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Assessment and analysis of IL-8, IL-10, IL-12, IFN-γ and TNF-α in the Immunopathogenesis of Chronic Hepatitis B Virus Genotype D Infection

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: The pathogenesis of hepatitis B virus (HBV) infection is based on the interactions between HBV replication and immune responses. The aim of this study was to look into the immune-regulatory functions of IL-12, TNF-α, IFN-γ, IL-8, and IL-10 in chronic hepatitis B (CHB) genotype D infection at different serological stages.
Materials and methods: The study included 125 chronic hepatitis cases. This research used a PCR-based genotyping method with type-specific primers. ELISA kit from Orgenium, Finland, measured TNF-α, IL-8, IL-10, and IL-12 levels, while ELISA, Diaclone, France, measured IFN-γ serum levels.
Results: Genotype D was detected in 123 cases (98.4 %) out of 125 CHB patients. HBsAg and anti-HBcIgG were positive in all cases, 59 (47.96%) cases were positive for HBeAg. The median

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levels of IL-8, IL-10, and TNF-α were significantly higher in this study than in healthy controls, at 304.78±174.22 pg/ml, 13.74±6.15 pg/ml, and 180.75 ± 14.29 pg/ml, respectively. The amounts of IL-12 and IFN-γ were 19.25 pg/ml and 9.32 pg/ml, respectively.

Conclusion: HBV infection may be persistent due to lopsided non-cytopathic antiviral mechanisms. Our findings suggest that lack of synchronicity of the cytokines may play a role in preventing non cytolytic elimination of the virus leading to chronicity. It's difficult to assign particular roles to any given cytokine in terms of HBV pathogenesis or clinical growth. Further research is necessary to further delineate the roles of individual cytokines.

Keywords: HBV; Genotype D; Chronic Hepatitis B.

1. INTRODUCTION

Chronic HBV infection is a hepatic necroinflammatory condition caused by a persistent HBV infection [1]. Immune-tolerant phase, immune clearance or active phase, inactive carrier phase, and reactivation phase are the four most frequently defined phases of Chronic Hepatitis B (CHB) [2,3]. CHB may be defined as HBsAg positivity for more than six months, serum HBV DNA levels greater than 20,000 IU per mL (lower values of 2,000 to 20,000 IU per mL are common in HBeAg-negative chronic hepatitis B), persistent or sporadic elevations in alanine transaminase (ALT) or aspartate transaminase (AST) levels, and liver biopsy showing chronic hepatitis with moderate or extreme necro-inflammatory disease [4].

Hepatitis B virus is a widespread non-cytopathic virus that can cause tissue damage of varying severity by triggering a defensive immune response that can be both damaging and protective [5]. HBV-specific T-cell responses are weak or undetectable in the peripheral blood in chronic hepatitis B virus infection [6]. It may be attributed to exhaustion of T cell responses and expansion of T cells generating Th2 cytokines, the high viral and antigen load in these patients. Thus, immune elimination of infected cells can lead to an end to the infection if it is effective, or to a persistent necro-inflammatory disease if it is not [7].

Nevertheless, the destruction of infected cells is not the only mechanism involved in the removal of the intracellular virus [8], as shown by studies carried out in animal models of HBV infection as well as in humans demonstrating the importance of cytokine-mediated, non-cytolytic anti-viral defense mechanisms [9,10].

Th1 and Th2 T cell subsets have been identified in studies, with distinct and mutually exclusive cytokine development patterns and functions [11]. In previous research, we found a connection between IFN-γ and IL-12 and a milder type of hepatitis and less severe liver damage. In non-necrotizing inflammation, IFN-γ and IL-12 play an important role [12]. Since HBV is non-cytopathic, liver damage is thought to be immune-mediated, but the molecular mechanisms that contribute to hepatocyte death in humans remain unknown [13]. Dissecting the ways in which different components of the immune response leads to liver disease in HBV infection is urgently needed.

Although it is well recognized that HBV genotypes play an important role in clinical outcome of hepatitis [14,15], possible immunological basis of this has not been explored completely. The aim of this study was to look into the immune-regulatory function of IL-8, 10 & 12 as well as TNF-α & IFN-γ in chronic hepatitis B (CHB) genotype D infection at different serological stages. Patients with chronic HBV genotype D positive for different serological markers were chosen for this study, and cytokine levels were assessed in each group of patients to perform a point cross sectional analysis of their possible function at each serological stage.

2. MATERIALS AND METHODS

The study was conducted from August 2012 to July 2014 in the Department of Microbiology, J.N. Medical College, A.M.U, Aligarh. Thirteen hundred and sixteen consecutive patients with hepatitis attending the Hepatitis clinic or admitted in the Medicine wards of Jawaharlal Nehru Medical College were screened for viral hepatitis. All patients were subjected to complete physical examination and a detailed clinical history was elicited from them.

2.1 Exclusion Criteria

This study excluded patients with autoimmune hepatitis, alcoholic hepatitis, drug induced

hepatitis, and patients with a history of recent infection, surgery, trauma within the preceding two months, renal insufficiency or other acute or chronic inflammatory diseases. Before or during this study, none of the participants had received any antiviral or immunosuppressive therapy. All cases of acute HBV infection were excluded for another study.

2.2 Necrotizing and Non- necrotizing Inflammation

If there was a major derangement of the liver function tests (LFTs) with extensive damage to liver parenchyma, a necrotizing inflammation (NI) is considered. In non-necrotizing inflammation (NNI), the LFTs were moderately elevated with minimal histopathological changes [12].

2.3 Clinical Grading

Clinical grading was performed using the Model End Stage Liver Disease (MELD) score [16], based on the patient's biochemical investigations and clinical status for mild moderate, severe and end stage liver disease.

2.4 Healthy Control Individuals

The control group consisted of 30 healthy people of comparable age, 22 (73.34%) among those were men and 8 (26.66%) women; mean age was 37 years, selected from the blood bank. For HBsAg as well as for anti-HCV and anti-HIV antibodies, these individuals were confirmed to be negative.

2.5 Collection of Specimen

Venous blood specimens are collected from a peripheral vein with informed consent from all participants for all serological assays. The serum was stored at -40 C following centrifugation before it was used for analysis.

2.6 Routine Investigations

Liver function tests (LFT) like serum amino alanine transaminase (ALT), aspartate amino transferase (AST) and alkaline phosphatase (ALP), bilirubin (direct & total), albumin, globulin, creatinine and international normalized ratio for prothrombin time were detected. Specific investigations like ultrasonographic examination of liver, upper GI endoscopy and liver biopsy were performed wherever feasible.

2.7 Primary Screening

All hepatitis patients were screened for HAV, HBV, HCV, HEV and HIV using commercially available ELISA kits using the manufacturer's instructions: HBsAg, third-generation anti-HCV, fourth-generation anti-HIV (J. Mitra& Co. Pvt. Ltd., India), anti-HAV IgM and anti-HEV IgM (DRG International, Inc., USA).

Patients positive for HBsAg for more than 6 months were enrolled for further study.

2.8 Secondary Screening

HBeAg, anti-HBe, anti-HBcIgM (DRG International, Inc., USA) and anti-HBcPlus (Monolisa TM, Bio-Rad, France) were used to determine the serological status of these patients.

2.9 Genotyping of HBV

DNA was extracted from 100*µ*l serum samples of HBV positive patients by phenol chloroform extraction method. Using a thermal cycler, a 125 base pair sequence of the HBV surface gene was amplified (Labnics, USA). In this study, a PCR-based genotyping process utilizing type-specific primers was used to establish genotypes D of the hepatitis B virus as described by Kirschberg et al. [17,18]. A 5-*µ*l DNA sample was mixed with the reaction mixture and a 25-*µ*l amplification reaction was performed. The reaction mixture contained 1x PCR buffer, MgCl2 (1.5 mM), 200mM dNTP's, a 20 pmol concentration of each primer; and 2.5 units of Dream Taq DNA polymerase; MBI Fermentas, USA). The primer sequences were synthesized by Operon, Germany (Genetix). The sequences of the HBV genotype specific primers were: HBV-GT1-D-s 5'-ACA GCA TGG GGC AGA ATC TTT CCA CCA G-3'; HBV-GT1-D-as 5'-CCT ACC TTG TTG GCG TCT GGC CAG G-3'.

Cytokine profile of individuals with chronic HBV genotype D infection was assessed.

2.10 IL-8,IL-10,IL-12, IFN-γ & TNF-α assay

ELISA kits were obtained from Orgenium, Finland, to measure IL-12 IL-8, IL-10 & TNF-α in serum of HBV infected patients and in healthy controls, while ELISA kits were obtained from Diaclone, France, to measure IFN-γ serum level. The tests were performed according to the manufacturer's instructions. Absorbance was read at 450 nm in an automated ELISA plate reader (Thermo scientific). Standard curves

corresponding optical densities and concentrations of IL-8, IL-10, IL-12, IFN-γ & TNF-α were plotted to determine their concentrations in serum samples. Results were expressed in picograms per milliliter (pg/ml). The detection limits of the IL-12, IL-8, IL-10 assay was 2 pg/ml while TNF-α and IFN-γ assay were 9 pg/ml & 5 pg/ml respectively. A point study was done to evaluate the levels of IL-8, IL-10, IL-12, IFN- γ & TNF-α in the patients in relation to the HBV markers in necrotizing and non-necrotizing inflammation.

2.11 Statistical Analysis

Correlations between continuous variables were assessed by the Spearman rank test, corrected for ties, where a value of p> 0.25 (combined with P < 0.05) was considered significant. The non-parametric Mann- Whitney U- test was used to determine the significance of differences in continuous variables. The level of significance in all cases was set at a two tailed P< 0.05. We used Microsoft Excel and MedCalc 10.2.0.0 soft wares to calculate the statistical analysis.

3. RESULTS

3.1 HBV Genotypes Detection

In 123 cases (98.4 %), genotype D was found out of 125 CHB patients. The cytokine profile was analyzed in HBV genotype D cases.

3.1.1 Clinical profile

Common complaints of patients with chronic hepatitis B infection (CHB) were anorexia, jaundice, nausea, splenomegaly and hepatomegaly. Among these 87 (70.73%) had NI and 36 (29.27%) had NNI. 47 (38.21%) had mild, 46 (37.39%) moderate and 30 (24.39%) had severe liver disease, 11 had End stage liver disease.

3.1.2 Biochemical profile

Table 1 shows the biochemical profile of liver parameters in patients with CHB. Hyperbilirubinemia was found to be three times higher in CHB than in the stable control group, whereas ALT and AST levels were two times higher in CHB.

3.2 HBV Serological Markers

Serological profile of CHB cases is given in Table 1. While HBsAg and anti-HBcIgG were positive in all cases, 59 (47.96%) cases were positive for HBeAg.

3.2.1 Serum cytokine levels

The median IL-8, IL-10, and TNF-α levels in this study were 304.78±174.22 pg/ml (p<0.001), 13.74±6.15 pg/ml (p0.05), and 180.75±14.29 pg/ml (p<0.001), respectively, compared to healthy controls. Fig. 1 and Table 2 show that IL-12 and IFN-γ levels were substantially lower than the control groups, at 19.25 pg/ml and 9.32 pg/ml, respectively.

Table 1. Biochemical and serological profile of 123 patients with CHB genotype D

Characteristics	Chronic HBV patients
Mean ALT levels(IU/L) *	46.17±40.86
Mean AST levels(IU/L) *	30.92±25.83
PT-INR*	1.65±.53
ALP (IU/L) *	26.88±.65
Serum Bilirubin(mg/100ml) *	3.042±0.23
MELD	15.14±6.6
HBsAg	123 (100)
Anti HBc IgG	123 (100)
HBeAg	59 (47.96%)
Anti Hbe/HbeAg -	53(43.0)
HbsAb	2 (1.62%)

**shows mean values*

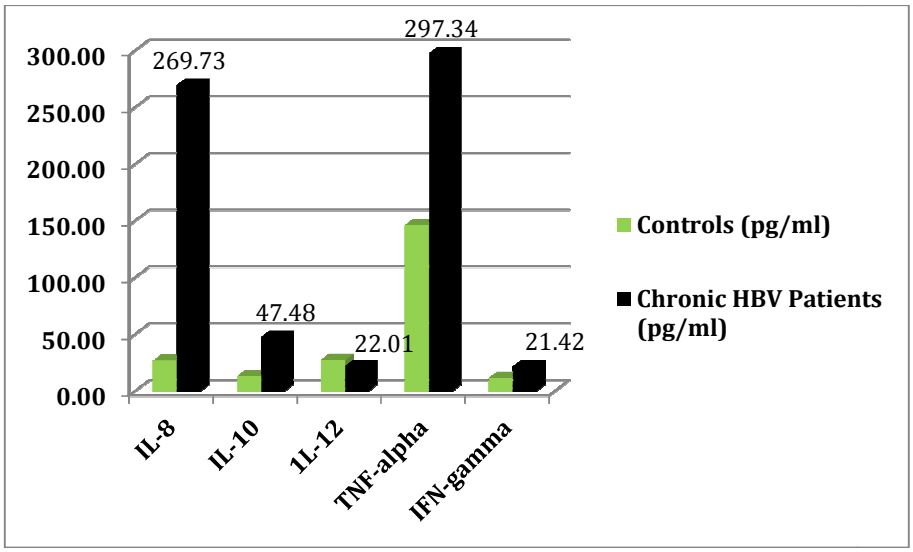

Fig. 1. Comparison of mean serum cytokines levels between Chronic HBV patients and healthy controls

Table 2. Cytokine profile of patients with CHB genotype D

Cytokines	Range and median levels of cytokines in Chronic HBV patients and healthy control				
	Healthy controls (n=20)		Chronic HBV patients (n=123)		F-Test
	Median+S.D.	Range	Median+S.D.	Range	p-value
IL-8 pg/ml	16.37±19.78	11.78-67.77	304.78±174.22	18.66-507.31	0.001
IL-10 pg/ml	8.37±15.07	0.39-51.98	13.74±106.15	0.78-471.86	<0.001
IL-12 pg/ml	25.44±12.46	10.76-50.92	19.25±14.78	1.00-53.36	0.504
TNF-α pg/ml	146.19±13.65	125.81-166.57	180.75±414.29	114.30-1818.09	<0.001
IFN-gamma pg/ml	11.81 ±3.67	5.47-17.05	9.32±43.54	1.42-144.87	0.039

3.2.2 IFN-γ, IL-12, TNF- α, IL-8, and IL-10 levels in NI and NNI

The median IFN-γ level in the NI category was 49.27pg/ml. The median IFN-γ level in NNI cases was higher, at 84.62pg/ml. Fig. 2 shows that IL-12 levels in NI cases were 44pg/ml, while IL-12 levels in NNI cases were slightly lower at 25.76pg/ml. TNF-α and IL-8 values in NI were 249.42pg/ml and 198.55pg/ml, respectively, while they were slightly lower in NNI at 169.08pg/ml and 168.43pg/ml, p<0.001. IL-10 mirrored the same picture although the elevations in levels were lower (18.86 and 14.99pg/ml in NI and NNI respectively) as shown in Fig. 3.

3.2.3 The relationship between cytokines and HBeAg status

In HBeAg seronegative individuals, mean levels of IL-8, IL-10, TNF-α, and IFN-γ were much higher, with mean levels of 257.43 pg/ml, 69.43 pg/ml, 365.48 pg/ml, and 31.18 pg/ml, respectively, as shown in Fig. 4. In HBeAg positive cases, however, IL-12 levels were higher.

The presence of HBeAg was found to be associated with IL-8 and IL-12 using multiple linear regression analysis. A negative correlation was observed with IL-10. Table 3 shows that none of the cytokines were independently linked to anti HBeAg. Although higher in HBeAg negative individuals as shown in Fig. 4, IL-8, IL-10, IFN-**γ**, and TNF-α were not individually linked to HBeAg.

3.2.4 Association of cytokines with transaminase levels

Multiple linear regression study of transaminase levels in relation to cytokines revealed that elevated AST levels were significantly correlated with IL-8 and IL-10. IL-10 and TNF-α together

were linked to elevated ALT and serum bilirubin levels. Interestingly, IL-10 was significantly associated with elevated ALT, AST, ALP and Serum bilirubin level as shown in Table 4.

4. DISCUSSION

The serum levels of immunoregulatory cytokines (IFN-γ, IL-12), pro-inflammatory cytokines (TNF-α, IL-8) and anti-inflammatory cytokine IL-10 were measured in genotype D CHB cases in this study. We also assessed the levels of cytokines in relation to NI and NNI, as well as HBeAg status.

IL-8, IL-10, and TNF- α levels were significantly higher in CHB genotype D patients than in healthy controls. IFN-γ and IL-12 levels, on the other hand, were largely suppressed. Their levels were indistinguishable from those of the controls. In acute hepatitis B (AHB), on the other hand, IL-10 was largely suppressed while IFN-γ and IL-12 levels were elevated. IL-8, IL-12, and IFN-γ were all significantly lower in CHB than in AHB, whereas IL-10 was significantly higher. TNF-α levels remained more or less the same [19]. Since it has been reported that HBV-specific CTLs may eliminate HBV replication in hepatocytes in HBV-transgenic mice [6,20].

A major reason for chronicity may be the low levels of both IFN-γ and IL-12 in CHB [10]. In acute HBV infection, a robust cell-mediated Th1 response, characterized by IL-2 and IFN-**γ**, is mounted against HBV and is involved in viral clearance [21,22]. IFN-γ is a significant contributor to viral removal by activating macrophages and locating neighbouring uninfected cells that are resistant to invasion [23]. In patients with hepatitis B or C infection, those who cleared the virus had higher IL-12 serum levels than chronic virus carriers [24,25].

In CHB, HBeAg seronegative individuals had substantially higher levels of IL-8, IL-10, IFN-γ, and TNF-α. This contrasts with our findings in AHB, where IFN-γ and IL-12 levels were significantly higher in HBeAg seronegative individuals, whereas HBeAg was linked to high IL-8 and TNF-α levels [12].The low levels of IL-12 may be pivotal in leading to chronicity. Recent research suggests that a decrease in IL-12 will cause the Th1/Th2 balance to tip forward Th2, resulting in a non-cytotoxic T-lymphocyte response against virus clearance and can cause persistent HBV infection [26]. It is well known that IL-12 plays a central role in the assembly of an active cellular immune response to intracellular pathogens [27].In other studies of chronic HBV, however, elevated levels of IFN-γ have been found [28,29,30]. It thus appears that both IL-12 and IFN- γ are essential to achieve HBeAg seronegative state.

NI was found to be correlated with high levels of IL-8, IL-12, TNF- α, and to a lesser degree, IL-10 in our analysis. Only IFN-γ levels were elevated in NNI which could partly explain the chronicity of HBV. In AHB, as in our previous research, high levels of IFN-γ, IL-12, and TNF-α were found in NNI, indicating that they play a major role in non-cytolytic HBV infection control [19]. TNF-α levels had the steepest elevation in AHB, indicating that it plays a dominant position in NNI. Low levels of IL-12 and TNF-α in CHB may be a major factor in the host's failure to remove HBV, resulting in chronicity [31].

In CHB NI, the levels of IL-8, IL-10, IL-12, and TNF- α were higher than in AHB NI [19]. However, higher levels of IL-8, IL-10, IL-12, and TNF-α in NI did not result in unusually elevated ALT and AST, suggesting that other immunoregulatory mechanisms are at work. Low viral load in CHB may also be a reason.

IL-10 levels were higher in CHB NNI than AHB NNI which could account for the low levels of liver enzymes. In non-viral hepatitis, high IL-10 levels are common, which may explain the low ALT and AST elevations, as well as lower NI levels, in this study population (in communication). Th2 cytokine IL-10 will inhibit Th1 cell responses, causing the body to be unable to effectively remove virus-infected liver cells, resulting in persistent HBV infection and inflammation [32]. The decrease in IL-12 and TNF-α in CHB NNI cases may be due to higher levels of IL-10. Since increased IL-10 has been shown to inhibit the production of pro-inflammatory cytokines [33,34].

TNF-α levels were substantially higher in CHB NI compared to AHB NNI and lower in CHB NNI compared to AHB NNI. This complete inversion of TNF-α levels in CHB appears again to play a major role in chronicity and damage to liver [35]. As mentioned in our previous study, decline of either IFN-γ or IL-12 levels, an immunoregulatory cytokine, which connects innate and adaptive immunity [36], lead to not only chronicity but also to poorer clinical profile [37].

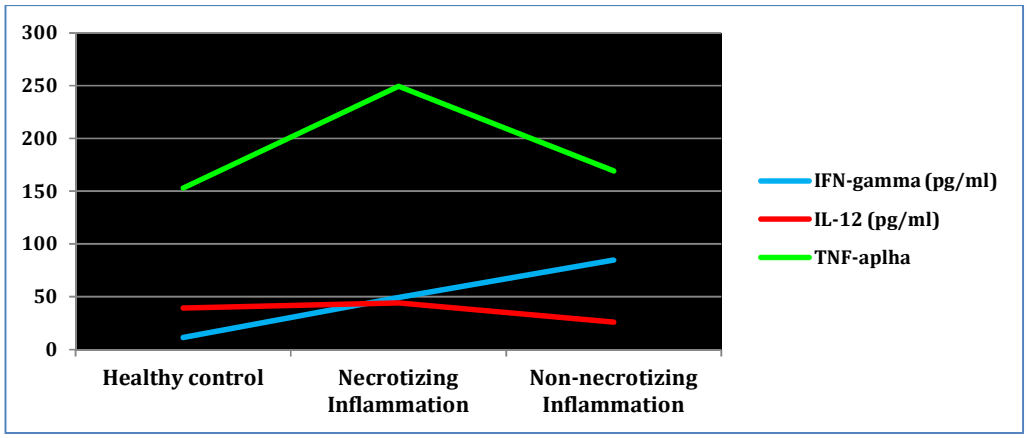

Fig. 2. IFN-γ, IL-12, and TNF-α levels in the blood of chronic HBV patients in NI and NNI

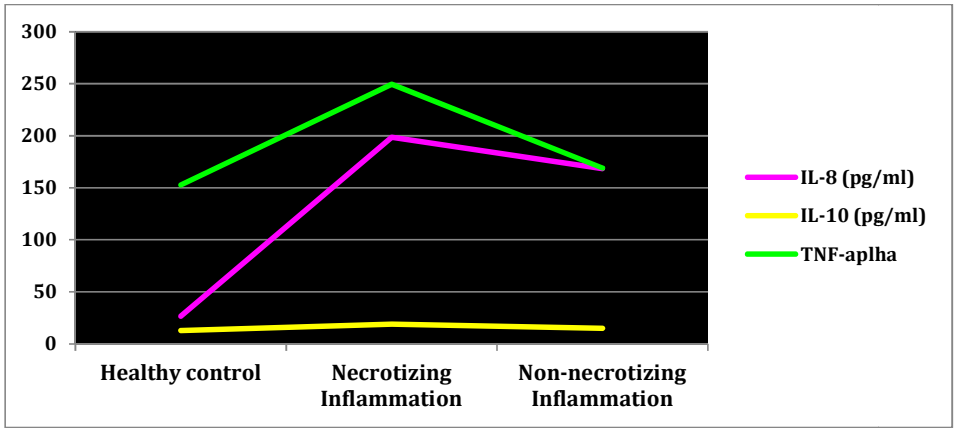

Fig. 3. TNF-α, IL-10, and IL-8 levels in the blood of chronic HBV patients in NI and NNI

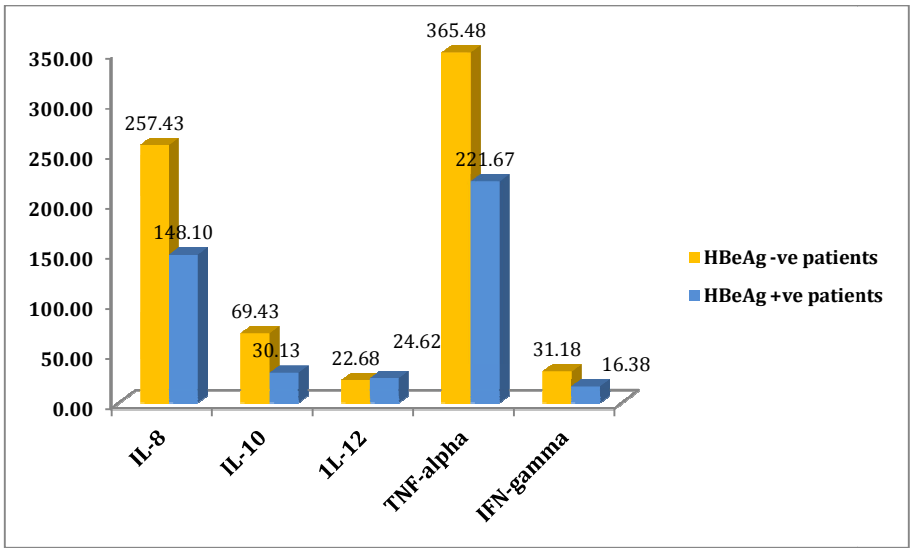

Fig. 4. Association of various cytokine levels with HBeAg status in Chronic HBV Patients

Table 3. Multiple linear regression analysis to study the independent variable predicting HBe Ag and HBe Ab in chronic HBV patients

Independent Variables	Dependent Variables			
	HBe Ag		Anti HBe	
	Coefficient	*p-value*	*Coefficient*	*p-value*
IL-8	0.002025	**0.0629**	-0.001215	0.2439
IL-10	-0.01257	**0.0168**	-0.005739	0.2519
IL-12	0.03033	**0.0256**	0.01508	0.2449
TNF-alpha	0.002029	0.1244	0.001435	0.2580
IFN-gamma	0.007299	0.0641	-0.001957	0.6035

Table 4. Multiple linear regression analysis to study the independent variable predicting ALT, AST, ALP and Serum bilirubin in chronic HBV patients

Independent Variables	Dependent Variables							
	AST		ALT		ALP		Serum Bilirubin	
	Coefficient	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*	*Coefficient*	*p-value*
IL-8	-0.0315	**0.0482**	-0.02587	0.1156	-0.02199	0.4771	0.003898	0.2112
IL-10	0.1893	**0.0140**	0.3323	**0.0001**	-0.3106	**0.0384**	0.03093	**0.0402**
IL-12	-0.1219	0.5347	-0.3328	0.1035	-0.1068	0.7809	0.01882	0.6260
TNF-alpha	-0.01257	0.5132	-0.04402	**0.0287**	0.06556	0.0836	-0.009224	**0.0162**
IFN-gamma	-0.06568	0.2523	-0.06266	0.2909	-0.1643	0.1443	-0.007205	0.5222

4.1 Limitations of the Study

One major limitation of this study is that we were unable to do HBV DNA quantification as ours is a resource limited setting because of which we could not classify the CHB patients in different phases. A range of factors, including host-related variables (e.g., genetic and immunological background), pathogen-related variables (e.g., viral load), and environmental variables (e.g., hygiene, diet, therapy, vaccination) may have impacted the outcome of this study.

5. CONCLUSION

Our findings suggest that lopsided non-cytopathic antiviral mechanisms can play a role in HBV infection chronicity. Our study suggests that lack of synchronicity of the cytokines may play a role in preventing non cytolytic elimination of the virus leading to chronicity. Dysregulated cytokine response leads to chronicity of HBV infection. Both IFN γ and IL-12 are essential in bringing about a non-cytolytic elimination of the virus. Alone neither of them can lead to viral elimination thus leading to chronicity. It is worthwhile to ponder that if IL-12 levels are elevated in CHB patients, will that lead to elimination of the virus, lead to reductions in exacerbation of chronic hepatitis (acute on chronic hepatitis). High levels of both these cytokines may direct the response towards NNI leading to lesser hepatic damage. The high IL-10 levels in CHB undoubtedly appear to contribute towards persistence of this virus. So interventions which lead to lowering of IL-10 and elevation of IL-12 may be useful in preventing chronicity and progression to cirrhosis. However, such interventions may need to be tailored to the individual patients' unique cytokine response as one size fits all principle will not work. Further research is necessary to further delineate the roles of individual cytokines.

CONSENT AND ETHICAL APPROVAL

The study was approved by the institutional ethical committee of J.N. Medical College, AMU, Aligarh. A written informed consent was obtained from each patient.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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