IL-10 AND IFN-γ IN PATIENTS WITH CHRONIC HEPATITIS B TREATED WITH ENTECAVIR – A CROSS-SECTIONAL PILOT STUDY

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ABSTRACT

Hepatitis B virus (HBV) is a major global health problem. An altered immune response against HBV is a core feature of chronic hepatitis B (CHB). Antiviral treatment with nucleoside or nucleotide analogues (NUCs) inhibits virus replication and partly restores HBV-specific immunity. The mechanism of action of NUCs is not completely understood, nor are the differences in the patient-specific immune responses to treatment.

The aim of this study was to determine if the biochemical, serological and virological response to Entecavir in CHB NUCs treated patients correlate with the immune response, as tested by IFN-γ and IL-10 production.

Methods. IL-10 and IFN-γ values were measured in 34 patients with CHB of whom 20 were treated with Entecavir, and studied in relation to the demographic features and response to treatment.

Results. Serum IL-10 levels were higher in Entecavir group, with a tendency for higher titers in treatment non-responder group. IFN-γ levels were higher in both treated and naïve CHB patients, with slightly higher levels in responder patients.

Conclusions. Treatment with NUCs seems to reinvigorate the immune system in CHB patients. IL-10 may be a bi-functional cytokine, increasing in all treated patients. IL-10 and IFN-γ may be inversely associated with prognosis.

Keywords: chronic hepatitis B, IL-10, IFN-γ, nucleoside or nucleotide analogues, immune response

Hepatitis B virus (HBV) is a member of the family Hepadnaviridae and a hepatotropic non-cytopathic DNA virus. HBV infection is a major global health problem (1,2). Vaccines against HBV are available since 1982 and had an important public health impact by decreasing infection rates, mortality from infant fulminant hepatitis, chronic carrier rates and incidence of hepatocellular carcinoma (HCC) in children (3-5). However, despite the effective vaccines, a third of the world's population have already been infected by this virus, and more than 350 million people are currently chronically infected (1,2,6).

The inability to control HBV infection and the consequent chronicity leads to a state of relative collapse of virus-specific adaptive immunity. The T-cell response is relatively mild and ineffective in chronically infected patients compared to acute hepatitis, suggesting the development of immune tolerance in these patients. The attempts to restore HBV-specific immunity by inhibiting virus replication through antiviral treatment results in partial immune restoration (7-9), yet inadequate to achieve full viral clearance.

The underlying immune mechanism for the antiviral response in patients treated with nucleoside

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or nucleotide analogs (NUCs) is not clearly understood. There is some evidence about a decrease in the number of regulatory T cells (Treg cells,Tregs), of increasing levels of Th1-proinflammatory cytokines and a decrease of Th2- anti-inflammatory cytokines during antiviral treatment with NUCs. Those findings suggest that the antiviral effect of NUCs may be attributed not only to their direct effect on virus suppression but also to their immunoregulatory capabilities (10).

To improve our knowledge about this complex interaction between NUCs and HBV, we performed a pilot study to investigate changes in values of two cytokines produced by CD4+ T cells: the Th1 proinflammatory Interferon-γ (IFN-γ) and the Th2 anti-inflammatory IL-10, under treatment with entecavir (ETV). The aim of this study was to determine if the biochemical, serological and virological responses to ETV correlate with the antiviral immune response, as tested by IFN-γ and IL-10 production. We have chosen these two cytokines because of their complex interactions and ubiquity. Both cytokines are known to be involved in the natural immune response to HBV (11-15) and the evolution under antiviral treatment (7,8,16-19). IFN-γ plays a key role in the management and elimination of HBV infection (20). IFN-γ level seems to be correlated with the degree of the necroinflammatory activity in CHB (2), and its production is increasing along the treatment period (10,17). IL-10 seems to be involved in HBV persistence (22,23) and is associated with necroinflammation and liver fibrosis in CHB (14,21,24,25). IL-10 was proposed as a predictor for HBeAg seroconversion in CHB patients (11) and its production increases under NUCs treatment (26).

Very recent data suggest that evaluating serum cytokines in concert with virologic and clinical parameters may help to identify CHB patients who can successfully discontinue nucleos(t)ide analogue therapy (27). In this study we performed a cross sectional examination of serum levels of IFN-γ and IL-10 in people with CHB treated with ETV. We aimed to detect if measuring those crucial cytokines would provide hints for prognosis and response to treatment. This approach would provide preliminary data to design a longitudinal study involving serial on-treatment immunological checks that would enable profiling responders and non-responders to NUCs.

PATIENTS, MATERIALS AND METHODS

A total of 34 patients with CHB, looked after in the National Institute of Infectious Diseases "Prof Dr. Matei Bals", with a median age of 33 years (range 15-59) were included in the study. Among them, 20 were treated with ETV (13 males and 7 females), and 14 of them were untreated (6 males and 8 females). The median duration of treatment was 21 months (range 12-24 months). Twelve healthy subjects (3 males and 9 females), 35 years age median (range 29-45), were included in the control group. Biochemical markers of disease activity (ALT, AST), viral load measured by a quantitative HBV-DNA PCR kit (COBAS AmpliPrep/ COBAS TaqMan HBV Test), quantitative HBs and HBe antigens (Abbott ARCHITECT Quantitative, expressed in IU/ml, compatible with EASL practice guidelines), and liver stiffness using transient elastography (FibroScan) have been assessed in all patients. Biochemical data and liver stiffness measurement were recorded for healthy controls. Serum samples were stored at -80°C for cytokine analysis from both patients and controls. IFNγ and IL-10 levels were measured from serum using commercial ELISA kit offered by R&D diagnosis.

According to the guidelines (28), the biochemical response to treatment is defined as normalization of ALT levels; the serological response for HBeAg (that applies only to patients with HBeAg-positive CHB) is defined as HBeAg loss and seroconversion to anti-Hbe. Serological response for HBsAg applies to all CHB patients and is defined as HBsAg loss and development of anti-HBs. Virological response is defined as undetectable HBV DNA by a sensitive PCR assay, and complete response is defined as sustained off-treatment virological response together with loss of HbsAg (28). Patients were divided in subgroups on the basis of response to treatment and the presence of HBe and HBs antigens. We considered the patients as 'non-replicative' or responsive (R) to treatment if the values of HBV DNA were less than 100 IU/ml and „replicative" or non-responsive (NR) if they were above this value; and positive if the viral HBe and HBs antigens were present and negative if absent.

Informed consent was obtained from all participants. The study protocol was approved by the National Institute for Infectious Diseases "Prof. Dr. Matei Bals" Ethics Committee.

For statistical analysis, SPSS 16 for Windows was used. Continuous data were compared using non-parametric tests (Kruskal-Wallis tests, Mann Whitney U tests) and binary data were compared using Fischer exact test. Correlations were determined using Spearman's correlation test. Results are given in median (range), unless specified otherwise. For all tests, value of $P < .05$ was considered statistically significant.

RESULTS

1. Patient characteristics

Data regarding demographics, viral load, ALT, AST, presence of HbsAg and HbeAg, liver fibrosis (FibroScan) are presented in Table 1.

TABLE 1

Parameters	Entecavir group (n = 20)	Naïve chronic hepatitis B (n = 14)	Healthy controls (n = 12)
Age (years)	40 (15-59)	33 (25-54)	35 (29-45)*
Sex (M/F)	13/7	6/8	3/9*
Replicative state enrollment (number (percent))	R 8 (40%)	13 (92.9%)**	N/A
	NR 12 (60%)		
ALT	54 (14-160)	43 (22-106)	13 (10-23)*
AST	36 (19-89)	29 (19-55)	20 (14-27)*
HbsAg positive (number (percent))	17 (85%)	13 (92.9%)	N/A
HbeAg positive (number (percent))	7 (35%)	2 (14.2%)	N/A
FibroScan: F1-2/F3-4	F1-2: 14 (70%)	F1-2:11 (78.6%)	N/A
	F3-4: 6 (30%)	F3-4 3 (21.4%)	N/A

N/A not applicable. *Differences between treatment groups and healthy controls volunteers were statistically significant ($p < 0.05$, Kruskal-Wallis test). **Differences between treatment groups statistically significant ($p<0.05$, Fisher exact test)

The levels of aminotransferases were strongly correlated ($p<0.001$) in CHB naïve patients, compared with treated patients and controls.

In the Entecavir group, 60% of the subjects achieved virological response versus only 7.1% in naïve CHB patients ($p=0.01$).

The NR patients (median age 28, range15-44) were younger than R (median age 44, range 28-59), with statistical significance, $p=0.01$.

HBeAg was present mainly in younger patients, median 29 years (range 15-39) versus median 44 years (range 25-59) in negative HBeAg patients.

Although 60% of patients were found R, only 3 of 20 (22%) treated patients achieved seroconversion of HBsAg.

Our study has not found correlations between the duration of the therapy and other parameters, like demographic traits, seroconversion or the viral load, however the treatment duration was short.

2. Cytokine levels in patients and controls are presented in Table 2.

Serum IFN-γ levels were higher in naïve HVB patients and also in entecavir treated patients compared to healthy controls ($p=0.05$, respectively $p=0.021$).

TABLE 2

Serum cytokine levels	Entecavir group	Naïve chronic hepatitis B	Healthy controls	P value
IFN-γ (pg/mL)	*2.38 (0-8.66)*	*1.73 (0-5.2)*	*0.64 (0-4.67)*	*0.03**
IL-10 (pg/mL)	*3.61 (0-20.56)*	*2.2 (0-8.06)*	*1.67 (0.53-3.56)*	*0.04**

Differences between treatment groups and healthy controls were statistically significant ($p < 0.05$, Kruskal-Wallis test).

Serum IL-10 levels were higher in entecavir patients when compared with naïve CHB patients ($p=0.05$). Serum IL-10 and IFN-γ levels did not correlate in CHB patients.

There were no relevant differences in serum IL-10 levels between NR and R status in the Entecavir group ($p=0.20$). However, in the NR group, there was a tendency for higher titers: the median value was 5 pg/mL (min 0 and max 20.56 pg/mL) versus 3.3 (min 0 maxim 20.10) pg/mL).

Although not reaching statistical significance ($p=0.08$), serum IFN-γ levels were higher for R patients, with median 3.68 pg/mL (min 0 max 8.66) versus NR patients, median 1.59 pg/mL (min 0 max 3.90 pg/ml).

Discussion in this cross-sectional study on HBV patients treated with NUCs, IL-10 and IFN-γ values were increased in all treated patients in comparison to untreated patients and healthy controls, and seemed to differentially correlate with prognosis.

Although many basic and clinical studies on the virology and pathophysiology of HBV have been attempted since 1965, when Baruch Blumberg et al. discovered the Australia antigen, to establish mouse models for HBV infection, the mechanisms of immune responses against HBV and the resultant clinical phenotypes have not yet been determined. Clinical outcomes in adults with chronic hepatitis B depend on patient and viral characteristics. Some studies have confirmed the relationship between age and poor outcomes, particularly for HCC (29-31), but overall, there was inconclusive evidence regarding the extent to which the association between age and clinical outcomes is explained by duration of infection, age at the time of infection, comorbidities in older individuals, and other factors (32).

In our study, in contrast with other papers, young age was slightly associated with NR status under treatment. It is also remarkable that the same group of younger subjects, were much more often HbeAg positive than the older patients. Several studies identified low HBV DNA and high ALT levels at baseline as strongly associated with favorable out-

comes under treatment in HBeAg-positive patients; and undetectable HBV DNA at week 24 as the strongest predictor for all outcomes at 2 years (33-36). The lack of data regarding the duration of infection and the small sample size don't allow to study significant associations between younger age, HBe Ag positivity and non-responsiveness to therapy. Since HBeAg seroconversion is a hallmark event of a durable clinical remission of liver disease, current treatment guidelines have adopted HBeAg seroconversion with sustained suppression of HBV DNA as an end point for treatment in patients positive for HbeAg. It is accepted that treatment with NUCs induces a much lower rate of HBeAg seroconversion and hence more prolonged therapy is required in CHB patients treated with NUCs (37). Our study has not found correlations between the duration of the therapy and seroconversion or the viral load, maybe because the duration of treatment was too short. It is therefore likely that these category of subjects (younger patients, NR and HbeAg positive), would remain at risk for future hepatic inflammation and fibrosis.

Immunological changes have been noticed during the course of HBeAg seroconversion in CHB patients treated with antiviral therapy, reflecting the restoration of the host immunity against HBV. For instance, the frequency of Toll-like receptors (TLRs) and programmed death-1 (PD-1), both regulated by the presence of HbeAg (38), an increase of active Th1 cytokines, IL-12 induced, and high serum levels of IL-12 and IL-10, have been associated with HBeAg seroconversion in HBeAg-positive CHB patients treated with alpha interferon, and with early, spontaneous, HBeAg seroconversion (11,39). Recently, IL-21 at 12 weeks of therapy was suggested as prediction marker for HbeAg seroconversion (40). IL-21 may be a critical factor in the control of persistent viral infections, mandatory for sustained CD8+ T cell effector activity and then, for maintaining immunity to resolve persistent viral infection (41).

Most of the patients from our study remained HBsAg positive, and no correlation could be obtained with the presence of HbeAg, demographic traits, viral load and cytokines levels. We didn't find correlations between the duration of the treatment and HbeAg loss or the cytokines serum levels, maybe because of the short period of treatment. In was proved that HBsAg levels decrease steadily in HBeAg-positive patients, especially in those with high baseline ALT, older age, and HBeAg loss, while only a marginal HBsAg drop was observed in HBeAg-negative patients (42).

HBsAg is produced from the translated messenger RNAs of the transcriptionally active cccDNA and integrated HBV DNA sequence. HBsAg levels indicate the presence of intrahepatic covalently closed circular DNA (cccDNA), which is the reason of incomplete clearance of HBV in chronic infection, mainly in HbeAg positive patients in an untreated population (43). Therefore, HBsAg might be used as a surrogate marker for the interaction between the immune system and the virus (44,45). NUCs therapy not only inhibits HBV reverse transcription step, but also shows a small effect on the reduction of intrahepatic cccDNA (45-47).

The lack of a vigorous, polyclonal and multispecific T-cell response in chronic HBV infection is accompanied by a weak, ineffective or undetectable virus-specific T-cell response. The mechanisms responsible for T-cell tolerance in chronic HBV infection are not completely understood. Previous studies indicated that CD4+ and CD8+ T cells mainly mediate the protective immune response against HBV infection. CD4+ and CD8+ T cells secrete IFN-γ and activated cytotoxic T lymphocytes, thus directly eliminating infected cells. (48,49) In addition, type 2 cytokines, may also be involved in the clearance of circulating virus by promoting the production of neutralizing antibodies against the HBV surface and core antigens (50).

Chronic HBV infection is characterized by an inefficient T helper (Th) cell response to hepatitis B surface antigen (HBsAg) and by a variable Th cell response to the HBV-related antigens such as hepatitis core antigen (HBcAg), and hepatitis e antigen (HBeAg). The class I- and class II-restricted T cell responses to VHB are vigorous, polyclonal, and multispecific in acutely infected patients who successfully clear the virus, but they are relatively weak and more narrowly focused in chronically infected patients who do not. (51) Also, the envelope-specific Th response is stronger in a proportion of vaccine recipients who have been immunized with plasma-derived or recombinant HbsAg, suggesting that differences in antigen load or presentation may influence the strength of the HBs-specific T cell response (52-55).

It is considered that permanent and profound suppression of viral replication achieved with actual treatments is beneficial for preventing complications of chronic hepatitis B (CHB), as persistent HBV viral load has been proven to be the most important predictor of progression to LC, hepatic failure, and development of HCC (31). NUCs act by suppressing HBV replication at the level of DNA synthesis, and may also enhance immune clearance

of infected hepatocytes (56). NUCs target the reverse transcriptase of HBV and are potent inhibitors of viral replication, resulting in a rapid decline of serum HBV DNA levels, and long-term therapy determines reduction in hepatic fibrosis, hepatic decompensation, and liver-related mortality (57, 58). Although the NUCs treatment has significantly improved the outcome of CHB, it remains largely unknown how immune system responds to the treatment.

Lamivudine (LMV), the first NUC used for the treatment of HBV infection, it was shown to restore the responses of HBV-specific T-cells, probably by restoring the function of exhausted T-cells, with reconstitution of CD4+ T cell activity and subsequent induction of the CD8+ T cell response, during the first few months of the treatment period (7,8,56,59, 60).

The Adefovir treatment led to an increase of Th1/Th2 cytokines producing T cells and serum cytokine levels in association with the decline of HVB DNA load, but the changes of serum cytokines were not associated with HBV DNA loads, ALT and AST. The levels of Th1 cells in responsive patients reached the peaks at the 36th week of the treatment, then started to drop and maintained the levels of normal healthy individuals at the 65th week of the treatment. The peak of the number of Th2 cells was at the 65th week and started dropping to the normal individual levels approximately at the 78th week of the treatment. IFN-γ showed much more increase in the response ADF treatment as compared to other cytokines. The enhancement of the immune response induced by ADF is compatible with the dissolution of the immune tolerance specific to the chronic HBV infection (17).

Telbivudine effectively suppress the HBV replication, decreasing the consequent liver injury (61). It was noticed that these effects are accompanied by marked increases in CD4+ and CD8+ T cell responsiveness (19), an increased frequency of peripheral blood CD4+T lymphocytes and an amplification of proliferative response of HBV-specific T cells to the hepatitis B core antigen (HBcAg) (18). This suggests a key role played by CD4+ T cells in HBV infection (19).

Few studies have done head-to-head comparisons between the immune response under therapy with potent NUCs, like TBV and ETV, with less potent ones, like LMV, respective ADF (10,62). Those studies reported an increase in Th1 cytokines with an decrease of Treg cells. This immune response seemed to be correlated with the levels of the viral load and antigens. Higher levels of Th1 cytokines were observed with ETV versus ADV group, suggesting that this drugs have different impact upon the immune system (10). The antiviral effect of the drugs may be attributed not only to their direct effect on viral suppression but also to their immunoregulatory capabilities, so the way of modulating the immune response motivates a future additional research.

In our study, serum IL-10 levels were higher in ETV patients when compared with naïve CHB patients (p=0.05). Within the ETV group, in the NR patients, there was a tendency for higher titers in serum IL-10 levels: [5 pg/mL (min 0 and max 20.56 pg/mL) versus 3.3 (min 0 maxim 20.10) pg/mL)]. The slightly increase of IL-10 in the non-responsive patients suggest active liver disease, in accordance with the recent evidence of the strong association of IL10 with the HBV infection mediated disease progression, from inactive carrier state to malignancy (21,24).

IL-10 is widely known as an immunosuppressive cytokine because of its ability to inhibit macrophage-dependent antigen presentation, T-cell proliferation, and Th1 cytokine secretion. (63-65) However, IL-10 has been shown to function as a cytotoxic T-cell differentiation factor, promoting a higher number of IL-2-activated cytotoxic T lymphocytes (CTL) to proliferate and differentiate into powerful cytotoxic effector cells (66), IL-12 may induce a stable phenotype in T-cell clones that coexpresses large amounts of IL-10 and IFN-γ (67). Moreover, in a murine system, systemic administration of IL-10 may exacerbate allograft rejection (68) while an anti-IL10 antibody prolongs allograft survival in normal as well as presensitized recipients (69). A recent study indicated a significant correlation between IL-10 and IL-12 in tolerance immune phase, suggesting that IL-10 may be bi-functional during the course of HBV infection and that its role may depend on serum levels and cooperative cytokines like IL-12 and downstream IL-2 (11). It will be a future task to investigate if the decrease of IL-10 level under successful antiviral therapy signifies a switch to Th1 and Th17 immune reaction or it is only a consequence of the improvement of the liver disease. Also its value as a marker for oncologic survey has to be validated.

In our study, IFN-γ levels were higher in naïve HVB patients and also in ETV treated patients compared to healthy controls (p=0.05, respectively p=0.021). In contrast with IL-10, IFN-γ levels were higher for R patients, with median 3.68 pg/mL (min 0 max 8.66) versus NR patients, median 1.59 pg/mL (min 0 max 3.90 pg/ml), indicating a reinvigoration

of the immune system, with a better prognosis, a better chance of viral clearance, as shown in other papers (20,70-73).

The action of IFN-γ on HBV elimination is thought to be pleiotropic. It was indicated that IFN-γ action results in the destabilization of HBV RNAs, by a La-dependent mechanism, facilitating the destruction of HBV RNAs by nuclear RNases (72, 74-76), it hinders the assembly of pregenomic HBV RNA-containing nucleocapsid protein, in a proteasome- and kinase-dependent manner (72,77), and effectively downregulates replication intermediates of HBV DNA in a nitric oxide (NO)-dependent manner (71,78,79). Direct effects of IFNs upon cccDNA have been deciphered, such as modifying the composition of HBV minichromosome, suppressing cccDNA transcription and accelerating cccDNA decay (80,81).These mechanisms suggest a very profound downregulation of HBV, most of which accomplished without destroying hepatocytes (20).

Most of the published studies indicate that IFN-γ is mostly not expressed in CHB patients at a level detectable by serology, whereas it is involved in the immediate immune response triggered by acute hepatitis; and that IFN-γ levels don't correlate with the clinical severity of CHB (15). Further studies with larger samples of subjects and baseline assay are warranted, to assess if the increase in IFN-γ levels in responsive patients could be used as a prognostic factor.

Virus-host immune interactions are versatile and complex. Repeated assessments in time are needed to establish a cost-efficient panel of immune biomarkers for the evaluation of the efficacy of the various anti-HBV therapies, to predict the decrease of viral load and antigenic pressure, and the clinical outcome for CHB patients.

In conclusion, treatment with ETV seems to reinvigorate the immune system. Although the serum levels of both cytokines increase during the treatment, they may differently correlate with prognosis. IL-10 may be a bi-functional cytokine, increasing in all treated patients, and may be associated with poor prognosis, while IFN-γ with a favorable outcome under treatment. This transversal investigation provides pilot data which can be used in designing a prospective study to assess the role of IL-10 and IFN-γ as prognostic markers in HBV NUCs-treated patients.

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