行政院國家科學委員會專題研究計畫 期中進度報告

先天性及專一性免疫在慢性B型肝炎病毒感染的角色-免疫病理機轉及治療上的意涵--慢性B型肝炎病人B型肝炎病毒特異性T細胞反應及調節型T細胞的分析（總計畫與子計畫一）(2/3)

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Analysis of HBV-specific T cell responses and regulatory T cells in patients with chronic hepatitis B virus infection

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Short Title: Regulatory T cell responses in chronic hepatitis B

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中文摘要

B 型肝炎在台灣是個非常嚴重的問題，大約 15-20% 的成年人是 B 型肝炎帶原者。B 型肝炎病毒的感染更是台灣地區慢性肝炎、肝硬化，肝細胞癌的主要原因。不少的研究顯示，B 型肝炎病毒本身並不會造成細胞毒性，而是經由引發宿主免疫系統而導致肝炎。另一方面，宿主對 B 型肝炎病毒免疫系統反應的強弱，也與 B 型肝炎病毒的清除有關。從文獻的回顧顯示，在急性 B 型肝炎的病人，其體內的 B 型肝炎病毒特異性 T 細胞反應較強。相反的，在慢性 B 型肝炎的病人，其體內的 T 細胞反應較為微弱。T 細胞反應的強弱似乎與 B 型肝炎病毒在體內的清除有關。

然而，以往的研究仍有美中不足的地方：(1) 慢性 B 型肝炎急性發作的免疫學研究資料仍然不足。(2) 在台灣最常見的 HLA haplotype 是 HAL-A11，其次是 HLA-A2。以往的研究都是著重在 HAL-A2，對於 HLA-A11 著墨較少。(3) B 型肝炎病毒特異性的 $CD8^+$ T 細胞反應，在肝硬化及肝細胞癌這些病人的研究較少。(4) 對於 $CD4^+$ $CD25^+$ 調節性 T 細胞在慢性 B 型肝炎病毒感染所扮演的角色，仍然是未知的。(5) 臍帶血的細胞，對於 B 型肝炎病毒特異性的 $CD8^+$ T 細胞反應仍然未知。因此，我們將在此計畫中對上述這些問題做研究。

在過去一年，我們已經採用 MHC-peptide 五合體來分析 B 型肝炎 HLA-A2 相關的 B 型肝炎病毒特異性的 $CD8^+$ T 細胞反應。今年的重點在於建立 HBV 特異性的 $CD8^+$ T 細胞株與 $CD4^+$ $CD25^+$ 調節性 T 細胞的分析。我們分析了 96 位 B 肝帶原周邊血液 $CD4^+$ $CD25^+$ 調節性 T 細胞的頻率，我們發現調節性 T 細胞的頻率(CD4+ CD25+/CD4+)的平均值是 1.79%±1.5。調節性 T 細胞的頻率與肝炎的病程(不活動性帶原、慢性肝炎、肝性化)並沒有統計上有意義的差異，與 e 抗原的狀態(陽性 vs.陰性)也沒有統計上有意義的差異。調節性 T 細胞的頻率與 HBV DNA 或 ALT 值，也沒有相關性。調節性 T 細胞的功能性分析正在進行中。

ABSTRACT

Taiwan is a hyperendemic area of hepatitis B virus (HBV) infection. HBV-specific T cell responses play important roles in the pathogenesis of chronic hepatitis B. However, these immune responses are still not well known in patients with acute exacerbation (AE) of CH-B and in patients with advanced liver diseases, such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC). The T cell responses might be related to the progression of HBV-related liver diseases. Besides, most of the previous studies focused on HLA-A2-restricted T cell responses. In Taiwan, HLA-A11 is the most common HLA haplotype. The HLA-A11-restricted T cell responses should be addressed. In addition, the $CD4^+CD25^+$ regulatory T cells might suppress immune responses against foreign antigens and pathogens. Thus, the specific aims of this project are: (1) To analyze HBV-specific T cells responses in patients with chronic HBV infection by both cross section study and longitudinal study. (2) To investigate the role of regulatory T cells in patients with chronic HBV infection of different disease stages (3). To investigate the fetal or neonatal immune responses to HBV.

In the past one year, we analyzed the HLA-A2-restricted HBV-specific CD8+ T cell responses. We focused on the generation of HBV-specific T cell line as positive control and the analysis of $CD4^+$ $CD25^+$ regulatory T cells in this year. Flow cytometry was used to analyze $CD4^+$ $CD25^+$ regulatory T cells in the peripheral bloods of 96 HBV (+) patients. The mean percentage of Treg/CD4+ T cells was 1.79%±1.5. There were no statistically significant differences in the Treg/CD4+ ratio between (1) inactive HBV carrier (2) chronic hepatitis B (3) HBV-related liver cirrhosis patients, between (1) HBeAg(+) and (2) HBeAg (-) HBV carriers. There were no correlations between percentage of Treg and ALT levels or HBV DNA levels. Functional studies of Treg are now undergoing.

INTRODUCTION

Taiwan is a hyperendemic area of hepatitis B virus (HBV) infection. Around 15-20% adults in Taiwan are chronically infected with HBV (1), which is the major cause of chronic hepatitis, liver cirrhosis (LC) and hepatocellular carcinoma (HCC) in Taiwan (2). Several lines of evidences suggest that HBV *per se* is not directly cytopathic. Instead, the immune response to HBV-encoded antigens is responsible both for viral clearance and for disease pathogenesis (3).

Previous studies demonstrated vigorous T cell responses to HBV-encoded antigens developed in patients with self-limited acute hepatitis B. In contrast, weak or no T cell responses could be detected in chronic hepatitis B (CH-B) patients (4-10). The $CD8^+$ CTL activity can lasts for decades following recovery and is thought to keep the HBV under control (11). The HBV virions can be cleared from the blood, by a series of antiviral signals initially delivered by MHC-I-restricted HBsAg-specific CTL (12). These results suggest that specific immunotherapeutic enhancement of the CTL response to HBV should be possible in chronically infected patients, and that it could lead to viral clearance in these individuals with resolution of chronic liver disease (13) (for review, see (14)).

However, there are several limitations on previous studies. First, these studies focused on either acute or chronic hepatitis B. First, few studies focused on acute exacerbation (AE) of CH-B (15). AE is common during the natural course of CH-B virus and has a great impact on the acceleration of progress into LC or HCC (16). However, the immunological responses before, during and after CH-B with AE are not well-known. This needs further investigation. Second, the HBV-specific T cell responses are not well-characterized in advanced liver diseases, such as LC and HCC. Third, most of these previous studies focused on

HLA-A2-restricted $CD8^+$ T cell responses. In Taiwan, the most common HAL is HLA-A11 (33%), followed by HLA-A2(30%)(17). The HLA-A11-restricted T cell responses in patients with CH-B were seldom studied. Tsai et al. (18) identified that HLA-A11-restricted $CD8^+$ HBcAg epitope (P2 sequence (YVNVNMGLK), HBcAg 88-96). However, the spectrum of this HLA-A11-restricted $CD8^+$ response from inactive HBV carrier to CH-B with AE, LC, or HCC are largely unknown. Fourth, the immune system is still not mature in the newborns, exposures to HBV antigens may lead the newborns tolerant to HBV. It has been shown that high dose of HBV DNA may increase tolerance to HBV (19, 20), the HBV-specific $CD8^+$ T cells responses in the cord blood has not been demonstrated before. However, few studies directly analyze the HBV-specific $CD8^+$ T cells in the cord blood.

In addition to the $CD8^+$ T cell responses, the $CD4^+CD25^+$ regulatory T cells (Treg) might also have important roles on the immunopathogenesis of CH-B. The $CD4^+CD25^+$ regulatory T cells were originally reported as a cell that suppresses $CD4^+$ T-cell induced organ-specific autoimmune disease, but recently it was shown to suppress immune responses against foreign antigens, pathogens and tumor-associated antigens. Augmentation of $CD4^+CD25^+$ T-cell numbers or proportions in peripheral blood and in tumor sites has been reported in variety of cancer patients (21-26). Similar scenario might also happen in patients with CH-B. However, no published study direct investigate this issue yet.

To address the above issues, we initiated this study. The specific aims of this project are (1) To analyze HBV-specific T cells responses in patients with chronic HBV infection, (2) To investigate the role of regulatory T cells in patients with chronic HBV infection of different disease stages (inactive HBV carrier, CH-B, LC and HCC) and in patients with acute exacerbation of CH-B, (3) To investigate the fetal or neonatal immune response to HBV.

PATIENTS AND METHODS

This is a 3-year project. In the first year, we investigated the HBV-specific T cell responses. In the second year, we focused on the regulatory T cells in patients chronically infected with HBV.

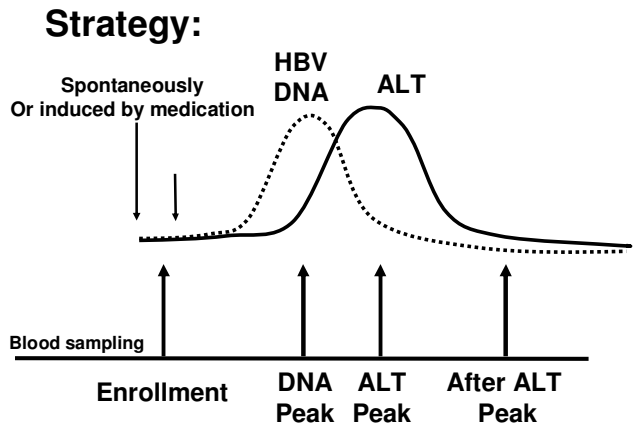
Patients

Cross section study

The purpose of cross-section study is to compare the HBV-specific $CD8^+$ T cell responses in HBV-infected patients of different clinical stages (inactive HBV carrier, CH-B, LC, HCC). The HBsAg (+) patients from National Taiwan University Hospital were included. Screening for HLA haplotype was performed by PCR method. HBsAg, anti-HBs, IgM anti-HBc, HBeAg, anti-HBe, anti-HCV, anti-HDV, anti-HIV were determined by commercial enzyme immunoassay kits and the data were obtained from the medical records. The serum levels of HBV DNA and genotypes will be done by quantitative real-time PCR. Patients who are also infected with HDV or HCV or HIV will be excluded.

Prospective study

The purpose of prospective study is to clarify the role of HBV-specific T cell responses during the course of acute exacerbation of CH-B.



Blood will be collected at four important time points, baseline, the surge of serum viral load, the peak of serum ALT, and after serum ALT peak activity.

Cord bloods

The cord bloods of HBsAg (+) mothers were collected and isolation of cord blood mononuclear cell (CBMCs) will be processed as for the PBMCs.

Blood samples

PBMCs were isolated from EDTA-containing blood samples by density gradient centrifugation on Ficoll-Hypaque. The PBMC were washed with phosphate-buffered saline (PBS) and resuspended in T cell medium. The PBMC and plasma were stored in the freezer using Program Freezer.

Flow cytometry

Analyses of CD4+CD25+ regulatory T cells (Treg) were done using by flow cytometry. Isolation of Treg were done by magnetic beads (Miltenyi Biotec) coupled with anti-CD4 and then anti-CD25 mAbs. Expression of Foxp3 was analyzed by flow cytometry using intracellular staining.

RESULTS

Patients

A total of 96 HBsAg (+) patients were enrolled, including 33 HBeAg (+) and 63 HBeAg (-) patients. There were 12 patients had serum ALT level higher than 5 x ULN. The diagnosis was asymptomatic HBV carrier in 14 patients, chronic hepatitis in 71 patients, liver cirrhosis in 8 patients and HCC in 3 patients.

Frequency of Treg

The mean percentage of Treg/CD4+ T cells was 1.79%±1.5. As shown in **figure 1**, the percentage of Treg is not correlated to disease status. The percentage of Treg is not correlated to serum ALT levels **(Figure 2)**. The percentage of Treg is not correlated to HBeAg status **(Figure 3)**. The percentage of Treg is not correlated to HBV DNA level **(Figure 4)**. More than 90% of the CD4+ CD25+ was also foxp3(+).

Figure 1. Correlation of frequency of regulatory T cells with disease status in patients infected with HBV

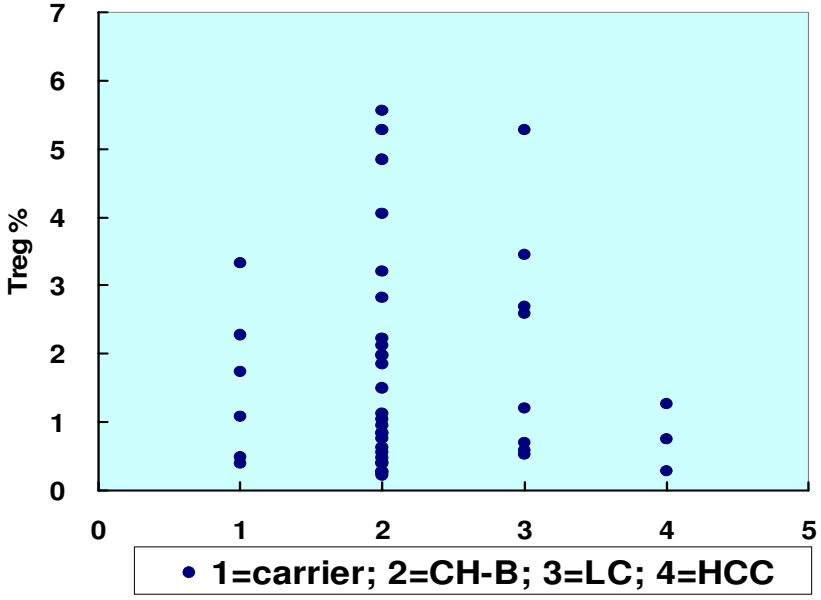


Figure 2. Correlation of frequency of regulatory T cells with ALT level

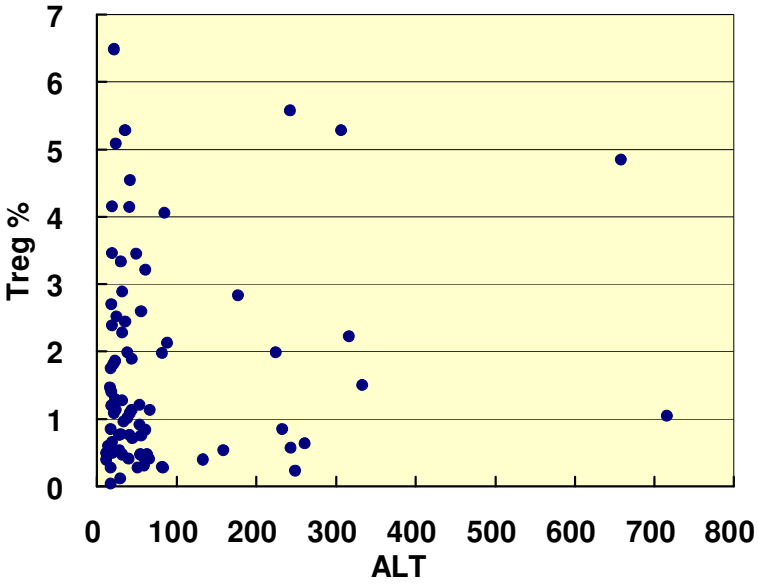


Figure 3. Correlation of frequency of regulatory T cells with HBeAg status

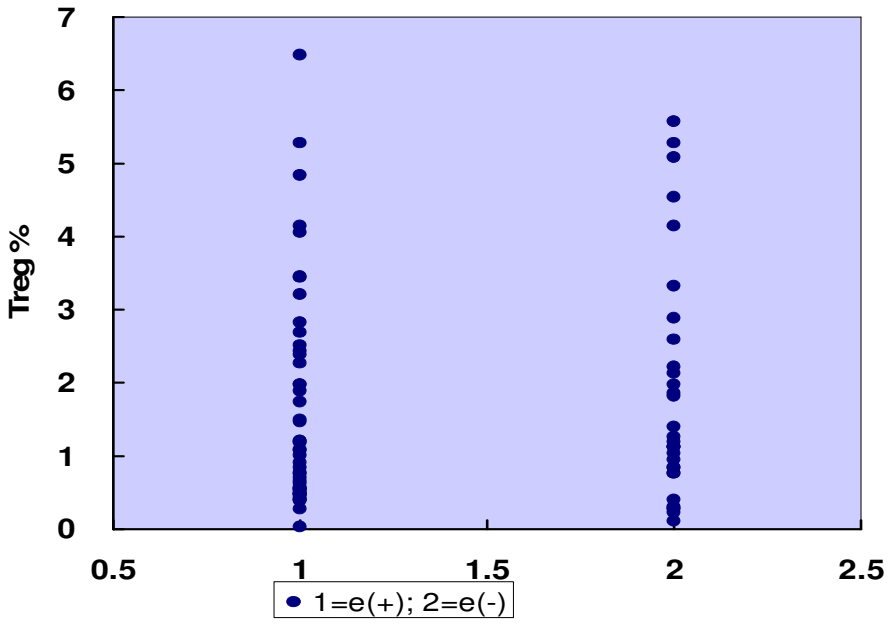
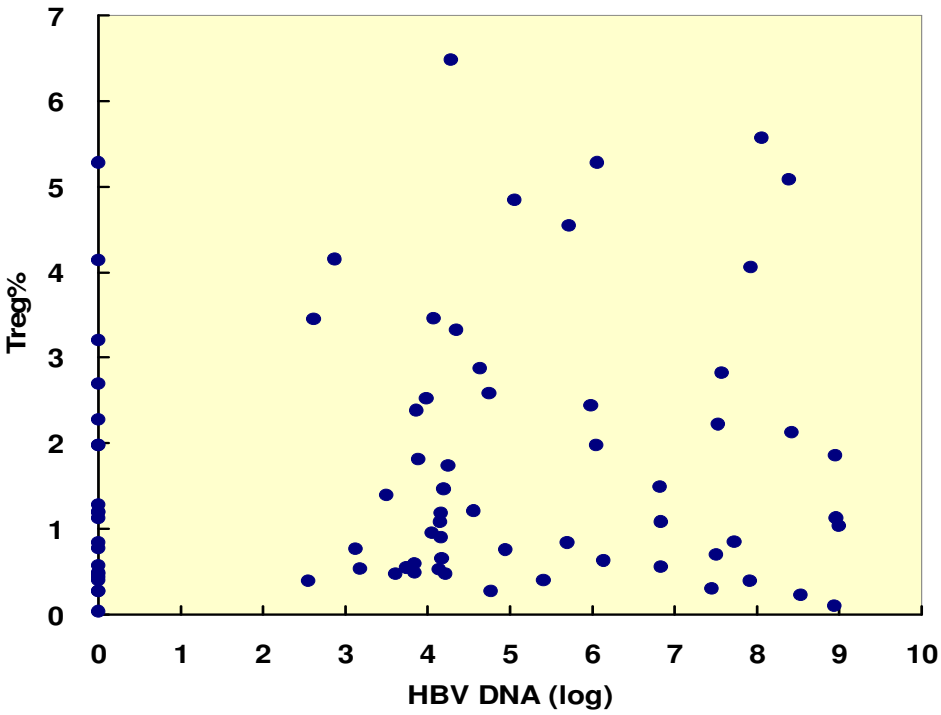


Figure 4. Correlation of frequency of regulatory T cells with HBV DNA level



DISCUSSION

In the second year study, we investigated the percentage of $CD4^+$ $CD25^+$ regulatory T cells in HBsAg (+) patients with different clinical stages. Unexpected, there were no correlations between the frequency of Treg and clinical status, HBeAg, ALT level and HBV DNA level.

One of the possible explanations was that not all $CD4^+$ $CD25^+$ T cells were regulatory T cells. Some $CD4^+$ $CD25^+$ T cells are activated T cells. If we used Foxp3 as the marker for Treg, more than 90% of the $CD4^+$ $CD25^{high}$ T cells expressed Foxp3. In contrast, only around 50% of the $CD4^+$ $CD25^{low}$ T cells expressed Foxp3 (data not shown). Therefore, the calculation of Treg should be restricted to $CD4^+$ $CD25^{high}$ T cells. In fact, a significant problem in comparing clinical studies in humans is the lack of a defined cut-off for $CD25^{high}$ expression. Thus, the reported frequency of Treg might vary in different studies. We are currently re-analyzed the percentage of $CD4^+$ $CD25^{high}$ T cells in the cross-section study and to see whether the percentage of $CD4^+$ $CD25^{high}$ T cells will be correlated with any clinical parameter.

We plan to continue enrollments of patients of CH-B with AE. Serial follow-up for the frequency of Treg will be done. The results of Treg will be correlated with the results of HBV-specific T cell responses done in our first year study. We hope that we can have clearer pictures on the T cell immune responses in the course of CH-B with AE.

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計畫成果自評

We have completed the study of Treg in the cross section study and collected the serial PBMC from patients with CH-B with acute exacerbation. The longitudinal study will be finished soon. However, we did encounter some difficulty in collecting the blood samples for cord bloods. Since the cord blood might be available only in the late evening or midnight, we did not the manpower to collect the blood in this time period. We are trying to figure out the solution for this problem.