Correlation of T-lymphocyte Subsets with Serum HBV DNA Loads and HBeAg Title among Different Clinical Types of Hepatitis B Patients

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ABSTRACT Objective: To investigate the relationship between T-lymphocyte subsets and HBV DNA levels and serum hepatitis B e-antigen (HBeAg) status in patients with chronic hepatitis B virus (HBV) infection. Methods: 103 patients with chronic HBV infection and 20 healthy blood donors were included in the study. The peripheral T-lymphocyte subsets in these patients were determined by flow cytometry. The serum HBVDNA levels were assessed by polymerase chain reaction (PCR) and HBeAg status were tested by enzyme immunoassay. Results: The percentages of $CD3^+$, $CD4^+$T lymphocytes in peripheral blood and $CD4^+/CD8^+$ ratios in Chronic Hepatitis B (CHB) patients and HBV carriers were significantly lower than those in control patients (all $P<0.05$ or 0.01); In contrast, the percentages of $CD8^+$T lymphocytes showed the opposite trend, and there were also statistically significant. HBV replication level had a positive correlation with the $CD8^+$T lymphocytes and negative correlation with the ratio of $CD4^+/CD8^+$T lymphocyte in HBeAg negtive patients. However, there was no corelational relationship between the HBV DNA level and the $CD3^+$T, $CD4^+$T, $CD8^+$T and the ratio of $CD4^+$T/$CD8^+$T lymphocytes subpopulations in HBeAg positive patients, nor did with the HBeAg level increased. Conclusion: Cellular immune function decreased and immune dysregulation in various clinical types of chronic HBV infected patients. HBV DNA level is positively correlated with the cellular immune status in the patients with HBeAg negative chronic hepatitis B.

Key words: Hepatitis B virus; Chronic hepatitis B virus infection; T lymphocyte subsets; Hepatitis B e Antigens; Viral load

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Introductions

Chronic hepatitis B virus (HBV) infection is a major health problem which may progress to liver failure, cirrhosis and hepatocellular carcinoma (HCC)[1]. The outcome of the chronic HBV infection depends on both viral and host-specific factors [2]. Apart from direct biological effects of viral variants, there is a growing consensus that the host immune response, especially the virus-specific T cell response, is the key determinant influencing the course of disease and the onset of liver disease [3]. Hepatitis B e antigen (HBeAg) may play an important role in the interaction between the virus and the immune system. CHB can be divided into HBeAg-negative CHB and HBeAg-positive CHB according to the e antigen. There are obvious differences in the pathogenesis, mechanisms historical process, clinical manifestation and anti-virus therapy[4-5]. At present, there are many studies of T-lymphocyte subsets, however, there were few researches which investigate peripheral T-cell subsets in HBeAg negative and positive HBV DNA infected patients dividedly. The aim of the present study was to investigate the relationship between T-lymphocyte subsets and HBV DNA levels and serum HBeAg status in different kinds of HBV infected patients with chronic hepatitis B virus (HBV) infection.

1 Materials and methods

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1.1 Plant materials

36 HBeAg-positive CHB patients, 45 HBeAg-negative CHB patients, 22 HBV carriers and 20 healthy blood donors were included in the study. All the patients were treated at the Affiliated Hospital of Qingdao Medical College from January 2010 to July 2011. The patients were diagnosed according to the criteria for viral hepatitis from "The guideline of prevention and treatment for chronic hepatitis B (2010 version)[6]" excluding other concomitant of liver disease, relatively rare liver disease (autoimmune hepatitis and metabolic liver disease), and treatment with antiviral therapy or immunosuppressive therapy for HBV infection within the past 12 mo before entry. None of the patients received antiviral, immunosuppressive therapy or exposed to hepatotoxin. Those who had liver cirrhosis were also excluded. 20 healthy individuals who were free of HBsAg were identified from physical examination center as the control group. The ages and genders were not significantly different among groups.

1.2 Experimental methods

1.2.1 Serological liver function tests and HBV markers evaluation Serum alanine aminotransferase (ALT), aspartate transaminase (AST) and total bilirubin were tested with routine automated techniques (upper limit of normal; 40 U/L, 40 U/L and 17.1 μmol/ml, respectively) (HITACHI7600.210, Japan). HBV markers were measured by chemuminescence method (Anthos 2010, Austria), according to manufacturer instructions (Sino-American Biotech Co., Ltd, Shanghai, China). According to serum HBeAg levels, patients were divided into 4 groups: negative (<1PEIU/ml), low (>1 PEIU/ml～100 PEIU/ml), medium (>100 PEIU/ml～1000 PEIU/ml) and high (>1000 PEIU/ml～10000

PEIU/ml) groups.

1.2.2 Detection of HBVDNA Levels The serum HBV viral loads were detected by the real-time quantitative PCR and HBeAg was detected by chemiluminescence method. According to serum HBV DNA viral loads patients were divided into 4 groups: HBV DNA negative (HBV DNA≤ 10^3 copies / ml), low (HBVDNA＞ 10^3 ～ 10^5 copies / ml), medium (10^5 ～ 10^7 copies / ml) and high (＞ 10^7 copies / ml).

1.2.3 Peripheral blood T-lymphocyte subsets measurement Blood samples were collected in heparinized vacutainer tubes. The peripheral T-lymphocyte subsets (the percentages of $CD3^+$, $CD4^+$, $CD8^+$ cells and the ratio of $CD4^+/CD8^+$) in these patients were determined by flow cytometry.

1.3 Statistical analysis

T test was performed for comparison among groups,the dates were summarized as mean± SD; Analysis of the correlation between variables was conducted using the Spearman's rank correlation coefficient. A final $P<0.05$ was considered statistically significant. Computations were carried out with the aid of SPSS17.0 software version.

2 Results and analysis

2.1 The percentages or ratios of T-cells sub-populations among groups

The percentages of $CD3^+$,$CD4^+$ T lymphocytes in peripheral blood and $CD4^+/CD8^+$ ratio in HBV carriers, CHB patients were significantly lower than those in control patients ($CD3^+$: t=2.56, 2.78; $CD4^+$: t= 2.57, 4.76; $CD4^+/CD8^+$: t=2.01, 4.48, all $P<0.05$ or 0.01). In contrast, the percentages of CD8+ T lymphocytes in peripheral blood in CHB patients, HBV carriers were significantly higher than those in control patients ($CD8^+$:t= -2.10, -2.40, $P<0.05$)(Table 1).

Table 1 Peripheral T -cell subsets broken down by clinical pattern in normal controls and chronic HBV infection patients (%,mean ± SD)

Groups	Patients(n)	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Normal control	20	69.6± 4.42	41.8± 4.60	24.8± 3.50	1.90± 0.42
Inactive carrier	22	64.6± 5.68[a]	37.2± 5.30[a]	27.8± 4.08[a]	1.75± 0.29[a]
CHB	81	60.5± 5.47[a]	32.0± 5.45[b]	28.3± 2.80[a]	1.58± 0.36[b]

Note: [a]$P<0.05$,[b]$P<0.01$ vs normal control.

2.2 Correlation between peripheral T-cell subsets and HBVDNA levels in HBeAg positive patients

There was no correlation between the levels of $CD3^+$T, $CD4^+$T, $CD8^+$T cells and $CD4^+$T/$CD8^+$T ratio and serum level of viral load in HBeAg positive patients (Table 2).

Table 2 Correlation between peripheral T-cell subsets and HBVDNA levels in HBeAg positive patients (%,mean ± SD)

HBVDNA levels	Patients(n)	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Low levels	6	62.14± 8.10	36.20± 8.59	20.80± 3.06	1.78± 0.42
Medium levels	19	63.90± 10.50	36.43± 6.18	21.09± 4.56	1.78± 0.30
High levels	11	63.60± 9.67	37.28± 8.48	19.60± 3.76	1.92± 0.36
r		0.072	0.100	-0.124	0.190
p		0.680	0.568	0.480	0.271

2.3 Correlation between peripheral T-cell subsets and HBVDNA levels in HBeAg negtive patients

There was positive correlation between the levels of $CD8^+$T cells while there was a negative correlation between $CD4^+$T/$CD8^+$T ratio and serum level of viral load in HBeAg negtive patients. However, there was no correlation between the levels of $CD3^+$T and $CD4^+$T cells and serum level of viral load (Table 3).

Table 3 Correlation between peripheral T-cell subsets and HBVDNA levels in HBeAg negative patients (%,mean ± SD)

HBVDNA levels	Patients(n)	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$
Low levels	22	63.50± 4.75	36.43± 4.61	21.88± 3.52	1.75± 0.40
Medium levels	18	62.51± 8.45	35.72± 5.28	23.80± 3.56	1.58± 0.26
High levels	5	65.41± 8.48	35.86± 5.43	26.60± 2.80	1.35± 0.15
r		-0.065	-0.156	0.567	-0.601
p		0.653	0.264	0.001	0.002

2.4 Correlation between peripheral T −cell subsets and HBeAg titer

There was no correlation between the levels of $CD3^+$T, $CD4^+$T, $CD8^+$T cells and $CD4^+$T/$CD8^+$T ratio and serum titer of HBeAg in the HBeAg positive patients (Table 4).

Table 4 Correlation between peripheral T-cell subsets and HBeAg titer (% mean ± SD)

Groups	Patients(n)	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+$/ $CD8^+$
Low titers	17	63.15± 5.67	36.35± 5.16	21.70± 3.41	1.75± 0.40
Medium titers	6	63.11± 9.23	36.03± 5.60	22.60± 4.26	1.64± 0.28
High titers	13	64.15± 9.20	36.84± 7.53	21.98± 4.60	1.75± 0.40
r		0.008	0.002	0.077	-0.026
p		0.943	0.967	0.779	0.818

3 Discussion

There are many reports about that immune function mediated by specific T cells play an important role in the disease progression and HBV clearing progress [7]. The composition of T cells settings include: $CD3^+$ T cells (Total T cells), which represent the whole body immune state; $CD4^+$T cells(Helper T lymphocyte) and $CD8^+$T (Cytotoxic T lymphocyte), which are mainly T cells; The $CD4^+$/ $CD8^+$ ratio is an important indicator of detecting the immune function. The unbalanced ratio will result in immune system disorders and series of immune pathological changes [8-9]. The clinical features and the severity of the illness were mainly caused by immune responses induced by HBV. By analyzing the percentages of $CD3^+$, $CD4^+$ T cells and the ratio of $CD4^+$/$CD8^+$ T cells, it was observed that the parameters of CHB patients and HBV carriers were significantly lower than those of control patients (all $P<0.05$ or 0.01). In contrast, the percentages of $CD8^+$T lymphocytes in peripheral blood in CHB patients, HBV carriers were significantly higher than those in control patients($P<0.05$). The percentages of $CD3^+$, $CD4^+$T cells and the ratio of $CD4^+$/ $CD8^+$ decreased, which meant that the dysfunctional T-cells hyporesponsiveness and disorder of cellar immune function in patients who infected with the HBV virus. These results was consistent with current reports from domestic and overseas [10-11]. One of the possible reasons of $CD3^+$ T cells decreased might be that T-lymphocytes have immune tolerance to HBV infection or apoptosis on account of antigenic stimulation when the people infected HBV and become HBV carriers, or T-lymphocytes subpopulations from activated to suppressed do not increase but decrease. The chronic antigenic stimulation might increase the percentages of $CD8^+$ T cells. The mechanism might include: The number of specific immune effect cells stimulated by HBV antigen increases; The $CD8^+$T cells specific expression increasing induced by HBV directly or promoted by some serum factors. The number of $CD8^+$T Lymphocytes increased but the activity decreased, and the possible reasons may be Central immunotolerance, T cells no reaction, immunological ignorance and so on [12]. The ratio of $CD4^+$/ $CD8^+$ T cells decreased mainly because of the increasing percentage of $CD8^+$ T cells. In the process of clearing HBV virus, the reduction of $CD4^+$T cells can lead to the dysfunction of NK and LAK cells, failure in clearing HBV in time. However, $CD8^+$T cells may play an important role in the process of clearing HBV virus, especially covalently closed circular DNA (cccDNA). The reductions of $CD4^+$T and $CD8^+$T cells and the low $CD8^+$T cells activity prevent getting rid of the virus in time, resulting persistent infection.

A lot of researches have showed that T-cell failure was significantly associated with viral replication [13], however, there were few researches which investigate peripheral T-cell subsets in HBeAg negative and positive HBV DNA infected patients dividedly. As is known to all, the levels of HBV DNA loads in both groups are significantly different. Once the composition of the two parts is different, the results may create a deviation, therefore, the conclusions would be more reasonable if analyzed respectively. Studies shows that, the severity of liver diseases was significantly associated with HBV replication status in HBeAg negative CHB [14], however, is this pathological damage caused by variation virus directly hurting the liver cells or by inducing abnormal immune response even immune injury? This study demonstrated that there was a positive correlation existed between the proportion of CD8+ T-cells and serum HBV viral load, whereas a negative correlation existed between the $CD4^+$/ $CD8^+$ ratio and serum HBV viral load, which was consistent with previous findings [15], namely, T-cell impairment appears to be related to the high level of HBV replication. The virus antigens usually present on the liver cell membrane or in the liver cell plasma, when the immune cells act on those antigens, it will induce liver cells damage by abnormal immune response [16-17]. The antiviral treatments have been used to inhibit HBVDNA duplication to improve the body cellular immune function, and the doctor should take the effective intervention strategies (such as anti-viral and /or immunotherapy) into account to prevent the progression, decrease long-term consequences and provide a train of thought and foundation in designing the specific ways of treatment. There was no significant difference for these parameters of T-lymphocyte sub-populations in the HBeAg-positive patients

with different levels of HBVDNA loads (Table 2). HBeAg may make the patients come to being tolerant to HBV DNA, decreasing the antiviral responses. The tolerance-inducing effect of HBeAg has been well characterized in mice [18-19] and likely contributes to the low level of core-specific T-cell responses present in HBeAg-positive CHB patients [20].

Therefore, According to serum HBeAg titer levels，patients were defined as low, medium and high groups in this study, however, there was still no correlation between the parameters and the difference HBeAg titer levels, which had also been demonstrated in the research from YOU et al[13]. One possible reason is that some subjects were infected with pre-C stop codon mutation virus (pre-C/C mutant), which resulted in a loss of HBeAg [21]. Although HBeAg are eliminated and conversed to anti-HBe, viral replication may have persisted in these patients, which may weaken the independent association between HBeAg and T-cell failure, so that we could not detect this magnitude of association. Another possible reason may be that infection from the mother and/or at young age predisposes to tolerance to HBV infection and thus higher viral load.

In conclusion, the decreasing of cellular immune function and immune dysregulation are noted in various clinical types of HBV infected patients. There was a strong independent correlation between serum HBV BDA load and T lymphocyte subpopulations, which suggested a causal relationship between viral load and T-cell failure. High viral might play an important role in T lymphocyte failure, and is more important than HBeAg in this regards. In addition, it might be possible to predict the variation of T lymphocyte subpopulations in peripheral blood in the future by measuring serum viral load in HBeAg negative HBV -infected patients.

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不同临床类型乙型病毒感染者外周血 T 细胞亚群与血清 HBV DNA 载量及 HBeAg 滴度相关性分析

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摘要 目的:探讨慢性乙型肝炎病毒(HBV)感染患者外周血 T 细胞亚群与血清 HBVDNA 载量及 HbeAg 滴度的关系。**方法**:选取 103 名 HBV 感染患者和 20 名健康者为研究对象。流式细胞术检测外周血 T 细胞亚群,聚合酶链式反应及酶免疫分析法分别检测血清 HBV DNA 载量及 HbeAg 滴度。**结果**:慢性乙型肝炎患者和慢性 HBV 携带者外周血 $CD3^+T$、$CD4^+T$ 淋巴细胞亚群百分数低于健康对照组,结果有统计学意义($P<0.05$ 或 0.01;而 CD8+T 细胞亚群则呈现相反趋势,结果亦有统计学意义($P<0.05$ 或 0.01)。HBeAg 阴性组中,HBVDNA 水平与 $CD8^+T$ 细胞亚群百分数呈正相关($r = 0.567$, $P < 0.01$),与 $CD4^+/CD8^+T$ 细胞亚群百分数比值呈负相关($r = -0.601$, $P<0.01$),而与 $CD3^+T$、$CD4^+T$ 细胞亚群百分数无相关性。HBeAg 阳性组中,HBVDNA 水平及 HbeAg 滴度与 $CD3^+T$、$CD4^+T$、$CD8^+T$ 细胞百分数及 $CD4^+/CD8^+T$ 细胞百分数均无相关性($P>0.05$)。**结论**:不同临床类型的慢性乙型肝炎病毒感染患者外周血 T 细胞亚群存在不同程度细胞免疫功能降低和细胞免疫调节异常。HbeAg 阴性的 HBV 感染患者,其血清 HBV DNA 水平与外周血 T 淋巴细胞免疫存在相关性。

关键词:肝炎病毒,乙型;慢性乙型肝炎;T 细胞亚群;HBeAg;病毒载量

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