

Proliferation and Function of CD4⁺ and CD8⁺ T Cells from Chronic Hepatitis B Patients

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ABSTRACT

Objective: Chronic hepatitis B virus infection (CHB) is involved in a deviated immune response to clear the virus. The disease process is caused by CD8⁺ T cell response. The mechanisms underlying different clinical manifestations of CHB are not clearly known. The present study investigated CD4⁺ and CD8⁺ T cell responses to hepatitis B virus (HBV) infection, in terms of cell proliferation and CD69, IL-4, IFN- γ , perforin and granzyme B expression.

Methods: A HBV peptide, FLLTRILTI and ionomycin/phorbol myristate acetate and phytohemagglutinin were used to stimulate peripheral blood mononuclear cells (PBMC) from patients with chronic HBV infection. The expression of CD69, IL-4, IFN- γ , perforin and granzyme B as well as cell proliferation was assessed.

Results: All subjects had similar CD69 expression and cell proliferation. However, the number of IL-4⁺ CD4 T cells increased in hepatitis B e-antigen (HBeAg)-positive CHB patients with increased alanine aminotransferase (ALT), HBV carriers, and HBeAg-positive CHB patients with normal ALT. IFN- γ ⁺ CD4 T cells increased in HBeAg-positive and -negative CHB patients with increased ALT and also in HBV carriers. However, granzyme B-positive CD8⁺ T cells decreased in HBeAg-negative and -positive CHB patients with normal ALT.

Conclusion: The present study demonstrated that the different CD4 and CD8 T cell responses may underlie the hepatitis outcomes of CHB.

Keywords: CD4 T cell, CD8 T cell, chronic HBV infection, granzyme B, perforin

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INTRODUCTION

Hepatitis B infection is the major health problem worldwide. There are two billion people infected with HBV and it has been estimated that 350 million people are HBV carriers.^{1,2} Most of these patients live in Asia-Pacific region and Africa. In Thailand, the prevalence of chronic hepatitis B infection is about 5-10%.³ The HBV infection results in various clinical outcomes including acute hepatitis, chronic hepatitis, HBV carrier and fulminant hepatitis leading to a morbidity and mortality.⁴ Chronic hepatitis B infection (CHB) patients include HBeAg-positive CHB and HBeAg-negative CHB patients and HBsAg carriers. These patients have significant risks to develop cirrhosis and hepatocellular carcinoma which result in health facility burdens.⁵⁻⁷ Efficacy

of HBV treatment nowadays is also limited.⁸⁻¹⁰ Many factors such as HBV-DNA level, HBV genotype, host immune response and underlying liver diseases can influence the disease natural history and treatment outcome.¹¹

The immune system plays a protective action in the human body to eliminate foreign substances. The function of innate and adaptive immune systems are believed to be major factors for different clinical manifestations in chronic hepatitis B infection.^{12,13} Innate immune response occurs predominantly in the acute phase of HBV infection. However, decreased cytokine production by NK cells has been observed in chronic HBV infection which may contribute to viral persistence.¹⁴ Adaptive immunity plays a pivotal role in chronic HBV infection. Impairment of CD4⁺ and CD8⁺ T cell responses has been observed in CHB patients.¹⁵⁻¹⁶ Nevertheless, T cell responses in different groups of CHB patients has rarely been reported. We hypothesized that HBeAg-positive, HBeAg-negative CHB patients and HBsAg carriers might have altered T cell function and response. Thus, T cell proliferation and cytokines and cytotoxic granules produced by T cells from CHB patients and HBV carriers were investigated and compared with normal subjects.

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MATERIALS AND METHODS

Subjects

The subjects were patients who had been HBsAg positive for at least 6 months and attended the Out-Patient Department of Naresuan University Hospital. They were divided into HBeAg-positive CHB with increased ALT (n=6), HBeAg-negative CHB with increased ALT (n=4) and HBV carriers (n=4), HBeAg-positive CHB with normal ALT (n=2), and HBeAg-negative CHB with normal ALT (n=6). All the patients were followed up for ALT at least twice in every three months. The mean HBV DNA levels in these patient groups were more than 17.16×10^6 IU/ml, 13.2×10^6 IU/ml, less than 54.5 IU/ml, 128×10^6 IU/ml and 4.68×10^5 IU/ml, respectively. HBV DNA levels were persistently more than 20,000 IU/ml in tests three months apart in all groups except in the HBV carrier group, in which the HBV DNA level was undetectable (less than 54.5 IU/ml). The phase of HBV infection was classified according to EASL Clinical Practice Guidelines 2009 published in the Journal of Hepatology 50 (2009) 227-242. Normal subjects (n=5) were healthy blood donors of the Blood Bank, Naresuan University Hospital. Exclusion criteria were pregnancy, lactation, alcohol drinking within 6 months, immunodeficient state such as tuberculosis, HIV infection, HCV infection, diabetes mellitus or renal failure, taking hepatotoxic drugs such as statins, metrotrexate, antituberculous drugs and herbal medicines. This study was approved by the Ethical Committee of Naresuan University and informed consents were obtained from all patients.

Stimulation of PBMC and proliferation assay

PBMC were isolated from whole blood samples using Lymphoprep (Alexis-Shied Poc AS, Oslo, Norway) and were plated at 1×10^5 cells/well of 96-well plates. For the proliferation assay, PBMC were stimulated with 10 μ g/ml PHA (Sigma, MO, USA) each in quadruplicate wells. They were cultured at 37°C with 5% CO₂ for 5 days before the proliferation was assessed using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Applied Science, IN, USA) according to the manufacturer's instruction.

Stimulation of PBMC and flow cytometric analysis

For the expression of CD69, intracellular cytokines, as well as granzyme B and perforin, PBMC were plated at 2.5×10^6 cells/well of 24-well plates. They were added with 10 μ g/ml FLLTRILTI peptide (Proimmune Ltd., Oxford Science Park, Oxford, UK) before they were incubated at 37°C with 5% CO₂ for 24 hours. Then, cells were stimulated with 40 ng/ml PMA, 1 μ g/ml ionomycin, and 10 μ g/ml brefeldin A (all from Sigma) for 5 hours before they were washed, fixed and stained with anti-CD4-PerCP, anti-CD8-FITC or anti-CD8-PerCP, anti-granzyme-B-FITC (BD Bioscience, San Jose, CA, USA), anti-IL-4-PE, anti-IFN- γ -FITC (BioLegend, San Diego, CA, USA), anti-CD69-PE, and anti-perforin-FITC (eBioscience, San Diego, CA, USA) monoclonal antibodies. The stained cells were then analyzed using FACScalibur and CellQuestPro software (Beckton Dickinson, San Jose, CA, USA)

Statistical analysis

All data were expressed as mean \pm standard deviation (SD). Differences between the two groups were analyzed with the Students' t-test using Microsoft Excel software. The *p* values of less than 0.05 were considered significant.

RESULTS

PBMC proliferation and CD69 expression

The proliferative response to PHA and HBV peptide by PBMC from CHB patients did not differ from healthy subjects. Similarly, HBV peptide induced CD69 expression by CD4 T cells and CD8 T cells from CHB groups was not different from healthy subjects.

Expression of cytokines, granzyme B and perforin by CD4 T cells

The numbers of IL-4⁺ CD4 T cells stimulated with HBV peptide were significantly increased in HBeAg-positive CHB patients with elevated ALT, HBV carriers, and HBeAg-negative CHB patients with normal ALT compared with normal subjects as well as HBeAg-negative CHB patients with normal and increased ALT (Fig 1A). The numbers of IFN- γ ⁺ CD4 T cells stimulated with HBV peptide were significantly increased in HBeAg-positive CHB and HBeAg-negative CHB patients with increased ALT, and HBV carriers compared with normal subjects and HBV carriers compared with normal subjects and HBeAg-positive CHB and HBeAg-negative CHB patients with normal ALT (Fig 1B).

As expected, very low numbers of CD4 T cells expressed granzyme B and the number of granzyme B⁺ CD4 T cells significantly decreased in HBeAg-negative CHB patients with normal ALT compared with other subject groups (Fig 1C). Perforin was almost undetectable in CD4 T cells from all subjects (Fig 1D).

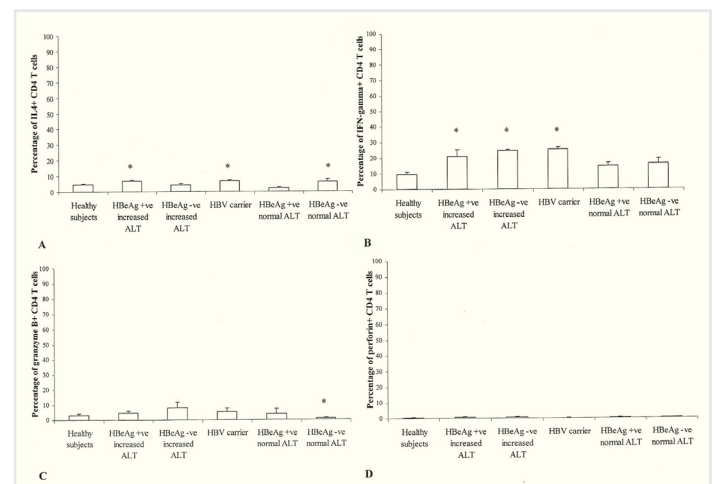


Fig 1. CD4 T cells from CHB patients and healthy subjects were stimulated with HBV peptide FLLTRILTI before they were assessed for (A) IL-4, (B) IFN- γ , (C) granzyme B and (D) perforin expression by flow cytometry. Each bar represents mean \pm standard error of the mean. *, *p* less than 0.05.

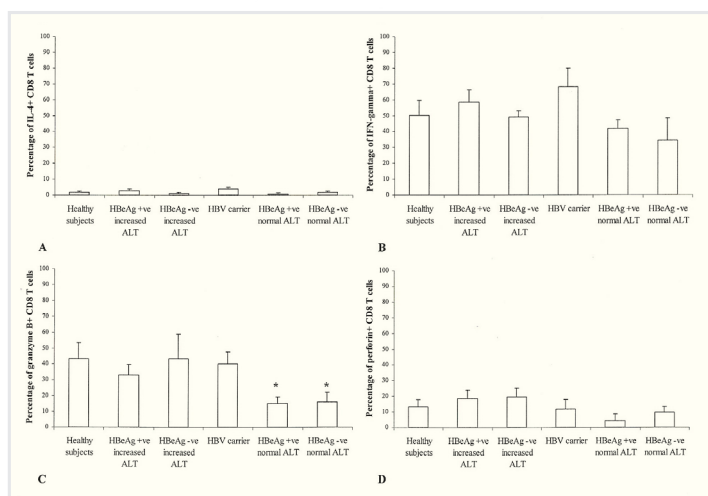


Fig 2. CD8 T cells from CHB patients and healthy subjects were stimulated with HBV peptide FLLTRILTI before they were assessed for (A) IL-4, (B) IFN- γ , (C) granzyme B and (D) perforin expression by flow cytometry. Each bar represents mean \pm standard error of the mean. *, p less than 0.05.

Expression of cytokines, granzyme B and perforin by CD8 T cells

The numbers of IL-4⁺ CD8 T cells were similarly very low in all subject groups (Fig 2A). The numbers of IFN- γ ⁺ CD8 T cells were comparable among all subject groups (Fig 2B). However, the number of these cells tended to be decreased in CHB patients with normal ALT (Fig 2B).

The numbers of granzyme B⁺ CD8 T cells significantly decreased in HBeAg-positive and HBeAg-negative CHB patients with normal ALT (Fig 2C). Although there was no difference in the number of perforin⁺ CD8 T cells among all subject groups (Fig 2D), the number of these cells tended to be decreased in HBV carriers and CHB patients with normal ALT.

DISCUSSION

Cytotoxic T lymphocytes (CTL) play major roles in the pathogenesis of hepatitis B viral infection. In chimpanzees, hepatocyte destruction occurs when the virus-specific CTL infiltrate into the infected liver and reduction of CTL with the highest amount of viral DNA in the bloodstream delays hepatic inflammation.¹⁷ There is also a correlation between virus-specific CTL response and the severity of HBV infection. Moreover, HBV-specific T cells can induce hepatic inflammation in rats, with a similar process in humans.¹⁸

In our present study, PBMC from HBV patients proliferated in the same magnitude as those from healthy subjects. It has been shown that lymphocyte proliferation in chronic HBV patients is lower than those with acute HBV infection.¹⁹ PBMC from chronic HBV patients may still have normal proliferative capacity as demonstrated by PHA induced proliferation, but they have suboptimal response to hepatitis B specific antigen.

IL-4 and IFN- γ are produced by T cells in response to HBV infection. In this present study, increased IL-4⁺

CD4 T cells in HBeAg positive patients with normal or increased ALT as well as in HBV carriers indicate up-regulation of Th2 activity in these patients. The Th2 cells promote antibody response to the infection, which is not directly correlated with hepatitis.²⁰⁻²² The cytokine IL-4 has also been reported to diminish CD8⁺ activity in vivo.²³ From our study, IL-4 might be only one factor involved in cytotoxic T lymphocyte activity.

The elevated IFN- γ ⁺ CD4 T cells might also involve in increased hepatitis when there is a high number of viral infected hepatocytes. IFN- γ can increase MHC class I expression in these cells thus causing them to be killed by specific CTL. Besides inhibition of viral replication, IFN- γ also recruits non-specific inflammatory cells that locally cause liver damage.²⁰ Previous studies have also suggested that CD4 T cells are essential for the maintenance and cytotoxic function of CD8 T cells that control chronic viral infections.^{24,25} The present result is consistent with another previous finding that lymphocytes from both acute and chronic HBV patients have the good response to PHA, but weak response to HBeAg in terms of interferon- γ and IL-4 production.²⁶

Perforin, granzyme B and Fas ligand (FasL) play a key role in T-cell mediated hepatocellular injury in viral hepatitis.^{27,28} Reduced numbers of granzyme B⁺ CD8 T cells in CHB patients with normal ALT may support a previous findings that PBMC from chronic hepatitis woodchucks have lower cytotoxic activity compared to controls.²⁹ Increased perforin⁺ T cells in viral hepatitis elderly patients are associated with severity of the disease.³⁰ However, there has been conflicting evidence about the correlation between granzyme B, perforin, and FasL and liver damage in CHB patients.^{28,31} The important role of this pathway in the pathogenesis of hepatitis B infection needs to be clarified.

CD69 is involved in early lymphocyte activation. CD69 can be detected in HBV-specific CTLs from patients who clinically and serologically recover from HBV infection for up to 23 years. This implies that HBV can still be kept under control in these patients for life even they have serological evidence of viral eradication.³² The comparable numbers of CD69⁺ T cells found in all patients indicate that early activation of their lymphocytes is not impaired.

Although this present study had a small sample size due to limitation of the time to recruit the subjects, we divided the patients into 4 subgroups in order to compare the different immune responses between groups and the immune response was measured quantitatively. Many factors have been proposed to influence the outcome of HBV infection such as dendritic cells,³³ regulatory T cells,³⁴ and PD-1 receptor,³⁵ which were not focused in our study. Further research may include the study of a variety of immunological parameters in correlation with different clinical outcomes of CHB.

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