

Role of IL-8, IL-10, IL-12, IFN- γ and TNF- α in the Immunopathogenesis of Acute Hepatitis B Virus Infection

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ABSTRACT

AIM: To study the role of IL-8, IL-10, IL-12, IFN- γ AND TNF- α in immunopathogenesis of HBV infection.

METHODS: Eight hundred and forty consecutive patients with hepatitis were screened by serological markers for acute HBV (AHB) and were genotyped using modified Kirschberg's method. Patients were graded clinically and biochemically. Serum IL-8, IL-10, IL-12, IFN- γ and TNF- α levels were estimated in them and in healthy controls by enzyme immunoassay.

RESULTS: Seventy six cases of AHB with genotype D were identified and cytokines estimated. IFN- γ , IL-12, TNF- α , IL-8 levels were significantly higher than in the healthy controls at 27.26 ± 7.39 pg/mL ($p < 0.001$), 54.6 pg/mL ($p < 0.05$), 176.32 pg/mL ($p < 0.001$), 202.32 pg/mL ($p < 0.0001$) respectively. In NNI cases, median IFN- γ , IL-12 and TNF- α levels were higher at 52.351 pg/mL, 57.12 pg/mL and 240 pg/mL $p < 0.001$ respectively. In contrast IL-8 and IL-10 were lower in NNI. All five cytokines appeared to play a role in seroconversion to anti-HBs. IFN- γ and IL-12 levels were highest in patients negative for HBV DNA, their median levels being 89.218 pg/mL and 67.23 pg/mL, respectively. HBeAg was independently associated with elevated levels of IL-8 and low levels of IL-12. Severe cases of liver disease, ESLD were significantly associated with elevated levels of TNF- α (190 pg/mL) and IL-8 (208 pg/mL) and depressed levels of IFN- γ (14.32 pg/mL), IL-12 (32.13 pg/mL) and IL-10 (16.52 pg/mL).

CONCLUSION: Both IFN- γ and IL-12 appear to play a dominant role in AHB and in causing HBV DNA and HBeAg negativity. Elevated levels were associated with best clinical status. IL-8 and

TNF- α was associated with presence of both HBV DNA and HBeAg pointing to its non-protective role. Low levels of IL-10 were an important finding in this study pointing to the patients inability to overcome the viral invasion. These results demonstrate that non-cytopathic antiviral mechanisms may contribute to viral clearance during acute HBV infection.

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Key words: Acute Hepatitis B Virus IL-8; IL-10; IL-12; IFN- γ ; TNF- α ; Immunopathogenesis; Necrotizing Inflammation; Non-necrotizing Inflammation; AntiHBs; HBeAg

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INTRODUCTION

Hepatitis B virus (HBV) is a non-cytopathic DNA virus that causes acute and chronic hepatitis as well as hepatocellular carcinoma^[1]. Patients who successfully clear acute HBV infection characteristically mount a strong, polyclonal and multi specific cytotoxic T lymphocyte (CTL) response to HBV^[2-6] that lasts for decades following recovery.

The immune determinants of successful clearance of HBV are not fully understood but it is clear that both cellular and humoral immune responses are important^[6,7,8]. Cytokines play an important role in the defence against viral infections, both directly, through inhibition of viral replication, and indirectly, through determination of the predominant pattern of host response. They may also lead to liver damage if involved in generation of an inflammatory response.

A complex interaction exists between HBV and the host in the initial clearance of HBV, the long term persistence of HBV and the pathogenesis of HBV liver disease. Recent studies have defined two T helper cell subsets, Th1 and Th2, which are characterized by distinct and mutually exclusive patterns of cytokine production and different functions^[9]. In our prior work, we have proposed an important association of IFN- γ and IL-12 with milder hepatitis and

lesser degree of damage to liver. We have also shown a pivotal role of IFN- γ and IL-12 in non-necrotizing inflammation^[10]. Genotypically, HBV is divided into eight groups, A-H. They are useful, clinical and epidemiological markers^[11]. Genotypic correlation has been associated with HBV core antigen, HBe antigen seroconversion and with activity of liver disease^[12,13].

The aim of the current study was to investigate the immune-regulatory role of IL-12, TNF- α , IFN- γ , IL-8 and IL-10 in various serological stages of acute hepatitis B (AHB) infection. For this purpose, we selected patients with acute HBV genotype D for different serological markers and assessed the cytokine levels in each category of patients to make a point cross sectional analysis of their possible role at each serological stage.

MATERIALS AND METHODS

The study was conducted over a period of one and a half years from August 2008 to July 2011 in the Department of Microbiology, J.N. Medical College, A.M.U., Aligarh. Eight hundred and forty 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 underwent complete physical examination and detailed clinical history was elicited from them. 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.

Exclusion criteria

Patients with autoimmune hepatitis, alcoholic hepatitis, drug induced hepatitis, patients giving history of recent infection, surgery, trauma within the preceding two months, renal insufficiency or with other acute or chronic inflammatory diseases were excluded from this study. None of the participants had received any antiviral or immunosuppressive therapy before or during the course of this study.

Necrotizing and non-necrotizing inflammation

Necrotizing and non-necrotizing inflammation were defined on the basis of biochemical and histopathological parameters^[10].

Necrotizing inflammation (NI): Liver function tests were considerably deranged and there was extensive damage to liver parenchyma. In NI significant necrosis (bridging necrosis and/or interface hepatitis) was seen.

Non-necrotizing inflammation (NNI): LFTs were either normal or mildly elevated and there was minimal to mild histopathological changes. In NNI insignificant necrosis (isolated dropout necrosis of hepatocytes) was observed.

Clinical grading

Clinical grading was done on the basis of biochemical investigations and clinical status of the patient into mild moderate, severe and end stage liver disease using the Model End Stage Liver Disease (MELD) score.

Healthy Control individuals

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

Collection of specimen

For all serological assays, venous blood samples were obtained after taking an informed consent. After centrifugation the serum was stored at -40°C until used for study.

Routine Investigations

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

Primary screening

All patients with hepatitis were screened for HAV, HBV, HCV, HEV and HIV by commercially available ELISA kits: 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). The tests were performed according to the manufacturer's instructions.

Patients positive for HBV were enrolled for further study. All cases of acute HBV infection were followed up for 6 months to assess seroconversion to anti-HBs (DRG International, Inc., USA). All cases which seroconverted to anti-HBs were included in acute HBV group. 83 cases of AHB were subjected to secondary screening.

Secondary screening

Serological status of the 83 AHB patients was determined using the following kits: HBeAg, anti-HBe, anti-HBc IgM (DRG International, Inc., USA) and anti-HBc Plus (Monolisa™, Bio-Rad, France).

Genotyping of HBV

DNA was extracted from 100 μ L serum by phenol chloroform extraction method. A 125 base pair sequence of the surface gene of HBV was amplified using a thermal cycler (Labnics, USA). A genotyping system based on PCR using type-specific primers was used in this study for the determination of genotypes D of hepatitis B virus according to previously described methods by Kirschberg *et al.*^[14]. 5- μ L DNA sample was mixed with the reaction mixture and a 25- μ L amplification reaction was performed. The reaction mixture contained 1 \times PCR buffer, MgCl₂ (1.5 mM), 200 mM 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 acute HBV genotype D individuals was assessed.

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

Cytokine profile of acute HBV genotype D individuals was assessed. To measure IL-12 IL-8, IL-10 & TNF- α levels in serum of HBV infected patients and in healthy controls, ELISA kits were procured from Orgenium, Finland while IFN- γ serum level was measured by ELISA kit from Diaclone, France. The ELISA kit protocols were followed as per the manufacturer's instructions. Absorbance was read at 450 nm in an automated ELISA plate reader (Thermo scientific). Standard curves of optical densities and corresponding concentrations of IL-8, IL-10, IL-12, IFN- γ and 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 were 2 pg/mL while TNF- α and IFN- γ assay were 9 pg/mL and 5 pg/mL. A point study was done to evaluate the levels of IL-8, IL-10, IL-12, IFN- γ and TNF- α in the patients in relation to the HBV markers in necrotizing and non-necrotizing inflammation.

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. ROC curve was used to analyze the cytokines using Med Calc programme. The level of significance in all cases was set at a two tailed $p < 0.05$.

RESULTS

Detection of HBV Genotypes

Genotype D was detected in 76 (90.78%) out of 83 AHB patients.

Clinical profile

Majority of these patients presented with nausea, fever, jaundice, vomiting, anorexia and hepatomegaly.

Of 76 AHB genotype D patients, 54 (71.31%) had necrotizing inflammation (NI) and 22 (28.69%) had non-necrotizing inflammation (NNI). According to MELD scoring 53 (69.73%) had mild, 15 (19.73%) moderate and 8 (10.52%) had severe end stage liver disease (ESLD).

Biochemical profile

Biochemical profile of liver parameters of the study group is given in table 1. All the parameters were significantly elevated. AST and ALT levels were approximately five fold higher than normal.

Table 1 Biochemical profile of 76 patients with AHB genotype D.

Parameters	Acute HBV patients
AST	160.33 (IU/L) ¹
ALT	127.16 (IU/L) ¹
ALP	19.91 (kau/100 mL) ¹
Serum Bilirubin	13.66 (mg/100 mL) ¹
Necrotizing Inflammation	22
Non-necrotizing Inflammation	54

¹ shows mean values.

HBV serological markers

Forty four (61.84%) cases were HBsAg and Twenty nine (38.15%) cases were Anti-HBs positive. Fifty five (73.5%) cases were positive for HBeAg. Thirty three (44.72%) cases of AHB were Anti-HBc IgM positive.

Serum cytokine levels

IL-8, IL-10, IL-12, IFN- γ and TNF- α were significantly elevated in the study group in comparison to the healthy controls as seen in table 2. The levels of individual cytokines were analyzed using receiver operative characteristics. The details are given in table 3.

Table 2 Cytokine profile of patients with AHB genotype D.

Cytokines	Healthy controls (n=20)		Acute HBV patients (n=76)		F-Test p-value
	Median+S.D.	Range	Median+S.D.	Range	
IL-8 pg/mL	16.37±19.78	11.78-67.77	202.32±88.37	20-271	0.001
IL-10 pg/mL	8.37±15.07	0.39-51.98	22.38±142.09	1-414	0.504
IL-12 pg/mL	25.44±12.46	10.76-50.92	54.60±16.74	17-76	<0.001
TNF-alpha pg/mL	146±13.65	125.81-166.57	176.32±62.58	124-325	<0.001
IFN-gamma pg/mL	11.82±7.39	5.47-17.05	27.26±7.39	9-32	0.039

Table 3 Analysis of IFN- γ , IL-12, TNF- α , IL-10 and IL-8 by receiver operator characteristics.

Cytokines	Sensitivity	Specificity	+PV	-PV	AUC	p-value
IFN- γ	52.94	100.00	100.0	52.9	0.814	0.0002
IL-12	80.77	57.14	87.5	44.4	0.706	0.0566
IL-8	90.00	85.71	97.3	60.0	0.936	<0.0001
IL-10	50.00	90.00	95.2	31.0	0.688	0.0361
TNF- α	62.50	100.00	100.0	34.8	0.772	<0.0001

+PV: positive predictive value; -PV: negative predictive value; AUC: area under curve; significant p-value is <0.05.

In the healthy controls, median IFN- γ , IL-12, TNF- α , IL-8, IL-10 levels were 11.82 pg/mL, 25.44 pg/mL, 146 pg/mL, 16.37 pg/mL, 8.37 pg/mL, respectively.

In the study group, median IFN- γ , IL-12, TNF- α , IL-8 levels were significantly higher than the healthy controls at 27.26±7.39 pg/mL ($p < 0.001$), 54.6 pg/mL ($p < 0.05$), 176.32 pg/mL ($p < 0.001$), 202.32 pg/mL ($p < 0.0001$) respectively. IL-10 levels at 22.38 pg/mL although higher, were not significantly elevated.

Comparison of IFN- γ , IL-12, TNF- α , IL-8, IL-10 levels in NI and NNI (Figure 1)

In the NI group, median IFN- γ level was 32.17 pg/mL. In NNI cases, median IFN- γ level was higher at 52.351 pg/mL. IL-12 levels in NI cases were 42 pg/mL while in NNI, IL-12 levels were higher at 57.12 pg/mL. TNF- α levels in NI were 150 pg/mL while in NNI they were highly elevated (240pg/mL) $p < 0.001$. In contrast IL-8 and IL-10 were lower in NNI. IL-8 levels in NI were 160pg/mL while they were extremely low in NNI cases (20 pg/mL) $p < 0.0001$. IL-10 levels in NI were 21.978 pg/mL while they were significantly low in NNI cases (11.51 pg/mL) $p < 0.0001$.

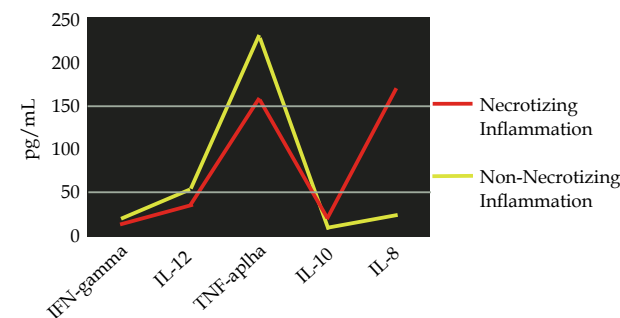


Figure 1 Serum levels of IFN- γ , IL-12, TNF- α , IL-10 and IL-8 in acute HBV in NI and NNI.

Association of cytokines with anti-HBs

All five cytokines appeared to play a role in seroconversion to anti-HBs as is seen in figure 2. IL-8 followed by TNF- α level was extremely high as compared to antiHBs negative patients. On statistical analysis of positive and negative anti-HBs patients by paired samples t-test, a strong association was observed in case of IL-8, TNF- α and IL-12. While IL-8, TNF- α and IFN- γ were independently associated with seroconversion to anti HBs when assessed by multiple logistic regression (Table 4).

Association of cytokines with HBV DNA

IFN- γ and IL-12 levels were highest in patients negative for HBV

DNA, their median levels being 89.218 pg/mL and 67.23 pg/mL, respectively. IL-8 levels were low in HBV DNA negative cases. IL-10 levels were largely suppressed in this group.

Association of cytokines with HBeAg status

IFN- γ and IL-12 levels were much higher in cases of HBeAg seronegative individuals, mean levels being 20.77 pg/mL and 36.53 pg/mL respectively as seen in figure 3. On statistical analysis of positive and negative HBeAg patients by paired-samples *t*-test, a strong association was observed in case of IL-8, TNF- α , IFN- γ and IL-12. Using multiple linear regression analysis, presence of HBeAg was independently associated with elevated levels of IL-8 and low levels of IL-12. IFN- γ , TNF- α and IL-10, although higher in HBeAg negative individuals was not independently related.

Association of cytokines with clinical status of patients

The patients were divided into three clinical groups: mild, moderate and severe liver disease. Severe cases of liver disease, ESLD were significantly associated with elevated levels of TNF- α (190 pg/mL) and IL-8 (208 pg/mL) and depressed levels of IFN- γ (14.32 pg/mL), IL-12 (32.13 pg/mL) and IL-10 (16.52 pg/mL). Cases with mild and moderate disease were associated with lower levels of IL-8 (112 pg/mL) and TNF alpha (160 pg/mL) and higher levels of IFN- γ (32.58 pg/mL), IL-12 (75.79 pg/mL) and IL-10 (35.82 pg/mL). It was further observed that in cases where both IFN- γ , IL-12 were elevated, prognosis was most favorable.

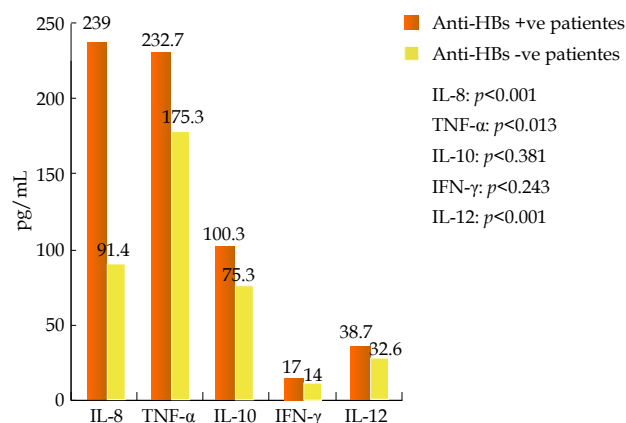


Figure 2 Association of various cytokines with Anti-HBs status and statistical significance was calculated by paired-samples *t* test; *p*<0.05 was considered significant.

Table 4 Multiple linear regression analysis to study the independent variable predicting HBeAg, anti-HBe and anti-HBs in Acute HBV Patients.

Independent Variables	Dependent Variables					
	HBe Ag		Anti-HBe		Anti-HBs	
	Coefficient	<i>p</i> -value	Coefficient	<i>p</i> -value	Coefficient	<i>p</i> -value
IL-8	0.004182	<0.0001	-0.006874	<0.0001	0.002196	<0.0001
IL-10	-0.0005662	0.3536	-0.0001337	0.8668	0.0005696	0.0745
IL-12	0.03918	<0.0001	0.0137	0.1757	-0.0035	0.3797
TNF-alpha	-0.0001574	0.9329	-0.0111	<0.0001	0.004794	<0.0001
IFN-gamma	-0.01615	0.2249	-0.02234	0.2007	0.03147	<0.0001

DISCUSSION

In this study we analyzed the serum concentrations of immunoregulatory cytokines (IFN- γ , IL-12), pro-inflammatory cytokines (TNF- α , IL-8) and anti-inflammatory cytokine IL-10 in genotype D AHB cases. We also assessed the cytokine levels in relation to NI and NNI, HBeAg status, presence of HBV DNA in

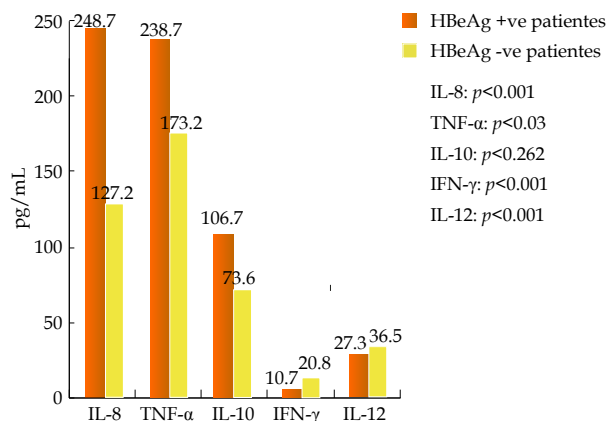


Figure 3 Association of various cytokines with HBeAg status and statistical significance was calculated by paired-samples *t* test; *p*<0.05 was considered significant.

serum and the clinical status of the patients.

Levels of IFN- γ , IL-12, TNF- α and IL-8 were significantly higher in AHB genotype D patients in comparison to the healthy controls. On the other hand IL-10 was largely suppressed and the levels were not significantly different from those of the controls.

As seen in our previous study IL-12 levels were higher than IFN- γ in AHB^[10] suggesting the former's pre-eminent role in genotype D as well. Interestingly all five cytokines appear to play a significant role in seroconversion from HBSAg to anti-HBs as the levels of all of them were significantly raised with that of IL-8 being maximally elevated.

Our observations are in agreement with other studies which observed elevated levels of IFN- γ in HBV patients with different clinical presentations^[15,16]. In acute HBV infection, a strong cell-mediated Th1 response, characterized by IL-2 and IFN- γ , is mounted against HBV and is involved in viral clearance^[1,4] and immunopathogenesis of liver disease^[1,5]. IFN- γ is also a major contributor of viral clearance by activating macrophages, as well as rendering neighboring uninfected cells resistant to infection^[6].

Among patients with hepatitis B or hepatitis C infection, those who cleared the virus had higher serum IL-12 levels than chronic virus carriers^[17], and their CD4 T cells preferentially produced IFN- γ and IL-2 in response to viral antigens^[7,18].

IFN- γ and IL-12 levels were highest in patients negative for HBV DNA. IL-8 levels in contrast were low in these cases. IL-10 levels were again largely suppressed in this group. The high levels of IFN- γ and IL-12 may be associated with absence of HBV DNA which points to their possible role in decreasing viral load. In a study by Ozkan *et al*^[19] a negative correlation was found between serum HBV-DNA copies and interferon gamma levels.

IFN- γ and IL-12 levels were much higher in cases of HBeAg seronegative individuals while presence of HBeAg was associated with elevated levels of IL-8 and TNF- α . However, a study by Song *et al* did not find evidence of altered IL-12 activity related to HBeAg positivity^[15]. The role of TNF- α is important not only in virus exclusion, but also in liver injury. A pro-inflammatory cytokine, TNF- α is thought to play a critical role in acute viral hepatitis^[20,21,22].

IL-8 was associated with presence of both HBV DNA and HBeAg pointing to its non-protective role. In addition, IL-8 has been examined as a marker of liver damage due to its ability to inhibit the antiviral activity of IFN- α ^[23,24].

In our study NI was associated predominantly with IL-8. Higher levels of IFN- γ , IL-12 and TNF- α were associated with NNI

suggesting their greater role in controlling HBV infection by non cytolytic mechanism. Steepest elevation in NNI cases was observed in case of TNF- α which points to its dominant role in this condition. TNF- α production is one of the earliest events in many types of liver injuries, and it triggers the production of other cytokines that together recruit inflammatory cells, kill hepatocytes, and initiate a hepatic healing response that includes fibrogenesis. In contrast, a sharp decline occurred in IL-8 in this group. IL-10 also declined in NNI although the difference was not significant. NNI cases had lower or normal levels of liver enzymes and patients were relatively stable. Thus higher levels of IFN- γ , IL-12 and TNF- α appear to tilt the clinical spectrum towards improved profile of liver markers and better clinical status. Low levels of IL-10 in NNI was surprising, as it is known that IL-10 may prevent development of severe liver damage by suppressing immune responses to HBV infection. It can be surmised that low IL-10 levels lead to the higher levels of liver enzymes and maybe to chronicity^[25]. IL-10 deficiency not only enhances liver injury but also results in lower viral clearance from this site. Similar low levels were observed in the study by Song *et al*^[15]. High levels of IL-10 are usually seen in non-viral hepatitis which may be the reason for the low elevations of ALT and AST and lower levels of NI in this group (in communication). Ineffective levels of IL-10 may translate to correspondingly higher levels of IFN- γ , IL-12, IL-8 and TNF- α as it is known that elevated IL-10 is capable of inhibiting synthesis of pro-inflammatory cytokines.

Our results demonstrate that non-cytopathic antiviral mechanisms may contribute to viral clearance during acute HBV infection. In our study it was seen that in mild liver disease IFN- γ , IL-12 and TNF- α were elevated and in conditions where any of the three were depleted, moderate to severe liver disease was observed. This finding suggests that together they are pivotal in stabilizing the clinical condition. Low levels of IL-10, another important finding in this study could be the reason for the inability of such patients to overcome viral invasion, leading to chronicity.

CONCLUSION

Attributing precise roles to any given cytokine in the context of HBV pathogenesis or clinical progression is complicated. However this study clearly highlights the protective role of IFN- γ and IL-12, non protective role of IL-8, and dual role of TNF- α . Interventions leading to elevated IL-10 levels may be beneficial in suppressing the pro-inflammatory cytokines and reducing liver damage. However, its dubious role in causing persistence of HBV needs to be explored further. Clinical stability, HBsAg seronegativity and HBV DNA absence were associated with high levels of IFN- γ and IL-12.

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