P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(9):2009-2011
World Journal of Gastroenterology
 ISSN 1007-9327

• VIRAL HEPATITIS •

Abnormal immunity and gene mutation in patients with severe hepatitis-B

Jing-Yan Wang, Pei Liu

Jing-Yan Wang, Pei Liu, Department of Infectious Diseases, the Second Hospital, China Medical University, Shenyang 110004, Liaoning Province, China
Supported by the National Natural Science Foundation of China, No.39370649
Correspondence to: Pei Liu M.D., Ph.D., Department of Infectious Diseases, the Second Hospital, China Medical University, Shenyang 110004, Liaoning Province, China. syliupei2003@yahoo.com.cn
Telephone: +86-24-83956962 **Fax:** +86-24-23891973
Received: 2003-03-10 **Accepted:** 2003-04-19

Abstract

AIM: To evaluate the abnormal immunity and gene mutation at precore 1896 site in patients with severe hepatitis-B.

METHODS: This study included 23 patients with severe hepatitis-B, 22 patients with acute hepatitis-B and 20 controls. Mutation at precore 1896 site of HBV gene was confirmed with restriction fragment length polymorphism (RFLP) analysis. Cytokines including TNF-α, IFN-γ, IL-6, and IL-8 were measured with ELISA, and T subgroups were detected with alkaline phosphatase anti alkaline phosphatase (APAAP) technique.

RESULTS: In patients with severe hepatitis-B, the infective rate of HBV mutant strain was 52.5 % (12/23), and only one patient with acute hepatitis-B was infected with the mutant strain. The percentage of CD8+ T lymphocyte was obviously lower (0.16±0.02 %) and the ratio of CD4+/CD8+ was obviously higher (2.35±0.89) in mutant group than in wild-type group (0.28±0.05 % and 1.31±0.18 %, respectively, $P<0.01$ or $P<0.05$). The levels of cytokines in patients with severe hepatitis-B were higher (TNF-α 359.0±17.2 ng/L, IFN-γ 234.7±16.5 ng/L, IL-6 347.5±31.3 ng/L, IL-8 181.1±19.6 ng/L) than those in acute hepatitis-B (TNF-α 220.6±8.9 ng/L, IFN-γ 174.9±12.0 ng/L, IL-6 285.8±16.5 ng/L, IL-8 118.4±5.1 ng/L, $P<0.01$ or 0.05). In patients with severe hepatitis-B, the levels of IFN-γ and IL-6 were higher in mutant group (273.4±26.6 ng/L, 387.7±32.5 ng/L) than in wild-type group (207.8±12.8 ng/L, 300.9±16.3 ng/L). The mortality of patients infected with HBV mutant strain was higher (100 %) than that with wild-type (0.9 %).

CONCLUSION: In severe hepatitis-B, the infective rate of HBV mutant strain was high. The mutant strain induces more severe immune disorders in host, resulting in the activation of lymphocyte and release of cytokines. HBV DNA mutates easily in response to the altered immunity. Ultimately liver damage is more prominent.

Wang JY, Liu P. Abnormal immunity and gene mutation in patients with severe hepatitis-B. *World J Gastroenterol* 2003; 9(9): 2009-2011
http://www.wjgnet.com/1007-9327/9/2009.asp

INTRODUCTION

HBV destroys hepatocytes by changing the host immune system after its invasion into the body. Various clinical types of hepatitis are associated with different immune status of the body. The serious damage of hepatocytes constitutes the pathological basis of severe hepatitis. At present, studies about the mechanism of hepatocytic damage in severe hepatitis B focus mainly on HBV gene mutation and immunologic abnormalities. HBV gene mutation at precore 1896 site has been already detected with restriction fragment length polymorphism (RFLP). In this study, we explored the relationships between HBV gene mutation and immune status in severe hepatitis. T subgroups in peripheral blood and levels of TNF-α, IFN-γ, IL-6, and IL-8 associated with severe hepatitis were measured.

MATERIALS AND METHODS

Patients

From 1995 to 1998, 45 patients with severe hepatitis or acute hepatitis were included in this study. There were 23 cases (17 men and 6 women) of severe hepatitis and 22 cases (15 men and 7 women) of acute hepatitis. The patients with severe hepatitis aged from 8 to 62 years and those with acute hepatitis from 11 to 65 years. The diagnosis was based on the diagnostic criteria revised at the National Infectious Disease and Parasitosis Conference in 1999 (Xian, china). All samples were obtained on the day of admission. The serological markers of HBsAg and HBV DNA were positive, and those of HAV, HCV, HDV, HEV, anti-EBV-IgM, and anti-CMV-IgM were all negative.

We also studied 20 healthy blood donors aged from 25 to 43 years, including 16 men and 4 women. They had no serological markers of HAV, HBV, HCV, HDV, HEV, and their liver functions were normal.

Analysis of restriction fragment length polymorphism (RFLP)[1]

Two pairs of primers were used in the analysis of RFLP. The primers used for the first round of PCR were 5'-GGCGAGGGAGTTCTTCTTCTAGGGG-3' (2 394 to 2 370 nucleotides) and 5'-CTGGGAGGAGTTGGGGGAGG AGATT-3' (1 730 to 1 754 nucleotides). The primers for the second round of PCR were 5'-CAAGCTGTGCCTTGGGTG GCCTT-3' (1 873 to 1 895 nucleotides), which was a mismatch primer, and 5'-GGAAAGAAGTCAGAAGGCAA-3' (1 974 to 1 955 nucleotides). All of the primers were synthesized by the Shanghai Cytobiology Institute. Huamei Company provided dNTP, PGEM-7Zf(+)Hae III, and the marker of DNA molecular weight. Bsu 36 I enzyme and relevant buffer, TaqDNA polymerase and PCR buffer were from Promega Company. HBV DNA was extracted from serum samples by using the standard method. Serum (100 μl) was treated with proteinase K, phenol and chloroform, and then precipitated with ethanol. The final products served as the amplification template of PCR. The fluorescent zone after electrophoresis was observed by ultraviolet light.

Staining of T subgroups

Monocytes were purified from the samples of anticoagulant blood using lymphocytic laminated fluid. The monoclonal

antibodies to CD4+ and CD8+ were purchased from DAKO (Denmark). The percentages of CD4+ and CD8+ were counted under microscope after staining with APAAP.

Detection of cytokines

The kits of TNF-α, IFN-γ, IL-6, and IL-8 were produced by the Genzyme Company (USA). The four cytokines were detected with ELISA. The first antibody was biological antibody and the second one was peroxidase-labelled streptavidin.

Statistical analysis

All values were expressed as means ± standard deviation. Chi-square test was performed. It was considered statistically significant when the *P* value was less than 0.05.

RESULTS

Mutation at precore 1896 site of HBV DNA from patients with severe hepatitis B

Among the 23 cases of severe hepatitis, 8 were simple infection of the mutant strain, 4 coinfection of the wild and mutant strains, and 11 simple infection of the wild strain. Only 1 case had mutation in precore region among the 22 cases of the acute hepatitis.

Effects of mutation at HBV DNA precore 1896 site on status of T subgroups in peripheral blood from patients with severe hepatitis

As shown in Table 1, the percentage of CD8+ in patients with severe hepatitis in mutant group (0.16±0.02) decreased more obviously than that in patients in the wild-type group (0.28±0.05), and the ratio of CD4+/CD8+ in mutation group (2.35±0.89) increased more significantly than that in wild-type group (1.31±0.18).

Table 1 Status of T subgroups in the peripheral blood from patients with severe hepatitis (%, $\bar{x}\pm s$)

Group	*n*	CD3+	CD4+	CD8+	CD4+/CD8+
Control	20	0.60±0.17	0.46±0.05	0.33±0.03	1.39±0.18
AH	22	0.61±0.06	0.38±0.04[a]	0.33±0.04	1.16±0.20
FH	23	0.53±0.07[a]	0.37±0.03[a]	0.25±0.06[a]	1.63±0.30[a]
Mutation	12	0.15±0.08	0.37±0.12	0.16±0.02[b]	2.35±0.89[b]
Wild	11	0.56±0.04	0.36±0.04	0.28±0.05	1.31±0.18

[a]$P<0.01$ *vs* control; [b]$P<0.05$ *vs* wild type. AH: acute hepatitis, FH: severe hepatitis.

Serum levels of TNF-α, IFN-γ, IL-6 and IL-8 from patients with mutation at HBV DNA precore 1896 site in severe hepatitis

Serum levels of TNF-α, IFN-γ, IL-6, and IL-8 in patients with mutation at HBV DNA precore 1896 site in patients with severe hepatitis are shown in Table 2.

The levels of IFN-γ and IL-6 in patients with severe hepatitis in mutant group (273.4±26.6 ng/L, 387.7±32.5 ng/L) increased more significantly than that in patients in the wild-type group (207.8±12.8 ng/L, 300.9±16.3 ng/L) ($P<0.05$).

The dynamic changes of the 4 cytokines are shown in Figure 1 and Figure 2. IFN-γ reached a peak and then decreased rapidly at the early stage of severe hepatitis with infection of mutant strain and acute hepatitis with infection of wild- type. TNF-α and IL-6 increased in acute stage and reached the highest level accompanied by the most severe jaundice, then decreased gradually. Both maintained a high level for a long time in severe hepatitis. IL-8, TNF-α, and IL-6 experienced the same change in severe hepatitis with mutation at HBV DNA precore 1896 site (173.5±10.7 ng/L, 356.6±18.1 ng/L, 387.7±32.5 ng/L) as in the course of acute hepatitis with infection of wild-type (173.1±11.3 ng/L, 328.4±14.6 ng/L, 300.9±16.3 ng/L).

All of the 12 patients in the mutant group died, and only 1 died among 11 patients in the wild-type group.

Table 2 The serum levels of TNF-α, IFN-γ, IL-6, and IL-8 in patients with mutation in HBV DNA precore 1896 site (ng/L, $\bar{x}\pm s$)

Group	*n*	TNF-α	IFN-γ	IL-6	IL-8
Control	20	146.7±9.4	65.0±7.7	231.1±16.4	110.2±2.9
AH	22	220.6±8.9	174.9±12.0	285.8±16.5	118.4±5.1
FH	23	359.0±17.2[bc]	234.7±16.5[bc]	347.5±31.3[bd]	181.1±19.6[a]
Mutation	12	356.6±18.1	273.4±26.6[e]	387.7±32.5[e]	173.5±10.7
Wild	11	328.4±14.6	207.8±12.8	300.9±16.3	173.1±11.3

[a]$P<0.05$ *vs* control; [b]$P<0.01$ *vs* control; [c]$P<0.05$ *vs* AH; [d]$P<0.01$ *vs* AH; [e]$P<0.05$ *vs* wild strain. AH: acute hepatitis, FH: severe hepatitis.

DISCUSSION

Severity of hepatitis caused by HBV varies greatly. The mechanisms determining the course and outcome of hepatitis B have not been known. Many investigations suggested that HBV was not directly cytopathic. The injury was mediated by the immune response[2]. Injury of hepatocytes in acute hepatitis was mediated by CD8+ CTLs recognizing the core protein of HBV presented in association with HLA class I proteins[3]. TNF-α and IFN-γ played indispensable roles in liver injury mediated by Th1 cells specific to HBV surface antigen[4]. Muto *et al*[5] suggested that the main immunologic abnormalities in severe hepatitis were due to TNF-α produced by monocytes in peripheral blood. Pretreatment with anti-TNF-α mAb in animal model strongly blocked Th1 cell-inducible liver injury[6]. TNF-α might be enhanced in animal models with impaired liver metabolism[7], and serum TNF-α took part in the pathogenesis of chronic hepatic failure of HBV infection[8]. The study of transgenic mice indicated that IFN-γ triggered the widespread hepatocellular necrosis. The cytopathic effect of IFN-γ was indirect, presumably due to recruitment and activation of antigen nonspecific host inflammatory cells. Immunopathological changes induced by CTL contributed to immunopathogenesis of viral hepatitis during hepatitis B virus infection in humans. IFN-γ activated the killing activity of CTL[9] and had killing activity to regenerating hepatocytes. Similarly, IL-6 was involved in the activation of NK cells and CTLs, inducing their killing activity to hepatocytes.

The serum levels of TNF-α, IFN-γ, IL-6, and IL-8 in patients with severe hepatitis were significantly higher than those in patients with acute hepatitis, and peripheral CD8+ was obviously lower, especially in mutant group. These findings suggest that cytokines and immunocytes associated with inflammation may play roles in the pathogenesis of severe hepatitis. TNF-α, IFN-γ and IL-6 may involve in hepatocytic necrosis and apoptosis. Remarkable increase of IL-8 leads to accumulation of CTL which gets direct and immediate assess to the target hepatocytes and the resident intrahepatic macrophages, and this constitutes the immunopathological basis of hepatocyte killing. CTLs that enter and reside in the liver, have the ability to bind to and kill the HBsAg-positive hepatocytes, and to activate an intrahepatic inflammatory response[4].

Omata *et al*[10] reported in 1991 that HBV DNA precore 1896 site mutation had some relationship with severe hepatitis B. Translational termination codon which was produced after

a point mutantion from G to A at nucleotide 1896 of precore region of hepatitis B virus DNA, converting tryptophan (TGG) to a stop codon (TAG) would interrupt the synthesis of HBeAg precursor protein, that may decrease or eliminate HBeAg. The presence of mutant viral strain is associated with and may be involved in the pathogenesis of severe hepatitis B and exacerbation of chronic hepatitis B. In our study the mortality was extremely high in patients with HBV DNA precore 1896 site mutation. It might be related to the immunologic abnormalities in severe hepatitis induced by HBV DNA precore 1896 site mutation[11]. It was also found that the percentages of CD8+ in the peripheral blood in patients with severe hepatitis in mutant group were much lower than those in patients in wild-type group, which was the result of the relative diminish of CD8+ infiltrating into the liver tissue in severe hepatitis. Meanwhile, the levels of IFN-γ and IL-6 increased significantly in severe hepatitis, indicating that HBeAg not only had effects on the regulation of CTL in killing hepatocytes, but also had certain effect on producing cytokines by the immunocytes. A cross-reactivition of c and e antigens at the T cell recognition sites was found by examining T cell responses to recombinant c and e antigens. When the expression of HBeAg on the surface of hepatocytes decreased or disappeared, the attacking action of specific CTL to c antigen and the target antigen through T cell-mediated cytotoxicity enhanced, resulting in massive necrosis of hepatocytes with HBV infection and deterioration of hepatitis because specific CTL bound to HBcAg which is the most possible main target for immune hepatocytolysis on hepatocytic nuclei was obviously increased[12-14]. HBV strains with mutation in the precore region that abort the translation of hepatitis B e antigen precursor, resulted in the formation of HBeAg-minus phenotype. Circulating HBeAg has been proposed as a viral strategy to induce immunotolerance[15]. Its absence, therefore, would accelerate inflammatory activity in the liver[16] which could be relevant to the pathogenesis of severe hepatitis. On the other hand, HBeAg and HBcAg were expressed simultaneously in hepatocytic membrane with HBV infection and became the target antigen of CTL[17]. Meanwhile, the activity of T suppressor cell (Ts) was weakened. As a result, the ability of Ts to regulate T cells specific to HBc/HBeAg was decreased. So the production of specific CTL to HBc/HBeAg was increased and hepatocytic necrosis exacerbated. Our investigation indicated that in severe hepatitis the abnormal immunity of patients in mutant group was due to the following aspects: (1) The levels of cytokines were abnormal compared with the wild-type, particularly IFN-γ. (2) Hepatocytic apoptosis induced by IL-6 produced by monocytes and vascular endotheliocytes might take part in the pathogenesis of fulminant hepatitis. (3) Overexpression of TNF receptors induced by IFN-γ in hepatocytes might correlate with the severe liver damage[18]. (4) Activation and aggregation of CTL in the liver enhanced the killing activity to hepatocytes and caused deterioration of liver damage. It is thus speculated that in severe hepatitis, HBV mutation might be resulted from response to immune defense reaction of the host body, or conversely, mutant HBV DNA might produce more significant immune disorders in the host body. Clinically, exploration for HBV gene mutation in patients with acute hepatitis might provide some useful hints for estimating the state of illness and predicting the prognosis.

REFERENCES

1 **Niitsuma H,** Ishii M, Miura M, Toyota T. Detection of HBV precore mutation by PCR-RFLP. *Nippon Rinsho* 1995; **53 Suppl (Pt 2)**: 316-320

2 **Chu CM.** Natural history of chronic hepatitis B virus infection in adults with emphasis on the occurrence of cirrhosis and hepatocellular carcinoma. *J Gastroenterol Hepatol* 2000; **15(Suppl)**: E25-30

3 **Bertoletti A,** Sette A, Chisari FV, Penna A, Giuberti T, Levrero M, De Carli M, Fiaccadori F, Ferrari C. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature* 1994; **369:** 407-410

4 **Ohta A,** Sekimoto M, Sato M, Koda T, Nishimura S, Iwakura Y, Sekikawa K, Nishimura T. Indispensable role for TNF-alpha and IFN-gamma at the effector phase of liver injury mediated by Th1 cells specific to hepatitis B virus surface antigen. *J Immunol* 2000; **165**: 956-961

5 **Muto Y,** Nouri-Aria KT, Meager A, Alexander GJ, Eddleston AL, Williams R. Enhanced tumour necrosis factor and interleukin-1 in fulminant hepatic failure. *Lancet* 1988; **2:** 72-74

6 **Tanaka Y,** Takahashi A, Watanabe K, Takayama K, Yahata T, Habu T, Nishimura T. A pivotal role of IL-12 in Th1-dependent mouse liver injury. *Int Immunol* 1996; **8:** 569-576

7 **Lehmann V,** Freudenberg MA, Galanos C. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. *J Exp Med* 1987; **165:** 657-663

8 **Zhang DF,** Ren H, Jia XP, Zhou YS. Serum tumor necrosis factor (TNF) in the pathogenesis of clinical hepatic failure of HCV and / or HBV infection. *Chin Med J* 1993; **106:** 335-338

9 **Ando K,** Moriyama T, Guidotti LG, Wirth S, Schreiber RD, Schlicht HJ, Huang SN, Chisari FV. Mechanisms of class I restricted immunopathology: A transgenic mouse model of fulminant hepatitis. *J Exp Med* 1993; **178:** 1541-1554

10 **Omata M,** Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N Engl J Med* 1991; **324:** 1699-1704

11 **Sato S,** Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, Tsuda F, Tanaka T, Okamoto H, Miyakawa Y. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. *Ann Intern Med* 1995; **122**: 241-248

12 **Yamada G,** Takaguchi K, Matsuda K, Nishimoto H, Takahashi M, Fujiki S, Mizuno M, Kinoyama S, Tsuji T. Immunoelectron microscopic observation of intrahepatic HBeAg in patients with chronic hepatitis B. *Hepatology* 1990; **12:** 133-140

13 **Hadziyannis SJ,** Vassilopoulos D. Hepatitis B e antigen-negative chronic hepatitis B. *Hepatology* 2001; **34(4Pt1):** 617-624

14 **Park YN,** Han KH, Kim KS, Chung JP, Kim S, Park C. Cytoplasmic expression of hepatitis B core antigen in chronic hepatitis B virus infection: role of precore stop mutants. *Liver* 1999; **19:** 199-205

15 **Milich DR,** Jones JE, Hughes JL, Price J, Raney AK, Mclachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? *Proc Natl Acad Sci U S A* 1990; **87:** 6599-6603

16 **Brunetto MR,** Giarin MM, Oliveri F, Chiaberge E, Baldi M, Alfarano A, Serra A, Saracco G, Verme G, Will H. Wild-type and e antigen-minus hepatitis B viruses and course of chronic hepatitis. *Proc Natl Acad Sci U S A* 1991; **88:** 4186-4190

17 **Yotsuyanagi H,** Hino K, Tomita E, Toyoda J, Yasuda K, Iino S. Precore and core promoter mutations, hepatitis B virus DNA levels and progressive liver injury in chronic hepatitis B. *J Hepatol* 2002; **37:** 355-363

18 **Ruggiero V,** Tavernier J, Fiers W, Baglioni C. Induction of the synthesis of tumor necrosis factor receptors by interferon-gamma. *J Immunol* 1986; **136:** 2445-2450

Edited by Zhu LH and Wang XL