in comparison with the normal control group [37-41]. Lu *et al*., [42] pointed that the expression of TLR2 was significantly increased in the disease progression, with the TLR2 expression rates of 2.60 ± 1.70%, 2.67 ± 2.89%, 3.53 ± 3.41% and 5.11 ± 4.93 in normal control, HBV, HBV-Liver Cirrhosis, and HBV-HepatoCellular Carcinoma, respectively. Furthermore, the HBeAg level was increased, while the amount of HBV-DNA exhibited a declining trend, along with the disease severity. Correlation analysis revealed that the expression of HBeAg was positively correlated with TLR2. The elevated expressions of TLR2/4 on DC cell surfaces in peripheral blood may synergistically promote the disease progression of chronic HBV infection. Indicating that, TLR2 and TLR4 might participate in the pathogenesis of HBV infection, probably through altering the innate immune responses during infection. In contrast, Chen *et al*., [43] indicated that TLR2 and TLR4 were downregulated to affect the innate immune responses during the HBV infection.

Lian *et al*., [44] also found that the expression of TLR2 was significantly upregulated in patients with liver cirrhosis and chronic hepatitis B patients. Interestingly, the therapeutic TLR strategy by Isogawa *et al*. [45] revealed that TLRs ligands except for TLR2 are able to induce antiviral cytokines (Interferon) at the site of HBV replication.

Also in this study, there was negative non-significant correlation (r = -0.025) between ALT concentration and TLR-2 levels in Hepatitis B patients groups as shown in Table-5.

Table 5-Correlation coefficient between ALT concentrations and TLR-2 levels in patient groups

Specimen groups	ALT conc. IU/ml	TLR-2 levels pg/ml	Correlation coefficient (r)
Acute hepatitis B patients	61.8± 67.0	3895 ±1032	- 0.3
Chronic hepatitis B patients with treatment	26.47± 15.0	4168 ±1128	-0.03
Chronic hepatitis B patients without treatment	64.1± 179.3	4947±1306	-0.05
Carrier	24.0±13.0	3467±1077	-0.3
Total patient groups	43.28±94.54	453.01±1234.01	-0.025
P value			NS

These results disagreed with Moradzadeh *et al*., [36] results who found that there was a significant correlation between serum ALT and TLR-2 (r=0.46; P=0.01). They speculated that the increased serum TLR-2 levels in hepatitis B patients may correspond to higher expression of cellular TLR-2 and consequently elevated TLR2 signaling leading to expression of proinflammatory cytokines that explain the chronicity of HBV infection that usually accompanies an increase in ALT. The differences in the correlation results between TLR-2 and ALT in this study and Moradzadeh *et al*, [36] study might be due to the low number of selected groups especially chronic hepatitis B patients without treatment group which was resembled to Moradzadeh *et al*, [36] studied group.

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