

Changes in Levels of Interleukin-8 in the Serum of Patients with Hepatitis B Virus Infection Correlate with HBe Seroconversion and Increased Levels of Interleukin-8 Indicate Resistance to IFN-alpha Therapy

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Abstract

Chronic hepatitis B (CHB) is a common and serious worldwide infectious disease of the liver that is caused by the hepatitis B virus (HBV). The number of chronically infected has been estimated to be 400 million people worldwide. Areas with high prevalence of HBV include China, Southeast Asia and Africa, where approximately 10% of the population are chronic carriers. Between 25 and 40% of those chronically infected with HBV are predisposed to developing cirrhosis of the liver and hepatocellular carcinoma (HCC). Mortality from HBV is estimated at 1 million deaths each year. Clearance of hepatitis B e-antigen (HBeAg) and loss of HBV DNA indicate transition to a state of low-level viral replication that is accompanied by biochemical remission of liver disease and prolonged survival.

Keywords: Interleukin-8; IFN-alpha therapy

Introduction

Chronic hepatitis B (CHB) is a common and serious worldwide infectious disease of the liver that is caused by the hepatitis B virus (HBV). The number of chronically infected has been estimated to be 400 million people worldwide [1]. Areas with high prevalence of HBV include China, Southeast Asia and Africa, where approximately 10% of the population are chronic carriers [2]. Between 25 and 40% of those chronically infected with HBV are predisposed to developing cirrhosis of the liver and hepatocellular carcinoma (HCC) [3]. Mortality from HBV is estimated at 1 million deaths each year [4]. Clearance of hepatitis B e-antigen (HBeAg) and loss of HBV DNA indicate transition to a state of low-level viral replication that is accompanied by biochemical remission of liver disease and prolonged survival [5,6]. The estimated yearly proportion of spontaneous HBeAg seroconversion is 2-15%, depending on such factors as age, serum alanine aminotransferase (ALT) concentrations and HBV genotype [7]. HBV replication is important for liver injury and disease progression and therefore the main aims of treatment are to suppress the virus to achieve HBeAg seroconversion or undetectable viral DNA levels, or both [8]. Interferon alpha and pegylated interferon alpha (peg IFN-alpha) have immunomodulating and viral suppression activity; the response rate is 30-40% in HBeAg-positive patients, with a risk difference of 23-25% against untreated controls [9,10]. The molecular mechanisms responsible for the ineffectiveness of INF- α -treatments in the majority of patients with CHB are not known. It has been shown that hepatitis C virus proteins induce the CXC chemokine interleukin-8 (IL-8), leading to partial inhibition of the Interferon-induced antiviral response and resistance to IFN therapy [11,12]. Recently, similarly to what was observed in hepatitis C virus infection, an inhibition of IFN-alpha signalling was found in cells expressing HBV proteins and in liver biopsies of patients with CHB [13]. Elevated IL-8 levels have been found in serum samples from HCC and chronic active hepatitis (CAH) associated with HBV infection [14]. Patients with HBeAg-negative CHB with liver inflammation had significantly increased levels of both IL-8 and IFN-alpha compared with a healthy control group, whereas patients with CHB without evidence of liver inflammation had no significant increased levels of IL-8 and IFN-alpha compared with healthy donors [15]. Because HBeAg is associated with immune tolerance in transgenic

mice [16,17], it has been suggested that this might be related to HBeAg suppressing the host innate response, including the production of cytokines and chemokines. Recently, it was confirmed that the IL-8 gene was significantly down-regulated in HBeAg-positive HepG2 cells (which stably expresses HBeAg) [18], which supports this notion.

The chemokine CXCL8/ IL-8 is a low molecular weight (84000-dalton) protein synthesised and secreted by a variety of immune and non-immune cells, including macrophages and monocytes, endothelial cells, fibroblasts, hepatocytes, polymorphonuclear leucocytes and T- and B-lymphocytes [19,20]. The chemokine IL-8 is produced *in vitro* in response to Interleukin 1 and tumour necrosis factor-alpha (TNF- α) [21]. IL-8 is also a potent chemoattractant for human neutrophils, monocytes, T lymphocytes and natural killer cells (NK cells) [22]. The transcription of CXCL8 is induced rapidly by virus infection, where IL-8 plays a causative role in acute inflammation and tissue destruction [23,24]. However, circulating levels of IL-8 in patients with HBeAg-positive CHB have been scarce and no longitudinal studies have been done to determine whether IL-8 regulation is associated with liver injury, viremia, HBeAg or HBsAg seroconversion in patients with acute HB or CHB. Thus, as part of a study of chemokines/cytokines and HB, we sought (1) to determine plasma values of IL-8 in patients with different clinical manifestations of HB and (2) to analyse the correlation between presence of circulatory levels of IL-8 and the levels of HBV DNA, ALT and serological response during the natural course of acute HB or IFN-alpha-induced HBeAg seroconversion in patients with CHB.

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Materials and Methods

Study population

Patients included 13 HBeAg-reactive and 43 anti-HBe-reactive patients with chronic HBV infection, 13 asymptomatic carriers of HBsAg (ASC) and 13 patients with naturally acquired immunity to HB. In addition, 43 healthy blood donors lacking hepatitis A-E markers were included as controls.

Treatment

Nineteen patients with chronic HBV infection (HBeAg reactive) were followed consecutively before, during and after treatment. Three patients were given prednisolone orally (40 mg daily for 2 weeks and 20 mg daily for another 2 weeks), followed by a 2-week rest without treatment and then treated with recombinant IFN- α -2b (IFN, Intron A, donation from Schering-Plough, Division of Essex Asia Ltd., Hong Kong). The patients were given 5 million international units of IFN-alpha during the first 2 weeks and thereafter 3 times per week for 14 weeks. Three patients were treated with Chinese IFN-alpha alone without a preceding course of prednisolone. These patients were hospitalised at Jing An Central District Hospital, Shanghai, China. Five patients were treated with prednisone for 6 weeks and thereafter with IFN- α (Intron A) three times per week for 4 months. These patients were hospitalised at the Memorial Hospital, Warsaw, Poland. Eight patients were treated with Wellferon (Glaxo, Wellcome) for 12 weeks at Sahlgrenska Hospital, Gothenburg, Sweden. Follow-up times were from 6 to 18 months. The clinical trials were approved by the Drug Administration Committee.

Response

Different criteria for response were used: a virological response was represented with viremia levels above or below 10^6 or above or below 10^5 copies/mL. A biochemical response was represented by an indexed aminotransferase (ASTi) value in relation to the upper limit of normal. Thus, a biochemical response was represented by an ASTi below 1.0. A serological response was defined as either confirmed loss of HBeAg (HBe response) or as confirmed seroconversion to anti-HBe (anti-HBe response).

Serology

Swedish serum samples were analysed for HBV markers by commercially available test systems (Abbott Laboratories, Chicago, Ill, USA). Chinese sera were tested by HBV markers using commercial enzyme-linked immunosorbent assay (ELISA) test kits (Shanghai Medical Reagent Centra).

Transaminase levels were monitored during treatment by routine screening procedures. HBV DNA serum levels were measured by a chemiluminescent molecular-hybridisation assay (Digene™ - Diagnostics). The sensitivity of the Digene assay is 5 pg/mL, which is equivalent to 2.87×10^5 genomes/mL. If samples were negative by the Digene assay, they were re-analysed using a polymerase chain reaction (PCR)-based assay (Cobas Amplicor HBV Monitor™ test; Hoffman La Roche). Professor G. Leroux-Roels at the Center for Vaccinology, Ghent University, Ghent, Belgium, performed these HBV DNA measurements.

Measurement of IL-8

Serum samples and culture supernatants were analysed for IL-8 content by a commercially available IL-8 ELISA kit (R&D Systems Europe Ltd.) and performed according to the manufacturer's suggestions. The limit of detection was 12.5 pg/ml.

Antigens

Purified HBsAg was provided by Dr M Einarsson, Research Department, Biochemistry, AB KabiStockholm, Sweden. Yeast-derived HBsAg (pre-S2+S) was provided by Dr. RW Ellis (Merck Sharp and Dohme Research Laboratories, West Point, PA, USA). HBcAg was provided by Dr. I Cayzer (Wellcome Diagnostics, Beckenham, England).

In vitro culture conditions

Peripheral B- or T-cells from two ASC and one HB-immune individual were co-cultured with autologous monocytic cells (5%), with or without antigen (HBsAg, HBcAg, recombinant S+preS (0.1-100 ng/ml)) for 1-6 days. The cell concentration of each fraction was adjusted to 1×10^6 cells per millilitre and cultured in RPMI 1640 (Biocult Laboratories, Paisley, UK) supplemented with antibiotics, glutamine and 2% heat-inactivated foetal bovine serum. B-, T- and monocytic cells were highly purified by gradient centrifugation, rosetting and adherence steps.

Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). For clinical study and the *in vitro* experiments, statistical significance was evaluated using the paired Student's t-test. Differences between HBV patients and healthy controls were tested with Student's t-test for unpaired samples. The correlation between serum IL-8 and HBV DNA was computed using Spearman's correlation test.

Results

We first measured the presence of IL-8 in circulation by a commercially available IL-8 enzyme-linked immunoassay in healthy blood donors (n=43), patients with naturally acquired immunity to HB (n=13), ASC individuals (n=13), anti-HBe patients (n=43) and HBeAg-reactive patients with CHB (n=13) (Figure 1, Table 1).

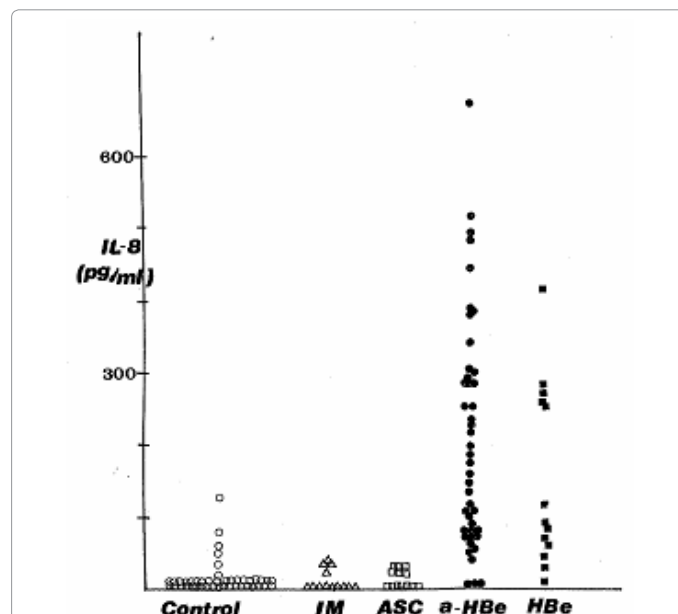


Figure 1: Serum levels of IL-8 in different groups of patients
Serum levels of IL-8 (pg/ml) were plotted for each individual in the healthy blood donor (control, open circles), HB-immune (IM, open triangles), ASC (open squares), anti-HBe reactive (a-HBe, filled circles) and HBeAg reactive (HBe, filled squares) group.

Groups	Number	IL-8 (pg/ml) (mean ± SEM)	Probability*
Healthy controls	n=43	9.3 ± 3.8	
HB-Immune	n=13	8.4 ± 3.6	ns
ASC (Asymptomatic HBsAg carriers)	n=13	13.7 ± 3.8	ns
anti-HBe reactive (HBV-DNA <10 ⁴ copies/ml)**	n=23	191 ± 30	p<0.0001
anti-HBe reactive (HBV-DNA >10 ⁴ copies/ml)**	n=20	223 ± 39	p<0.0001
HBeAg reactive chronic HB infection	n=13	149 ± 35	p<0.0001

*Healthy control group compared with the other groups

**Quantification of HBV-DNA in serum was performed by the Cobas Amplicor HBV Monitor TM test

ns= not significant

Table 1: IL-8 serum reactivity in different groups of patients.

Markers	RESPONDERS (n=11)	NON-RESPONDERS (n=8)	Probability
IL-8 (pg/ml, mean ± SEM)	34 ± 4	139 ± 34	p<0.005
IgM anti-HBc (S/CO)	1.7 ± 0.4	3.7 ± 1.8	ns
ALT (ukat/L)	85 ± 20	154 ± 43	ns
HBV-DNA (genomes/ml)	7.9 ± 2.8×10 ⁷	14.4 ± 9.5×10 ⁷	ns

S/CO= S/cut-off

ns=not significant

Table 2: Comparison between pre-treatment levels of serum IL-8 in hepatitis B in responders compared with non-responders patients.

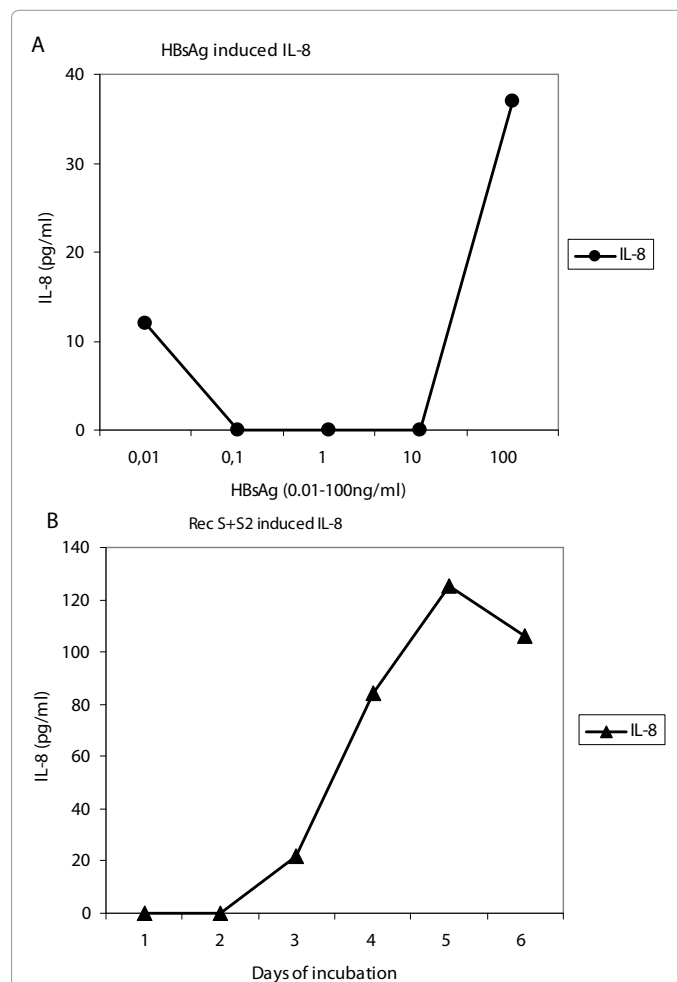
The mean levels of IL-8 in sera did not significantly differ between the healthy controls (9.3 ± 3.8 pg/ml, mean ± SEM), the HB-immune patients (8.4 ± 3.6) and the ASC patients (13.7 ± 3.8 pg/ml). In contrast, when HBV-infected patients were stratified to HBeAg status and HBV DNA levels, anti-HBe (HBV DNA <10⁴ copies/ml, 191 ± 30 pg/ml p<0.0001), anti-HBe (HBV DNA >10⁴ copies/ml, 223 ± 39, p<0.0001) and HBeAg-reactive (149 ± 35 pg/ml, p<0.0001) patients with symptomatic chronic HBV infection had significantly elevated levels in circulation compared with the healthy control group (9.3 ± 3.8 pg/ml). In HBeAg-positive patients with CHB no correlation was found between the levels of serum IL-8 and liver disease activity as measured by ALT (R=0.24) (data not shown).

Table 2 shows the basal levels of IL-8 in serum in comparison with three traditional pre-treatment markers of response in the 19 individuals who were treated grouped by the response to therapy. Patients with a sustained response to treatment exhibited significantly decreased IL-8 levels (34 ± 4 pg/ml, mean ± SEM) compared with non-responders (139 ± 34, p<0.005). In addition, the responders had significantly lower levels of IL-8 than patients who were not treated (p<0.001). None of the other pre-treatment markers tested could discriminate the responder from the non-responder group in this small cohort of patients (Table 2). The positive predictive value (PPV) of IL-8 serum levels below 69 pg/ml (mean value ± 2.5 SD) in determining a virological response was 92% and the negative predictive value (NPV) was 100%. These data demonstrate that chronic HBV infection is associated with elevated levels of IL-8 in serum and that high levels of IL-8 are strongly associated with lack of a therapeutic response to IFN-alpha treatment (p<0.001).

Next, we examined whether IL-8 secretion could be antigen specifically induced in peripheral blood mononuclear cells *in vitro* that were obtained from individuals sensitised for HBV *in vivo*. Both HBsAg and HBeAg induced IL-8 secretion in cell cultures of autologous B/Mo cells, T/Mo cells or T/B/Mo cells in a dose-dependent manner as demonstrated in Figures 2a, 3a and 3b. When analysing the kinetics of chemokine IL-8 production in Mo/B-cells in response to recombinant

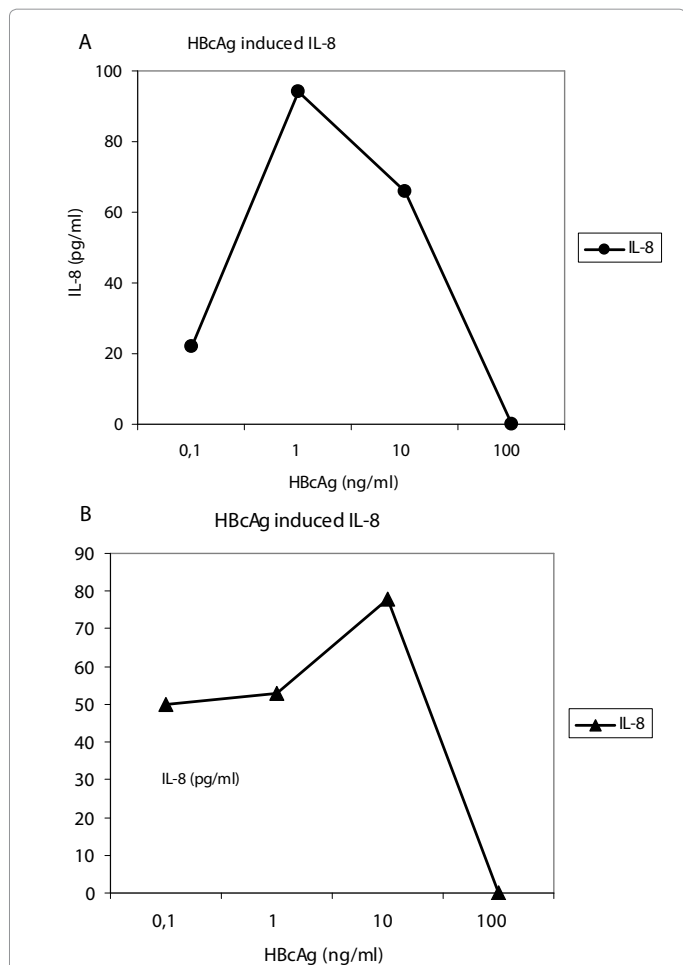
S+S2 stimulation, a time-dependent secretion of the chemokine was demonstrated. Maximal IL-8 secretion *in vitro* was obtained after 5 days of co-cultivation (Figure 2b). These data suggest that hepatitis B surface antigen-derived antigen and core-derived proteins can be immunogenic at the T- and B-cell level in hepatitis B-sensitised individuals and induce production of the chemokine CXCL8.

Patients (n=10) with acute HB infection were consecutively followed and assayed for the kinetics of serum IL-8 during the acute phase of the disease. Figures 4 and 5 demonstrate that the IL-8 levels were high during the acute phase of the infection and declined during the resolution of the disease. The peak response of IL-8 was always preceded by the peak level of the transaminases (Figure 4) and the reduction of HBV DNA (Figure 5). Moreover, the peak level of IL-8 coincided with seroconversion of HBe and HBsAg. In patients in whom the time interval between the HBe and the HBsAg seroconversion was lengthy a biphasic pattern of IL-8 secretion was observed, with one peak coinciding with the HBe seroconversion and the second peak with the HBsAg seroconversion (Figures 4 and 5). These data demonstrate



Figures 2a and 2b: Hepatitis B surface antigen induced IL-8 secretion *in vitro*

Peripheral B- and monocytic- cells from one ASC-A (2a) were cultured with 0.01-100 ng/ml HBsAg for 5 days. Peripheral T-cells and monocytic cells from one HB-immune (2b) were cultured with 100ng recombinant S+S2 antigen/ml for 1-6 days. Culture supernatants were analysed for IL-8 content by ELISA. IL-8 levels (pg/ml) in antigen stimulated cultures are corrected for backgrounds in cultures without antigen.



Figures 3a and 3b: Hepatitis B core antigen induced IL-8 secretion *in vitro* Peripheral T/B- and monocytic cells from one ASC-B individual were cultured with 0.1- 100 ng/ml HBcAg at a ratio of T/B of 8:1 (Figure 3a) or 0:1 (Figure 3b) for 5 days. Culture supernatants were analysed for IL-8 content by ELISA.

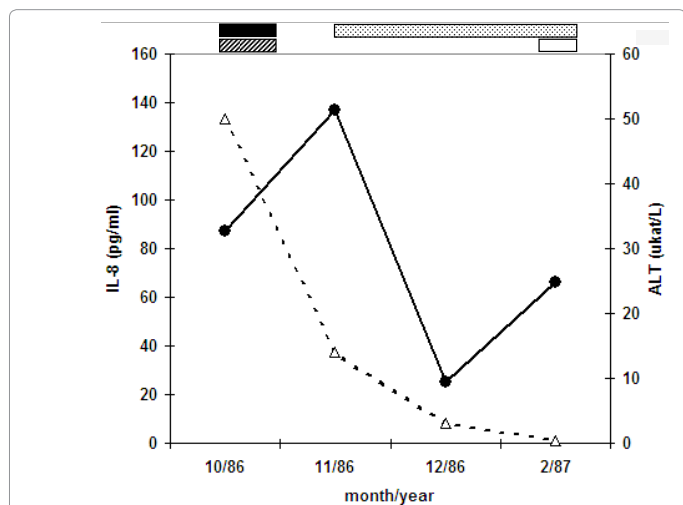


Figure 4: Serological profiles of IL-8 and ALT during acute HB infection. IL-8 (pg/ml; filled circles) and ALT (ukat/l; open triangles) levels were measured in sera from one patient with acute HB infection. The X-axis gives month/year. HB serology is given at the top of the figure. Solid bar=HBe positive, hatched bar=HBs positive, spotted bar=anti-HBe positive and open bar= anti-HBs positive.

that the chemokine CXCL-8, which is capable of suppressing the anti-viral effect of type I IFN and influencing NK cell activation [25], is significantly elevated and detectable in serum after a decrease in viremia and normalisation of transaminases during acute hepatitis B.

CXCL-8, HBV DNA and transaminases in serum were measured consecutively in patients with chronic HBV infection before, during and after treatment. Examples are given for two prednisolone/IFN-alpha-treated patients in Figures 6 and 7. The presence of IL-8 in circulation was dynamic during treatment. One patient had a marked increase of IL-8 secretion during the steroid arm of therapy (Figure 7), whereas

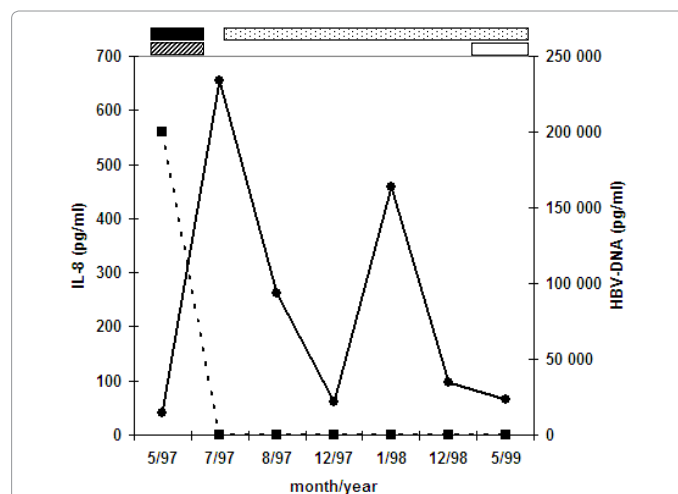
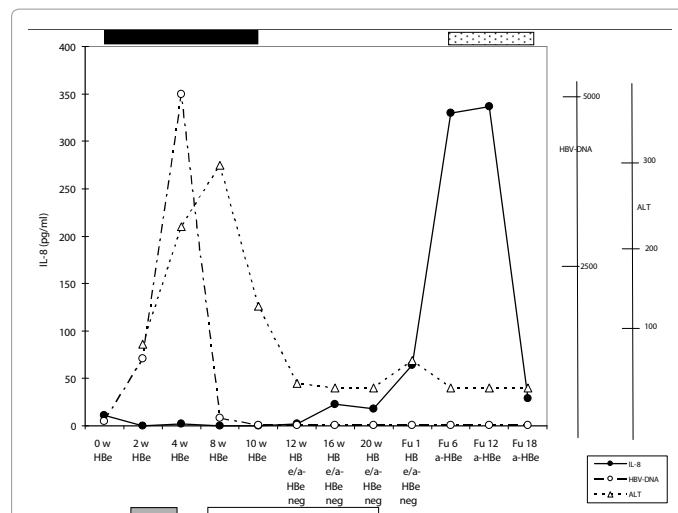
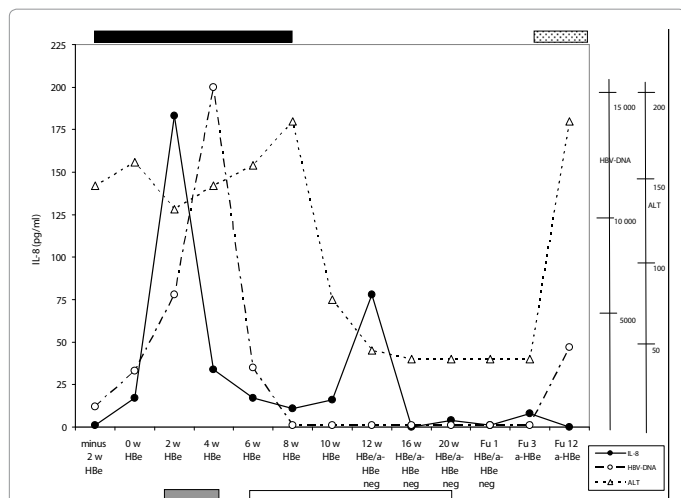


Figure 5: Serological profiles of IL-8 and HBV-DNA during acute HB infection IL-8 (pg/ml; filled circles) and HBV-DNA (pg/ml; filled squares, dotted line) was measured in sera from one patient with acute HB infection. The X-axis gives month/year. Serum HBV-DNA was quantified by HBV-DNA Digene and given as pg/ml (1pg=2.87x10⁵ genomes/ml). HB serology is given at the top of the figures, as indicated in Figure 4.



Figures 6: Serological profile of IL-8 during prednisolone/interferon alpha treatment IL-8 (pg/ml; filled circles), HBV-DNA (pg/ml; open circles) and ALT (ukat/l; open triangles) were measured in sera from two patients with chronic HB infection. The X-axis gives weeks (2-20w) after start of treatment and months of follow-up (Fu1-Fu18) after end of treatment. HB serology is given at the top of the figures. Solid black bar=HBe-positive and spotted bar=anti-HBe positive. At the bottom of the figures, the grey filled bar=time of prednisolone and open bar= time of interferon-alpha treatment.



Figures 7: Serological profile of IL-8 during prednisolone/interferon alpha treatment

IL-8 (pg/ml; filled circles), HBV-DNA (pg/ml; open circles) and ALT (ukat/l; open triangles) were measured in sera from two patients with chronic HB infection. The X-axis gives weeks (2-20w) after start of treatment and months of follow-up (Fu1-Fu18) after end of treatment. HB serology is given at the top of the figures. Solid black bar=HBe-positive and spotted bar=anti-HBe positive. At the bottom of the figures, the grey filled bar=time of prednisolone and open bar=time of interferon-alpha treatment.

the other patients' IL-8 secretion exhibited corticosteroid insensitivity. Detectable levels of IL-8 were always measured after the reduction of HBV DNA and transaminase levels. Note that both patients exhibited an increase in HBV DNA levels during the prednisolone arm of therapy and a marked decline after prednisolone withdrawal and a further ALT decrease and normalisation after starting IFN-alpha treatment. The reduction of HBV DNA and transaminases was followed by HBeAg seroconversion to anti-HBe. Similarly to some patients with acute HB, the IL-8 peak response was biphasic with one peak at the loss of HBeAg and/or HBsAg and the second peak response at the time of the emergence of anti-HBe and/or anti-HBs reactivity.

Three HBeAg-reactive patients with chronic HB infection were consecutively followed over a 9-year period during which spontaneous HBe/anti-HBe seroconversion was observed. The serum levels of IL-8 and ALT fluctuated during the observation period. Peak responses for IL-8 were noted before the HBeAg/anti-HBe and HBs/anti-HBs seroconversion with reduced levels thereafter (Figure 8).

Discussion

CXCL8/IL-8 can be induced by bacteria, bacterial products, viruses and viral products [26]. We demonstrated a significant increase in the levels of IL-8 in HBV-infected patients compared with levels in uninfected individuals and patients with naturally acquired immunity to hepatitis B. Similarly, IL-8 levels in serum have been found to be significantly higher in patients with acute and chronic hepatitis B virus infection [27] and IL-8 production has been shown to be correlated with increased inflammation in inflammatory bowel disease [28]. In addition, IL-8 levels are associated with the severity of tuberculosis [29]. Finally, IL-8 increased with exacerbation of liver damage [27], suggesting that chronic induction of IL-8 may cause pathology in CHB as in the case of chronic illnesses, such as arthritis [30], Helicobacter pylori infection [31] and HIV infection [32].

Previous studies have shown that protein antigen can induce IL-8

in a time-dependent manner in peripheral blood mononuclear cells [33]. We found that IL-8 was induced specifically by HBV envelope and core-derived proteins *in vitro* in T- and B-lymphocyte cultures obtained from individuals with acquired immunity to HB and ASC. Kinetic studies have revealed that IL-8 production by T- and B-cells *in vitro* is relatively long-lived, as evidenced by the profound accumulation of IL-8 in cell culture supernatants obtained for up to 6 days of cell cultivation. Our finding of the time kinetics of IL-8 release is similar to that of CMV infection of human peripheral blood mononuclear cells, demonstrating that the IL-8 protein level reached a plateau on days 3-5 [34]. The expression of IL-8 from T- and B-cells was highly regulated by the dose of antigen used for stimulation. These results are in agreement with previous studies suggesting that human B lymphocytes can express chemokines that belong to the CxC and CC gene superfamilies [35]. Our results also suggest that IL-8 expression is important for B lymphocytes undergoing Ag-dependent responses and that its secretion may facilitate cellular interactions and be important for the attraction of cognate T-cell help [20].

Several studies have examined the flares associated with HBeAg seroconversion and those found in patients undergoing therapy, demonstrating increases in serum IL-12 [36] and activation of TH1 immunity [37,38]. We have recently demonstrated that there was a significant increase in IL-18 production after the ALT flare in IFN-alpha-treated patients with CHB in which the peak of IL-18 preceded or coincided with the time of HBe seroconversion in patients who cleared the virus [39]. In our study we longitudinally investigated the fluctuations of IL-8 in serum obtained from patients with CHB during IFN-alpha therapy. We observed that high serum levels of IL-8 were temporally associated with HBe and HBs seroconversion rather than with fluctuations in HBV DNA levels or ALT flares.

Many independent studies indicate that several CXC chemokines are expressed together with IL-8 in a variety of cells in response to viral infection [40]. Moreover, data suggest that, in addition to their well-defined role in antibody production, B cells may regulate immune responses to infectious pathogens through their production of cytokines [41].

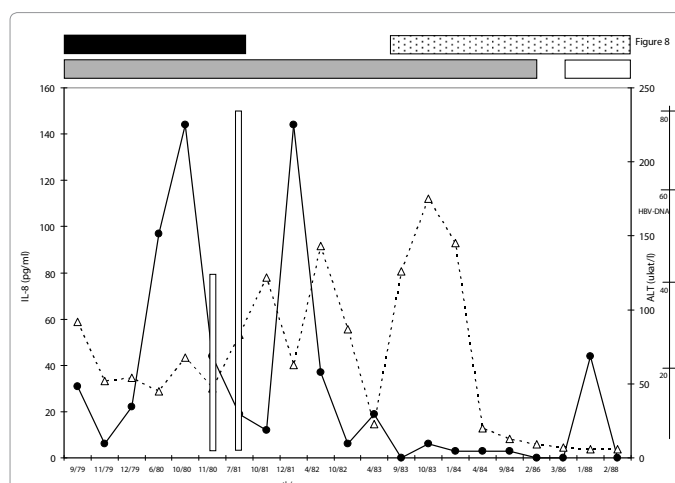


Figure 8: Serological profiles of IL-8 and ALT in one chronic HBeAg-reactive patient who spontaneously seroconverted

IL-8 (pg/ml; filled circles) and ALT (ukat/l; open triangles) levels were measured in serum samples from one HBeAg-reactive patient over a 9-year period. X-axis gives month/year. HBV-DNA (genomes/ml) was measured occasionally as indicated by upright open bars. HB serology is given at the top of the Figures as indicated in Figure 4.

We therefore speculate that treatment with IFN-alpha, in addition to activating an anti-viral effect and a pathway to hepatocyte damage also via IL-8 secretion, contributes to chemotaxis of T-cells [42] to participate during Ag-dependent humoral responses. The *in vivo* production and secretion of IL-8 by antigen-driven B-lymphocytes could result in the generation of a gradient to attract antigen-specific T-cells into the histological germinal centres in which effective B- and T-cell interactions can take place [20] and specific antibody production occur. This notion is supported by the finding of a temporal association of the secretion of high levels of IL-8 in human neonates and the development of both humoral- and cell-mediated immunity during acute CMV infection *in utero* [43]. Taken together, these findings represent an indication of an immunoregulatory function exerted by IL-8 on B- and T-cells and that IFN-alpha therapy can modulate the production of IL-8 in CHB patients.

We found that patients who were non-responders to IFN-alpha therapy had significantly higher pre-treatment levels of IL-8 in serum compared with patients who were responders. Patients having an IL-8 level in circulation below 69 pg/ml had a PPV and NPV for achieving sustained response after treatment of 92 and 100%, respectively. Further studies are needed to identify and validate optimal cut-off levels for IL-8 in pre-treatment sera in order to identify those patients with CHB that could benefit from IFN-alpha therapy.

For HCV, it has been shown *in vitro* that the core protein activates the IL-8 promoter [44] and HCV-E2 up regulates IL-8 production [45]. Protein X of the HBV (HBV-X) can induce the IL-8 gene as evidenced by the enhanced IL-8 mRNA expression and IL-8 production observed in HBV-X-transfected cells [46]. Recently, the expression of HCV-NS5A protein in human cells was observed to induce IL-8 and enhanced IL-8 was associated in a dose-dependent manner with inhibition of the antiviral effects of IFN-alpha *in vitro* [11,25,47]. Taken together, the capacity of HBV to specifically induce enhanced levels of IL-8 may represent a strategy similar to HCV to evade the host immune system and also inhibit the effect of IFN-alpha-based antiviral therapy and thereby aggravating the viral infection [48,49].

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Author Disclosure Statement

No competing financial interests exist.

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