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Estimation of cellular immune response by evaluation of some cytokines in immunopathogenesis of chronic hepatitis B and C pre- and post- treated Iraqi patients (*in vivo* and *in vitro*)

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Abstract:

Two groups of chronic hepatitis B and C virus patients were divided into Pre-treated patients (25 CHB patients with positive HBs Ag for more than 6 months and 40 CHC patients), and post-treated patients [12 CHB patients (4, 6, and 2 were treated with lamivudine, IFN-α and combination of LMV + IFN-α respectively), and 27 patients for CHC (3, 13 and 11 patients were treated with Ribavirin, IFN-α and combination therapy (RBV+ IFN-α) respectively].These patients were followed up for 6 months.

By using ELISA technique, levels of IL-6, IL-10, IFN-γ and TNF-α were measured *in vivo* and *in vitro* (supernatant of PBMCs stimulated with PHA) and compared with healthy control. The mean level of IL-6, IL-10 and TNF-α in CHB patients showed significant differences ($P<0.05$) between pre- and post-treated patients *in vivo* and *in vitro,* while there was no significant difference in IFN- γ between pre- and post-treated patients *in vivo* and *in vitro*. The difference between control and CHB patients was highly significant ($P<0.0001$) in IL-6 , IL-10 and TNF-α levels *in vivo* and *in vitro*. In CHC patients the mean levels of IL-6, IL-10, IFN-γ and TNF- α showed significant difference between pre- and post-treated patients *in vivo* and *in vitro*. There was highly significant difference ($P<0.0001$) between patients and control in IL-10 levels. Hence, these observations indicate the predominance of Th2 cytokine, which promote the persistency of the CHB and CHC virus.

Key words: cellular immune response, cytokines, chronic hepatitisB, chronic hepatitisC, IFN-γ,TNF-α

Introduction:

Cytokines are small molecules that act as a means of communication between cells, thus have many roles within the liver. Cytokines play an important role in the defense against viral infections, both indirectly, through determination of the predominant pattern of host response, and /or directly, through inhibition of viral replication. However, within the context of an inflammatory response against a virus, Cytokines may also lead to liver damage [1].

It was reported that HBV infection is associated with the production of a broad range of proinflammatory cytokines and chemokines such as IL – 1 β, IL – 6, IL – 8, IL – 12 and IFN - γ. As well as the anti-inflammatory cytokine IL – 10 [1,2,3].In HCV, the cytokines present at the initiation of immune response are the most clearly defined factors determining Th1 and Th2 differentiation from Tho [4].Pro-inflammatory cytokines such as IFN – γ and TNF – α are increased and they

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attributed to an imbalance in Th1, and Th2 CD4+ cell activity with a predominant T helper 1 cell response [5].Tumor necrosis factor – α (TNF – α), interleukin – 6 (IL –6) are important mediators of the inflammatory response. Monocytes are considered to be the main – producers of these monokines, but along list of potential cell sources have been reported. Some of these reports indicate that peripheral T cells can produce TNF – α, IL – 6 and IL – 1 [6,7] demonstrated that monokines release was totally inhibited by anti - IFN – γ treatment indicating that TNF-α and IL – 6 were both induced by IFN – γ.Production of IL – 6 and TNF – α is certainly stimulated Th1 derived lymphokines, and IFN – γ act as macrophage – activating factor and to induce monokines release [6].

Proteins whose expression was reported to be elevated in chronic HCV infection are IFN – γ [8] , and class I and class II major histocompatibility complex (MHC) [9], which are known to be regulated by IFN – γ , as well as the accessory molecules CD80 , CD40 , and B7 . In addition to $CD8^+$ lymphocytes and monocytes, preferentially antigen – non specific activated Th1 lymphocytes are attracted to the liver. By secreting IFN – γ, the latter cells are thought to activate monocytes, macrophages, thereby initiated a delayed type hypersensitivity reaction [8].

The cytokines may down regulate HCV specific cellular immunity and promote viral persistence [10]. Higher serum IL – 10 levels correlate with a poor response to treatment , a sustained treatment response has been associated with decreased IL –4 and IL – 10 [11] .

So that the purpose of this study was:

Evaluation of possibe role of Th1 cytokine IFN-γ and Th2 cytokines: IL-6, IL-10, and other inflammatory cytokines such as TNF-α, in immunopathogenesis of chronic hepatitis B and C pre- and post- treated patients (*in vivo* and *in vitro* (supernatant of PBMCs stimulated with PHA).

Materials and Methods:

Subjects

Across sectional study was conducted on the following study groups for the period from September 2004 to October 2005:

A total of sixty five (65) patients of both sexes were divided into two major groups:

* Twenty- five (25) patients with chronic hepatitis B- virus (CHBV) (marked with appearance of hepatitis B surface antigen HBs-Ag and persisted more than 6 months). This marker in the sera was detected by using Enzyme- Linked Immunosorbant Assay (ELIZA), these patients consisted of 18 males and 7 females, with age ranged between (25-73) years.
* Forty (40) patients with chronic hepatitis C- virus (CHCV) (marked with appearance of anti- hepatitis C antibodies in their sera and persist more than 6- months which was detected by using ELIZA- technique). These patients included 21-males and 19- females and were of age ranged between (6-60) years.

All these patients showed increasing levels in liver function tests (TSB, SGPT, SGOT, and SALP) and they had been clinically diagnosed according to the previous laboratory test and the clinical examination when they were inpatients admitted in Gastroenterology and Hepatology Teaching Hospital.

Normal control group: Fifteen (15) healthy persons of different age and sex were randomly selected as normal control (NC) during the period of this study.

Collection of Blood Samples

Ten ml of venous blood was drawn from each subject aseptically.

* Serum from 4 ml of blood sample was used for the evaluation of interleukins .
* A volume of 3.75 ml of blood sample was collected in sterile evacuated tubes containing heparin for lymphocytes transformation assay using ″3ml blood for purified lymphocytes ″ and ″0.75ml blood for Whole blood ″.

Lymphocyte transformation assay by using Separated lymphocytes.

A. Lymphocyte separation

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient method, principally as described by Boyum [12].

B. Viability test: The viability was determined by trypan blue dye exclusion test [13].

C. Preparation of culture: This assay was done according to Kanto *et al*[4].

* A volume of 2.75 ml of separated lymphocytes (1×10^6 cell/ml RPMI 1640) was cultured in sterile silicon coated tubes**.**
* A volume of 250µl of mitogen (PHA) was added to one of these tubes (test), while the other tube left without any addition of mitogen (control).

- The ratio of transformed cells was calculated by the following equation:

$$\text{Transformed cells \%} = \frac{\text{No. of transformed cells}}{\text{Total No. of cells count}} \times 100$$

Determination of Cytokines

Cytokines levels in both serum and supernatant of separated lymphocytes culture were determined. The cytokines that used in this assay were IFN-γ, IL-6, IL-10 and TNF-α, and they were measured by means of enzyme linked immunosorbant assay, using ELISA kits (Mabtech AB, Sweden) as recommended by the manufacture:

Statistical analysis

Student's *t* test of significance was used for two- groups' comparison when the data were quantitative in nature. The Chi- square (X^2) test for significance was adopted for the comparison and calculation of association in qualitative data.

The statistical significance of difference in mean of variable between more than two groups was assessed by ANOVA test [14].Probability values of $P<0.05$ were considered statistically significant.

Results and Discussions:

Estimation of cytokine levels *in vivo* and *in vitro* pre- and post- treatment

To know whether an imbalance between Th1 and Th2 cytokines are present in patients with CHB and CHC infection, with and without treatment *in vivo* and *in vitro*(PBMCs supernatant harvested after 3 days of incubation stimulated by PHA) in comparison to that of healthy control group, levels of Th1 cytokines (IFN-γ) and Th2 cytokines (IL-6, 1L-10) and inflammatory cytokine (TNF-α) were measured using enzyme-linked immunosorbent assay in this study.

1Level of IL-6

The result of this study revealed high mean values of IL-6 levels pg/ml in CHB patients pre-treatment that are shown in table (1), as compared to healthy controls both *in vivo* (P= 0.000) and *in vitro* (P= 0.000) (PBMCs supernatant stimulated with PHA). Also the present study showed that there was highly significant difference in IL-6 levels between pre- and post-treated CHB patients *in vivo* and *in vitro* (stimulated with PHA and non stimulated) ($P < 0.05$), this results was coincided with the results of Lee *et al* [15]who reported that IL-6 can be induced by HBV-X protein as evidenced by high levels of serum IL-6

in patients with CLD with HBV infection.

Preliminary experiments determined that lymphokine levels in the culture supernatants of both normal subjects and chronically infected HBV patients peaked after 3 days [16]. They showed that the patients who failed to mount a proliferative response also failed to secrete any of the cytokines under study.

Table (1): - Comparison in cytokine levels between (25) pre- and (12) post-treated CHB patient *in vivo* and *in vitro*.

cytokine	Source	Pre-treatment* (25)	Post-treatment*			P Value	Control (15)	P
			lamivudine (4)	IFN-α2b (6)	lamivudine IFN- α2b (2)			
IL-6	Serum	902.9 ±392.40	53.55 ±18.17	78.50 ±26.16	46.0 ±8.48	0.008[a]	38.83 ±27.65	0.000[c]
	Supernatant stimulated withPHA	896.23 ±663.66	87.50 ±9.57	125.05 ±76.70	95.0 ±4.24	0.011[a]	42.67 ±10.60	0.000[c]
	Supernatant without stimulation	8.81 ±5.60	0.45 ±0.23	0.55 ±0.35	0.45 ±0.01	0.07[b]	0.33 ±0.11	0.4[d]
IL-10	Serum	489.0 ±42.34	76.75 ±48.94	51.50 ±24.44	46.25 ±5.30	0.001[a]	18.63 ±16.02	0.000[c]
	Supernatant stimulated withPHA	585.88 ±44.65	63.25 ±50.88	38.50 ±4.94	30.1 ±0.000	0.002[a]	17.86 ±12.01	0.000[c]
	Supernatant without stimulation	13.77 ±3.26	11.3 ±0.00	10.2 ±0.07	5 ±0.000	0.027[a]	0.1 ±0.05	0.12[d]
TNF-α	Serum	149.76 ±24.34	76.75 ±48.94	51.50 ±24.44	55.25 ±5.30	0.003[a]	46.63 ±27.93	0.012[c]
	Supernatant stimulated withPHA	145.70 ±45.77	103.25 ±38.15	95.0 ±7.07	80.00 ±28.28	0.027[a]	61.93 ±19.79	0.043[c]
	Supernatant without stimulation	79.38 ±73.20	3.05 ±2.75	0.000 ±0.000	0.00 ±0.00	0.342[b]	0.00 ±0.00	_
IFN-γ	Serum	487.17 ±175.05	822.5 ±144.85	983.0 ±721.09	995±353.55	0.432[b]	990 ±291	0.2[d]
	Supernatant stimulated withPHA	401.087 ±204.57	687.37 ±89.50	700.0 ±70.10	750.0 ±275.7	0.488[b]	563.40 ±0.00	0.38[d]
	Supernatant without stimulation	131.73 ±60.12	5.50 ±1.29	60.0 ±56.56	200 ±0.00	0.60[b]	0.00 ±0.00	_

*** mean ±SD.**

a: The difference between pre- and post-treatment is significant (P < 0.05).

b: The difference between pre- and post-treatment is non significant (P < 0.05).

c: The difference between control and CHB patients is highly significant (P < 0.05).

d: There is no significant difference between CHB patients and control (P < 0.05).

Kakumu *et al*[17] reported that CHB patients who received IFN- α2b and 1L-2 therapies showed depressed IL-6 activity during treatment, and rebound beyond pre-treatment values after cessation of therapy. These findings suggest that IL-6 plays a role in the development of CHB, and it may contribute, at least in part, to the elimination of HBV.

The present study also showed no significant difference of IL-6 levels pg/ml in CHC patients that shown in table(2), as compared to their levels in healthy controls both *in vivo* and *in vitro* ($P < 0.05$). Also this study showed that there was significant difference of IL-6 levels between pre- and post-treated CHC patients *in vivo* and *in vitro* (stimulated with PHA) ($P < 0.05$). This difference may be due to the effectiveness of IFN- α treatment as Quadri *et al*[18] reported that serum IL-6 concentration was initially elevated in chronic HCV, increased level was detected before IFN- α therapy followed by decreased level after 6th IFN- α dose.

Ren *et al*., [19] showed a significant increase in IL-6 levels in patients with HCV as compared with healthy control. Falasca *et al.*, [20]

found that HCV positive and HBV positive patients have higher levels of Th2 cytokines, particularly in the course of CHB, and that IL-8 and IL-6 levels may have important roles as markers of both inflammation and hepatic injury particularly in the course of HC.

2- Level of IL-10

In the present study, there was an increased levels of IL-10 (Pg/ml) was observed in patients with CHB infection pre-treatment, as compared to their levels in healthy control ($P < 0.0001$) both *in vivo* and *in vitro* (with and without simulation by PHA). Also this study showed that there was a highly significant difference in IL-10 levels between pre- and post-treatment ($p < 0.05$) (table 1). These results were coincided with other results of Vingerhoets *et al*(21); Hsu *et al*[2] who reported that the levels of IL-10 in CHB patients were recorded a significant elevation as compared with healthy control.

Also the present study showed that there was significant difference of IL-10 levels (Pg/ml) in CHC patients pre-treatment as compared to their levels in healthy controls both *in vivo* and *in vitro* (with stimulation by PHA) ($P < 0.05$) (table 2). In addition, this study showed a significant difference in IL-10 levels between pre- and post-treated CHC patients *in vivo* and *in vitro*(with and without stimulation by PHA) ($P < 0.05$) (table 2). These results coincided with the results of Abayli *et al*[22].

Kakumu *et al*[23] reported that the levels of IL-10, and IL-2 secreted by PBMCs taken from chronically infected patients with HCV were significantly elevated than in control group. Radkowski *et al*[24] found that IL-10 concentration was higher in supernatant from infected macrophages stimulated by PHA, but this difference didn't reach statistical significance($P< 0.05$).

Table (2): Comparison of cytokine levels between (40) pre- and (27) post-treated CHC patients *in vivo* and *in vitro*.

Cytokine	Source	Pre-treatment* (40)	Post-treatment*			P Value	Control (15)	P
			ribavirin(3)	IFN-α2b(13)	ribavirin IFN-α2b (11)			
IL-6	Serum	197.38 ±81.23	126.66 ±36.71	86.38 ±38.27	46.81 ±43.25	0.014[a]	38.83 ±27.65	0.2[d]
	Supernatant stimulated withPHA	365.53 ±103.12	69.33 ±60.34	62.72 ±32.01	55.9 ±47.69	0.005[a]	42.67 ±10.60	0.5[d]
	Supernatant without stimulation	0.3 ±0.28	5.45 ±2.43	0.50 ±0.10	0.06 ±0.05	0.017[a]	-	-
IL-10	Serum	211.69 ±52.42	42.33 ±27.74	27.92 ±15.84	27.45 ±16.62	0.017[a]	18.63 ±16.02	0.000[c]
	Supernatant stimulated withPHA	217.33 ±26.50	43.33 ±24.32	31.61 ±18.26	33.36 ±13.61	0.036[a]	17.86 ±12.01	0.000[c]
	Supernatant without stimulation	0.65 ±0.49	15.05 ±11.14	0.1 ±0.14	0.03 ±0.01	0.422[b]	-	-
TNF-α	Serum	147.15 ±21.28	59.33 ±49.32	57.76 ±48.89	55.18 ±29.69	0.031[a]	46.63 ±27.93	0.1[d]
	Supernatant stimulated with PHA	131.15 ±52.87	83.33 ±4.04	92.79 ±48.89	77.18 ±29.69	0.025[a]	61.93 ±19.79	0.2[d]
	Supernatant without stimulation	1.55 ±1.05	0.05 ±0.02	0.25 ±0.15	3.66 ±2.35	0.974[a]	-	-
IFN-γ	Serum	445 ±349	685.33 ±488.99	519.23 ±279.83	699.09 ±275.15	0.214[b]	750 ±297.11	0.2[d]
	Supernatant stimulated withPHA	500 ±72.99	633.33 ±282.01	723.15 ±207.45	840.90 ±306.70	0.002[a]	563.40 ±215.79	0.1[d]
	Supernatant without stimulation	10 ±4.14	100 ±0.000	75 ±69.65	36.66 ±15.07	0.062[b]	-	-

*** mean ±SD.**

a: The difference between pre- and post-treatment is highly significant ($P < 0.05$).

b: The difference between pre- and post-treatment is non-significant ($P < 0.05$).

c: The difference between CHC patient and control is significant ($P < 0.05$).

d: The difference between CHC patient and control is non-significant ($P < 0.05$)

3-Level of Tumor Necrosis Factor – α (TNF – α)

In the present study there was an increased level of TNF – α in CHB patient's pre – treatment as compared with control subjects with significant differences ($p < 0.05$) (table 1) both *in vivo* and *in vitro* (supernatant of PB MCs stimulated by PHA) . Also there was significant differences ($p < 0.05$) of TNF – α levels between pre and post treated CHB patients *in vivo* and *in vitro* (supernatant of PB MCs stimulated by PHA but not without stimulation) (table 1) . These results are in agreement with other results of (2,3) who reported that in response to HBV infection a broad range of proinfammatory cytokines and chemokines which required for viral clearance were produced , such as IL – 1β , IL – 6 , IL – 8 , IL – 12 , TNF – α and IFN – γ.

In addition , in the present study there was an increased levels of TNF – α in CHC patients , as compared with the control group but with no significant differences ($p < 0.05$) (table 2) both *in vivo* and *in vitro* (PBMCs stimulated by PHA) . Also there was a significant difference of TNF – α levels (pg / ml) between pre – and post – treated CHC patients *in vivo* and *in vitro*(PBMCs stimulated by PHA but not that were without stimulation (table 2) ($p< 0.05$) . This result coincided with the result of Hsu *et al*[2].

Radkowski *et al*[24] found that exposure of primary macrophage to HCV positive sera (before treatment with IFN- α2b) resulted in enhanced production of TNF – α and IL – 8 and their respective mRNA.

4 - Levels of Interferon – γ (IFN– γ)

The results of this study showed the decreased of IFN – γ levels(pg/ml) of CHB patients pre-treatment ; and no significant difference has been shown between this patients and control and between pre – and post – treated patients (table 1) both *in vivo* and *in vitro* (with and without stimulation by PHA) .

This result is in agreement with Livingston *et al*[16] who reported that the secretion of the Th 1 – associated cytokine IL – 12 and IFN – γ were considerably reduced in patients with CHB infection compared to normal subjects , while secretion of Th2 cytokine IL – 5 appeared to be elevated , although not significantly . Also they showed that the secretion of IFN – γ in supernatant of PBMCs from CHB patients (that stimulated by PHA as a mitogen stimulus) was decreased in PBMCs supernatant in comparison to normal subjects.

In addition, in this study the IFN – γ estimation showed decreased level in mean ± SD(pg / ml)in CHC patients with no significant difference as compared with control *in vivo* and *in vitro* (stimulated by PHA) (table 2) and between pre – and post – treated CHC patients *in vivo* ($p < 0.05$) . While in comparison with *in vitro* (supernatant of PBMCs stimulated by PHA) showed significant difference between pre – and post – treated CHC($p < 0.05$).

This result is in agreement with Abayli *et al*.[22] who studied the serum profile of Th1 and Th2 cytokines, in chronically HCV infected patients and found that the Th2 cytokines were increased remarkably include IL – 10 , IL – 4 , while the Th1 cytokine IFN – γ revealed no difference and they suggested that Th1 response decreased during CHC infection .

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قياس الاستجابة المناعية الخلوية من خلال تقديربعض السايتوكينات في المرضى العراقيين المعتلين مناعيا والمصابين بالتهاب الكبد الفيروسي نمط ب وج قبل وبعد العلاج (داخل وخارج الجسم الحي).

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الكلمات المفتاحية: الاستجابة المناعية الخلوية, السايتوكينات, التهاب الكبد الفيروسي نمط ب, التهاب الكبد الفيروسي نمط ج, انترفيرون كاما.

الخلاصة:

تم تبني هذه الدراسة لغرض تقييم بعض السمات السريرية والمناعية والفايروسية لدى المرضى العراقيين المصابين بالتهاب الكبد الفايروسي المزمن نمط (ب ,ج) وتحديد مدى استجابتهم للعلاج بعقار الانتفيرون, اللاميفودين, والريبافرين.

مجموعتان من المرضى المصابين بالتهاب الكبد الفيروسي المزمن تم تصنيفهم الى مجموعتين رئيسة : مجموعة المرضى قبل العلاج وتضمنت (25) مريضا مصابا بالتهاب الكبد الفايروسي المزمن نمط (ب) والذين اظهروا نتائج موجبة لفحص المستضد السطحي لفايروس الكبد نمط (ب) HBs Ag ولمدة اكثر من 6 اشهر و (40) مريضا مصابا بالتهاب الكبد الفايروسي المزمن نمط (ج). ومجموعة المرضى بعد العلاج وتضمنت (12) مريضا مصابا بالتهاب الكبد الفايروسي الزمن نمط (ب) واعدادهم 2,6,4 مريضا عولجوا بعقاقير اللاميفيدين, النترفيرون-الفا, والاثنان معا على التتالي و (27) مريضا مصابا بالتهاب الكبد الفايروسي المزمن نمط (ج) واعدادهم 3,13 ,11 مريضا عولجوا بعقاقيرالريبافرين, النترفيرون-الفا والاثنان معا على التتالي, وقد تم متابعة حالة المرضى لمدة 6 اشهر.

باستخدام تقنية الامتزاز المناعي المرتبط بالانزيم (ELISA), تم قياس مستويات النترلوكين 6 , 10, انترفيرون-كاما وTNF-الفا تم قياسها داخل وخارج الجسم الحي (راشح الخلايا اللمفاوية المنقاة المحفزة بالمشطر النباتيPHA) وتم مقارنتهم مع مجموعة السيطرة.لقد اظهر معدل مستوى IL-6 , IL-10 , TNF-الفا في المرضى المصابين بنمط (ب) ارتفاعا معنويا ($P<0.05$) عند مقارنته في المرضى قبل وبعد العلاج داخل وخارج الجسم الحي, في حين لا توجد فروقات معنوية في مستوى IFN-كاما ($P<0.05$) في المرضى قبل وبعد العلاج داخل وخارج الجسم الحي.

في المرضى المصابين بالتهاب الكبد الفايروسي المزمن نمط (ج) فان الدراسة اظهرت فروقات معنوية ($P<0.05$) في مستوى كل من IL-10,IFN-γ, TNF-α وIL-6 ما بين المرضى قبل وبعد العلاج داخل وخارج الجسم الحي, اظهرت الدراسة بان الفروقات ما بين المرضى ومجموعة السيطرة كانت معنوية عالية($P=0.000$) في مستوىIL-10 وكذلك تشير تلك الملاحظات الى سيادة السايتوكينات للخلايا المساعدة نوع-2 / والتي تزيد من بقاء الفايروس.