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## Some Immunological Aspects of Chronic Hepatitis B Virus Infection among Iraqi Patients

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#### Abstract

**Background:** Hepatitis B virus HBV infection is a serious public health problem in Iraq, and HBV is a major cause of acute & chronic hepatitis.

**Objectives:** The main target of this study is to estimate and evaluate the role of Interleukin -1 $\alpha$  and IL-8, in addition of the T helper cells CD $_4^+$  and cytotoxic – T-cells CD $_8^+$  in patients with chronic hepatitis B (CHB) compared with acute hepatitis B patients and apparently healthy individuals.

**Patients and methods:** This study was conducted from May 2010 to October 2010 in teaching laboratories of Medical City. involved 60 patients, (30) patients with acute HBV infection (22 male , 8 female) and (30) patients with chronic HBV infection (21 male, 9 female) referred to gastroenterology and hepatology teaching hospital & medical consultant department of Baghdad Teaching hospital ,compared with 20 apparently healthy individuals. Enzyme linked Immunosorbent assay technique (ELISA) was performed to estimate (IL- 1 $\alpha$  and IL-8) in 60 patients compared with (20) healthy control group .T cells (CD $_4^+$ ) and T (CD $_8^+$ ) cells were estimated by using indirect immunofluorescent assay ( IFA ) .

**Results:** The most incidence of HBV infection in males is more than in females with significant differences  $p = 0.0001$ . The mean level of (IL- 1 $\alpha$  and IL-8) increases in patients with (CHB) with significant differences compared with acute & healthy control groups ( $P = 0.001$ ).The ratio of CD $_4^+$  / CD $_8^+$  T cells was significantly decreased ( $P = 0.0001$ ) in CHB patients compared with acute hepatitis B patients, and healthy control group.

**Conclusions:** The result of this study indicates that the cytokines (IL-1 $\alpha$  –IL8) play an important role in the pathogenesis of chronic HBV infection. Also this study confirms that T (CD $_4^+$ ) cells and T (CD $_8^+$ ) cells play an important role in host defense against HBV.

**Keywords:** Chronic HBV infection, IL $\alpha$  - IL8, TCD $_4^+$  cells' TCD $_8^+$  cells.

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#### Introduction

Hepatitis B virus (HBV) is a serious global health problem, with 350 million suffering from chronic HBV infection. Approximately 15-40% of infected patients will develop cirrhosis, liver failure or hepatocellular carcinoma (HCC).<sup>[1]</sup>

The 10<sup>th</sup> leading cause of death worldwide, caused by chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).<sup>[2]</sup>

Most of acutely infected adults recover spontaneously and completely from infection, only a small fraction (3-5%) of HBV infected adults becomes chronic carriers with HBV surface antigen (HBsAg) <sup>[3]</sup>

The acute and chronic HBV infections are also comprised the health problems in Iraq. The prevalence of hepatitis B is 2–4 % among normal Iraqi population, as reported by Omer and Al-Douri, 1984<sup>[4]</sup>. Availability of highly effective vaccines and recommendation with new strategies of treatment, this percentage is reduced to 1.6 % in 2006 as reported by Ataallah <sup>[5]</sup>

Hepatitis B virus replicates in hepatocyte and it is a non cytopathic virus, so the liver damage is mainly mediated by the host immune response <sup>[6]</sup>. Cytokines play an important role in the defense against viral infections, both indirectly (through determination of the predominant pattern of host response), and directly (through inhibition of viral replication). Cytokines may also lead to liver damage. <sup>[7]</sup>

In patient with an acute HBV infection, specific CD $_4^+$  and CD $_8^+$  T-cell response are important for control of the infection <sup>[8]</sup>. However, if there is a defect in the acute response, HBV becomes chronic; in that case, the presence of an ongoing suboptimal

inflammatory response can activate the process of hepatic fibrosis. <sup>[9]</sup>

#### Aim of Study

1. Evaluate the role of certain cytokines (IL-1 $\alpha$  ,IL-8) in immunopathogenesis of chronic HBV infection compared to control group using ELISA technique .
2. Assess CD $_4^+$ cell/CD $_8^+$  cell ratio in acute & chronic HBV and determine their importance in controlling of HBV infection.

#### Subjects and Methods

##### Subjects

Sixty Iraqi patients with hepatitis B infection, (30) patients with acute HBV infection, whom age ranged (14-54) years, 22 males and 8 females, while the other (30) patients with chronic HBV infection, with age ranged from (18-65) years, 21 males and 9 females, were referred to gastroenterology and pathology teaching hospital and medical consultant department of Baghdad Teaching hospital . Twenty apparently healthy individuals were selected as a control group; their age and sex were matched.

##### Blood Sampling

2.5 ml of blood was transferred into plain tube to obtain serum.

This serum was used for cytokine estimation, the other 2.5 ml of blood was added into heparinized or EDTA tube for CD $_4^+$  and CD $_8^+$  estimation.

##### Laboratory Investigation

Serum cytokines (IL-1  $\alpha$ , IL-8) were measured by using ELISA technique while estimation of CD $_4$  and CD $_8$  ratio were done by Indirect immune

Fluorescent (IFA) technique using kits supplied by immunotech / Company –France.

### Estimation of IL-1 $\alpha$ & IL-8 by ELISA:

#### Procedure of the test:-

1. Standard was constituted to 1000 pg / ml with standard diluents buffer. A serial diluents of the standard (250, 125, 62.5, 15.6 and 0 pg / ml ) for IL-1 $\alpha$  and 2000,500,125,31.2 and 0 pg/ml for IL-8 were prepared from original standard.
2. One hundred  $\mu$ l of standard or sample was added per well, then 100  $\mu$ l of diluents 1 added into standard wells or 100  $\mu$ l of diluents 2 added into sample wells. The plate was incubated for 4 hours at (2 – 8) °C.
3. The contents of wells were discarded and washed three times with washing solution.
4. Two hundred  $\mu$ l of conjugate reconstituted was added into each well and incubated overnight at 18–25°C, and then washed 3 times.
5. Two hundred  $\mu$ l of substrate lyophilized was added into each well, followed by incubation for 15 minutes at 18 – 25°C with continuous shaking in the dark.
6. Fifty  $\mu$ l of stopping solution (H<sub>2</sub>SO<sub>4</sub>) was added in to each well and mixed gently.
7. Absorbance was measured by spectrophotometer at 450 nm within 2 hours.

### Assessment of CD<sub>4</sub><sup>+</sup>cell/ CD<sub>8</sub><sup>+</sup>cell by indirect IFA:

#### 1. Procedure of the test:-

##### A. lymphocyte Separation

The isopaque ficol lymph prep technique described by Nilson., *et al* .<sup>[10]</sup> was used for lymphocyte separation.

- \* 2.5 ml of heparinized blood was diluted 1:1 ratio in PBS and were layered over 4 ml of Ficoll and centrifuged for 20 min, at 3000 rpm at room temperature.
- \* The buffy coat layer was collected using Pasteur pipette, then cells washed 3 times with PBS for 10 min. at room temperature by centrifugation, first at 1000 rpm to separate the platelet then at 2500 rpm.
- \* Cells were then suspended in 0.5 ml of the PBS.

- \* Cell viability was determined by trypan blue 0.4%. This method is based on the principle that live (viable cell do not taken up certain dyes), whereas dead (non viable cells) do staining facilitates the visualization cell morphology.
- \* Cells were resuspended and adjusted to the concentration of  $2 \times 10^6$  cell/ml.
- \* 10  $\mu$ l of the cell suspension was applied to each well on teflon coated microscopic slide, air dried, fixed with acetone, foiled with parafilm and stored at -20°C until they used in immunostaining .

#### Procedure:-

- The parafilm was removed from the slides allowed to reach room temperature, and then 50 $\mu$ l of diluted anti- CD marker were added to each well incubated 30 minutes at (18–25) °C in humid dark container.
- The slide was washed in a slide jar containing PBS under continuous gentle agitation for 5 minutes.
- Fifty  $\mu$ l of conjugate diluted 1:160 in PBS were added to each well and incubated for 30 minutes at (18–25) °C in a humid dark container.
- The slides were washed in a slide jar containing PBS under continuous gentle agitation for 5 minutes.
- Slides were mounted by glycerol/PBS 70%. Covered by cover slips, then examined under UV microscope.

#### Cell counting:-

The fluorescent cell were counted under a fluorescent microscope with high power magnification, compared with 100 cells were counted in each spot under the same microscope but with light filter. This will give the percentage of cell with each marker.

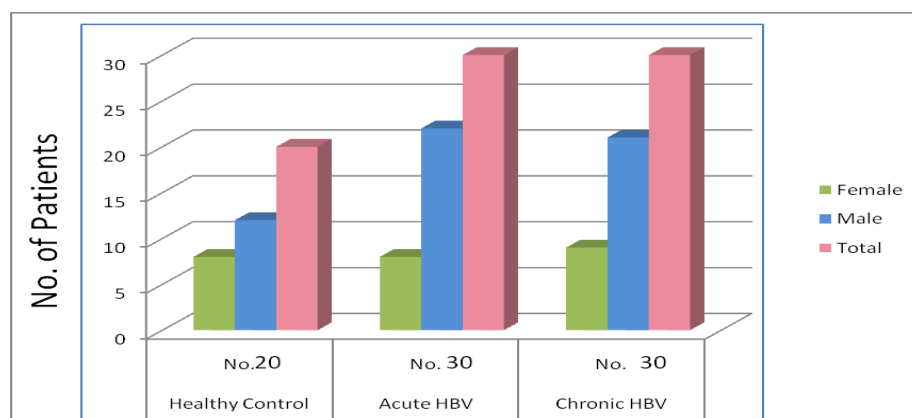
#### Results:

There is a significant difference between males and females of studied groups

P =0.0001 as showed in table (1) and figure (1)

Table (1) Gender distribution of studied groups

Gender	Studied groups					
	Healthy Control		Acute HBV		Chronic HBV	
	No.	%	No.	%	No.	%
Female	8	40	8	27	9	30
Male	12	60	22	73	21	70
Total	20	100%	30	100%	30	100%
P ( NOVA )	0.0001					

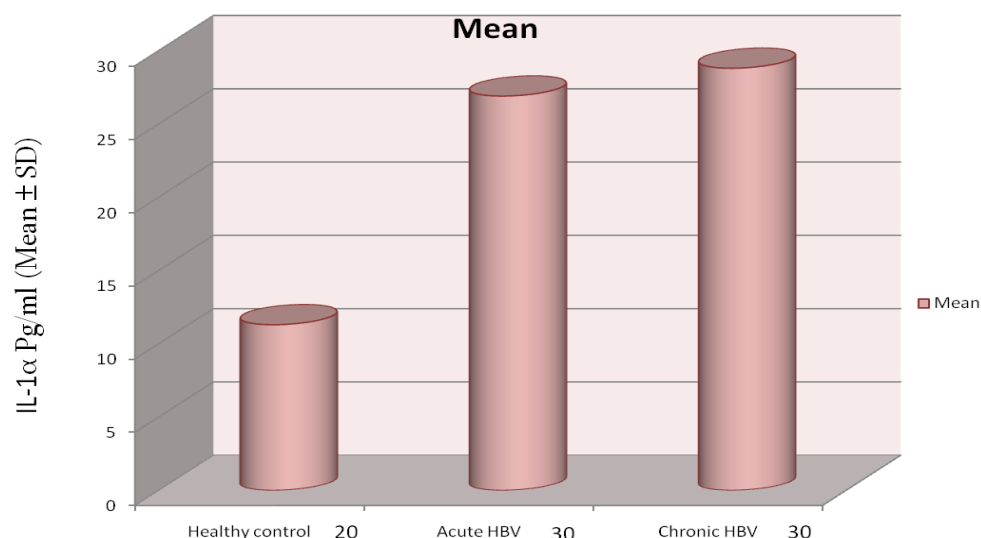


**Figure (1):** The gender distribution of the studied groups.

Levels of IL-1  $\alpha$  in the studied groups were revealed significant elevation among acute group  $26.9 \pm 13.01$  pg/ml while  $28.8 \pm 16.02$  pg/ml in chronic group compared with control group  $11.3 \pm 2.03$  pg/ml with  $p = 0.001$  as shown in table (2) and figure (2)

**Table (2):** The difference in mean serum IL-1 $\alpha$  levels (pg/ml) between the studied groups

Table ( 2 ): The difference in mean serum IL - 1α levels (pg/ml) between the studied groups				
	Study groups			
Values pg/ml	Healthy control	Acute HBV	Chronic HBV	P value
Minimum	8.7	10.	16	=0.001
Maximum	14	75	80.0	
Mean	11.3	26.9	28.8	
SD	2.03	13.01	16.02	
SD : Standard deviation				

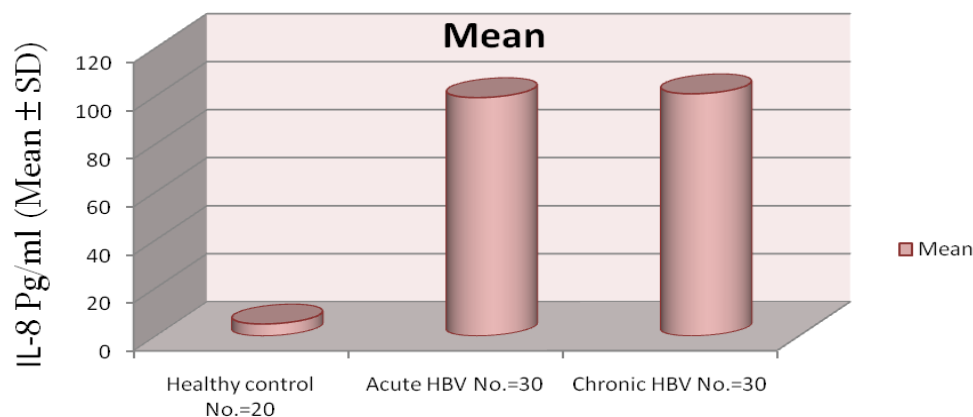


**Figure (2):** Mean of IL1 $\alpha$  levels (pg/ml) in studied groups.

Statistical analysis revealed that there was a significant elevation of IL-8 levels in acute & chronic HBV groups  $99.3 \pm 17$  pg/ml &  $100.9 \pm 53.7$  pg/ml, respectively, compared to control group  $5 \pm 2$  pg/ml with  $p = 0.001$  (Table 3) (fig 3)

**Table (3):** The difference in mean serum IL-8 levels (pg/ml) between the studied groups

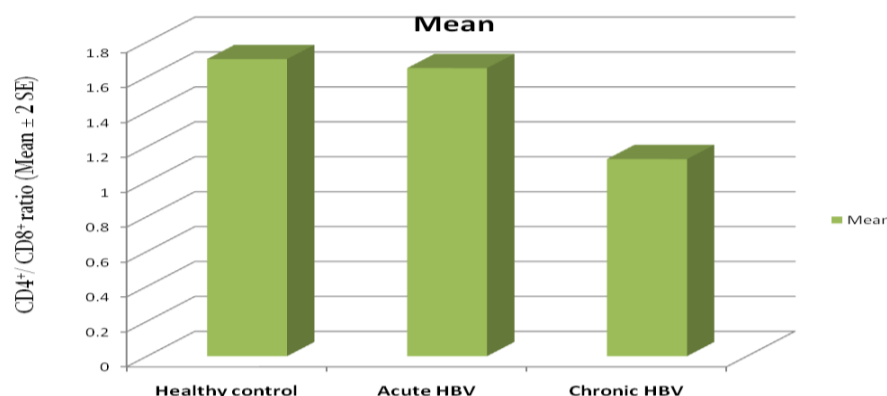
Values Pg/ml	Studied Groups			P value For mean
	Healthy control No.=20	Acute HBV No.=30	Chronic HBV No.=30	
Minimum	1.1	60	53	0.001
Maximum	16	135	350	
Mean	5	99.3	100.9	
SD	2	17	53.7	

**Figure (3):** Mean of IL-8 levels (pg/ml) in studied groups.

The ratio of CD4<sup>+</sup> Th cell / CD8<sup>+</sup> T Cytotoxic cell was estimated by using direct immunofluorescent technique. There is no significant difference in mean of CD4 cell / CD8 cell ratio among acute cases compared to the healthy subjects, while in chronic cases there is significant decrease ( $p=0.0001$ ) in CD4<sup>+</sup> cell / CD8<sup>+</sup> cell ratio ( $1.129 \pm 0.293$ ) compared with ( $1.65 \pm 0.24$ ) and ( $1.7 \pm 0.24$ ) in the acute cases and control group, respectively .as shown in table (4) and figure (4) .

**Table ( 4 ):** The difference in mean ratio CD4<sup>+</sup> cell / CD8<sup>+</sup> cell between the studied groups

Values	Study groups			P value
	Healthy control	Acute HBV	Chronic HBV	
Minimum	1.24	1.32	0.67	0.0001
Maximum	2.03	2.01	1.668	
Mean	1.702	1.65	1.129	
SD	0.24	0.24	0.293	

**Figure (4):** Mean of CD4<sup>+</sup> cell / CD8<sup>+</sup> cell ratio in studied groups.

## Discussion:

In this study 73% of acute patients were males and 27 % were females, with male predominance with ratio of males to females 2.7 : 1, while in chronic cases were males 70% and 30% females, with ratio of males to females 2.3:1 These results were agreed with Sherlock <sup>[11]</sup>.

Al – Shamary, 2004, detected a higher prevalence rate of HBV markers among males than females,<sup>[12]</sup>, on the other hand, Hassan, 2004 reported no different incidence of HBs Ag positive contacts in male or females <sup>[13]</sup>.

Possible explanation may be due to males may have a greater chance to come in contact with risk factor of HBV infection than female ,or alcohol intake being common in males, which may enhance the liver damage caused by HBV infection or may be due to the hormonal differences between them and ,their effects on the immune responses <sup>[11]</sup>.

The total number of lymphocyte in male and female is similar but there is a greater proportion of Natural Killer T-cell NK-T and TCR, cells in females, those make female normally tend to amount robust immune response. <sup>[14]</sup>

Cytokines act as a key role in regulation of the immune response to hepatitis B virus infection, both directly, through inhibition of viral replication or indirectly through determination of predominant pattern of the host response <sup>[15]</sup>.

Therefore, increased concentration of IL-1  $\alpha$  in acute and chronic groups compared to control groups which reflect the action of IL-1 $\alpha$  that enhance B and T cells activation, fibroblast proliferation and induce a hormonal immune response to HBV infection,<sup>[16]</sup>.

This finding is in agreement with Vlassopolas *et al.* 2003 who demonstrated the role of IL-1 $\alpha$  in response to HBV infection, reported that IL-1  $\alpha$  and other cytokines modulate type of immune response to HBV infection <sup>[17]</sup>.

The results were in agreement with the previous results reported by de Gastillo *et al.*, 2002<sup>[18]</sup>

Actually this finding is attributed to the production of IL-1 $\alpha$  by activated monocyte and macrophages<sup>[19]</sup>.

Also, Kolarski, *et al.* 2002, reported that IL-1 and other cytokines modulate liver metabolism in health and disease, physiological and pathological liver function, and the evolution of liver inflammation and injury to hepatic fibrosis and liver cirrhosis <sup>[20]</sup>.

On the other hand, Interleukin -8 is important cytokine associated with liver damage .It is a chemotactic factor of neutrophils and T- cells and play a role in hepatic injury in patients with Chronic hepatitis B –remarkable increase of IL-8 lead to accumulation of CD8 which get direct & immediate access to the target hepatocyte and the resident intrahepatic macrophages ,subsequently causing damage hepatocyte <sup>[21]</sup>.

In present study table (3) and figure (3) showed significant elevation of IL-8 serum level in acute group and chronic HBV group compared to healthy control.

This result is in consistent with Radkowskin *et al.*, 2004 who reported that IL-8 levels were elevated in patients with (HCC) and chronic hepatitis, they were concluded that IL-8 is increased in some patients with Liver disease, they play a significant role in host defense and disease <sup>[22]</sup>.

This result also supported by Masumoto *et al.*, 1998 who demonstrated IL-8 positive cells in the liver tissue of patients with (CHB), <sup>[23]</sup>

Interleukin – 8 is an extremely potent chemotactic factor for neutrophil enhance expression of adhesion molecules generation of oxygen radicals and release of lysosomal enzymes, also stimulate acute phase protein production, as a mechanism contributing to inflammation and local tissue damage,<sup>[24]</sup> in support with this hypothesis .Arend,2000. <sup>[25]</sup>

The current work indicates that IL-8 may account for some pathogenesis of disease progression seen in patients with CHB infection.

Assessment of CD<sub>4</sub><sup>+</sup> cell & CD<sub>8</sub><sup>+</sup> cell : Helper T-cells CD<sub>4</sub><sup>+</sup> are major function subgroups of T-cells .An antiviral cellular immune response of T cell CD<sub>4</sub><sup>+</sup> and T cell CD<sub>8</sub><sup>+</sup> is the important mechanism of hepatocyte injury induced by HBV ,the specific responses of CD<sub>4</sub><sup>+</sup> and CD<sub>8</sub><sup>+</sup> to the viral antigens is closely related with elimination of virus <sup>[26]</sup>.Helper T-cells play an important regulatory function by secreting of cytokines that can facilitate B- cells maturation .

Cytotoxic T cells CD<sub>8</sub><sup>+</sup> can kill infected cells by direct contact .They also secrete cytokines which have the potential to inhibit HBV replication <sup>[6]</sup>.

The present study revealed that CD<sub>4</sub><sup>+</sup> cell /CD<sub>8</sub><sup>+</sup> cell ratio among the acute HBV group as compared to the healthy control subjects were statistically non – significant .Actually, this finding is in accordance with report by Eyigum *et al.* ,1999 who found that longitudinal analysis of 15 patients failed to show any consistent pattern of changes in the immunophenotypic profile during acute flare and seroconversion phase, Suggesting an active role of T-cells subpopulation in maintaining low virus levels during seroconversion<sup>[27]</sup>.

The decreased of CD<sub>4</sub><sup>+</sup> cell and increased CD<sub>8</sub><sup>+</sup> cell in chronic HBV patients resulted a significant decrease in CD<sub>4</sub><sup>+</sup>/ CD<sub>8</sub><sup>+</sup> ratio p= 0.0001 when compared with ratio observed in healthy control group .

The results of our study is in agreement with Genel *et al.*, 2003 who reported increase the frequency of CD<sub>8</sub> subsets in chronic hepatitis cases<sup>[28]</sup>.

This results attributed to the HBs Ag overload present in chronically infected subjects ,when HBV specific CD<sub>8</sub><sup>+</sup> responses is unable to control viral

replication and causing the recruitment of non virus specific T-cells<sup>[29]</sup>.

Moreover, the results of the present study are consistent with Lebray *et al.*, 2003 who found that CD4<sup>+</sup> cells decreased in chronic HB cases<sup>[30]</sup>.

Our study is in agreement with Chen *et al.*, 2005 who discovered the level CD4<sup>+</sup> cell was low whereas the level of CD8<sup>+</sup> cell was higher in patients with chronic HB compared with normal persons suggesting that disorder of cellular immune function and pathologic damage occur in chronic hepatitis B patients<sup>[26]</sup>.

On the other hand, our study is disagreed with Eyigun *et al.*, 1999, who reported that CD4<sup>+</sup> cell/ CD8<sup>+</sup> cell ratio in chronic HBV cases was not reduced<sup>[27]</sup>.

This may reflect redistribution of CD4<sup>+</sup> cells to the liver and CD4 cells are important subset because the cells needed to support the production of neutralizing antibodies by HBV specific B-cells<sup>[31]</sup>.

Also high antigen loads after viral spread may induce a general activation of all available virus – specific CD4<sup>+</sup> T- cells. These cells then would die of activation induced apoptosis proceeded by a period of anergy Ciurea *et al.*, 2001 presented evidence that the failure of HBV patient to mount effective humoral response correlates with the loss of CD4<sup>+</sup> T-cell responsiveness during the viral persistence<sup>[32]</sup>.

### Conclusions:-

- \* Cytokines play an important role in the pathogenesis of HBV infection, this study provides a significant elevation in serum levels of IL-1α and IL-8 in patients with chronic HBV infection .
- \* Adaptive cellular immune response plays important role in host defense against HBV infection, and successful control of HBV infection, requires T (CD4<sup>+</sup>) cells and T (CD8<sup>+</sup>) cells that due to the hepatotropic nature of HBV in patients with chronic HBV .The ratio of CD4<sup>+</sup>cell/ CD8<sup>+</sup> cell was significantly decreased in patients with chronic HBV infection .

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