Alteration of Peripheral Blood Lymphocytes (PBLs) Profile with HBsAg Level in Patients with Chronic Hepatitis B Infection

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***Abstract*---**Chronic hepatitis B infection is associated with dysfunction of cell- mediated immunity. Little is known about the changes of immune response during chronic hepatitis B infection, particularly in correlation between sequential alterations in peripheral blood immune cells population and hepatitis B surface antigen (HBsAg). AIM: to examine the dynamic changes in the population of peripheral blood lymphocyte (PBL) subsets (T cells subsets, B lymphocytes, and NK cells) in healthy donors and patients with CHBI and their correlation with the level of HBsAg (qHBsAg). METHODS: the immunophenotype profiles of PBL of 50(HCV, HDV and HIV negative) chronic hepatitis B patients and 25 healthy controls were analyzed by Flowcytometry (FCM). In addition the serum HBeAg status was determined by ELISA and the HBsAg level was quantified by Elecsys assay (Roche Diagnostics, Germany). Results: significant reduction in both the percentages of CD4+($p \leq 0.05$), CD8+ T lymphocyte subsets and the CD4+/ CD8+ ratio ($p<0.01$) was found in chronic hepatitis B patients as compared with the healthy donors. Meanwhile, there were no significant differences between patients and healthy controls with regard to other PBL parameters (total T, B, and NK cells), but a significant correlation was observed between HBsAg level and the percentages of T and NK cells ($r=0.366$; $p<0.01$, $r=-0.462$; $p<0.05$) respectively. Conclusion: our findings confirmed that CHB patients may have adversely affected cell-mediated immunity which is significantly correlated with a higher HBsAg level that leading to progress of the disease these patients.

***Keywords*---**Peripheral blood cells, chronic hepatitis b virus infection, HBsAg quantitation

I. Introduction

THE human hepatitis B virus (HBV) is one of the most common pathogens. It is a small enveloped DNA virus causing acute and chronic hepatitis. The infection with HBV still represents a chief global health burden even though an effective vaccine has been available. Many reports referred to the fact that approximately 350 million people in the world are chronically infected with this virus, and more than 1 million deaths per year occur due to HBV– associated liver pathologies such as liver failure, cirrhosis and hepatocellular carcinoma [1, 2].

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Principally, when the human body is infected by hepatitis B virus (HBV), complicated innate and adaptive immune responses are triggered to overcome the virus and ultimately terminate the infection. It is generally acknowledged that the humoral antibody response helps to remove circulating virus particles and prevent the viral spread within the host, while the cellular immune response is the most important factor in eliminating infected cells and is thought to be in charge of both viral clearance and disease pathogenesis during hepatitis B virus infection [3]. The antiviral immune response, partially, the T cell arm represent a vital factor in determining the outcome of infection and it has been shown that chronic HBV infection correlates with the occurrence of dysfunctional immune responses [4].

Peripheral blood mononuclear cells (PBMCs) containing the combination of immune – competent cells, such as T lymphocytes, B , and natural killer cells act an important role in the control or persistence the HBV infection[5, 6]. It is believed that the expression of viral antigens on the surface of infected hepatocytes can invoke a cytotoxic T–cell (CTL) response [7] and different subsets of T cells lymphocytes have distinct responses to viral antigens and effects on the clinical course and prognosis of infection [8]. Recently, the results of studies performed on animal models Illustrated that activated virus –specific T lymphocytes are critical for the pathogenesis of bothHBV infection and hepatocellular carcinoma[9].

On the other hand, many important studies reported that there is a strong correlation between T lymphocyte response, liver damage cells and HBV clearance[10, 11],as a results, the most investigations concentrated on lymphocyte subsets alteration in peripheral blood of chronic HBV infection, which demonstrated that there was imbalance or impaired balance of the T cells subsets like decrease in CD4+/CD8+ ratio[12], while other approaches showed an increase or no significant changes in these cells[13, 14] and ultimately the dysfunction of the immune responses. These disruptions are thought to be a consequence of prolonged display the body to large amounts of viral antigens, like hepatitis B e antigen (HBeAg)[15, 16], while the effects of Hepatitis B surface antigen (HBsAg) in this dysfunction is still more controversial. Detection of HBsAg is the first serological marker of the hepatitis B infection and its persistence in patients for more than six months consider chronic infection. Currently, HBsAg quantification is receiving renewed attention for its diagnostic-clinical role, due to its importance as a helping factor to

differentiate disease status in chronic HBV infection; moreover, it is a good indicator of response to antiviral therapy [17, 18]. Overall, HBsAg is created by many pathways, including i) translation of transcriptionally active cccDNA molecules, the intrahepatic virus reservoir acting as a template for replication and ii) from the translation of viral genes transcribed from integrated HBV sequences in the host genome [16]. Moreover, soluble HBsAg is present in the serum of HBV patients as sub- viral, non-infectious particles, exceeding the number of virions by a factor of 102–105 [19]. Some reports have demonstrated that HBsAg could be involved in the synchronization of the immune response, partially in disturbances of the appropriate immune response, and the quantity of HBsAg in peripheral blood might influence the HBV–specific CTL response[20, 21].

Because the influence of HBsAg serum levels on HBV specific cellular and humoral immune responses has not been yet clarified, in this study, we aimed firstly, to assess the differences in PBL subsets between chronic hepatitis B patients and normal controls at baseline, and between HBeAg positive and negative patients. Secondly, to define the relationship between peripheral blood T cell subsets and serum HBsAg levels.

II. Material & methods

A. Subjects

50 consecutive CHB patients (31 males and 19 females, age 18 to 70 years) from Gastroenterology Department at Hospital Tengku Ampuan Afzan (HTAA) in Pahang, Malaysia were recruited as study group, and twenty-five healthy individuals were used as a control group. Informed consent of individuals in both groups was obtained prior to their enrolment in the study. All patients were sero-negative for hepatitis C virus, delta virus and human immunodeficiency viruses. The patients were positive for HBsAg for more than six months, and have clinical features of chronic HBV infection according to the hospital records and clinician report.

B. Serological and biochemical assays

The serum HBeAg, anti-HBe, HBsAg, and anti-HBs status of the subjects and control were checked by commercial third –generation ELISA (MONOLISA® Bio-Rad) conducted as routine assays to follow up the status of the patients at screening laboratories in HTAA. Serum aspartate transferaselactate dehydrogenase and alanine transferase were tested by routine automated techniques.

C. Quantification of peripheral blood lymphocytes

Two panels of antibodies were used in fluorescence –activated cell sorter (FACS) analysis to determine the percentage and absolute counts of lymphocyte cells. One panel had three colour direct immunofluorescence reagent TriTEST CD4 fluorescein isothiocyanate (FITC)/ CD8phycoerythrin (PE) / CD3 peridinin chlorophyll protein (PerCp). This panel was used to measure the percentage and absolute counts of mature human T lymphocytes (CD3+), Helper/inducer (CD3+CD4+), and (CD3+CD8+) T lymphocyte. The second panel had four-color immunofluorescence reagent MultiTEST CD3 FITC/CD16, CD56 PE/CD45 PerCP/CD19 allophcocyanin, these reagents were used to measure the percentage and absolute counts of mature human T lymphocytes (CD3+), NK cells (CD4-, CD16+, CD56+), and B cells lymphocyte (CD3-, CD19+) in erythrocyte –lysed whole blood samples. All reagents were from Becton Dickinson (San Jose, CA), and they are used as per the manufacturer's instructions.

D. Quantification of serum HBsAg assay

Elecsys assay was used in serum HBsAg quantification (Roche Diagnostics, Germany) following the manufacturer's protocol for HBsAg II assay. If the results of cut off index (c.o.i) are between 1 and 1000, the final result is the c..o..i X 400, if c.o.i > 1000, the sample is retested at a 1:8000 dilution and the final result is calculated as c.o.i X 8000. While, if the c..o..i is < 1 the sample is retested undiluted. This method was validated by others and a very strong correlation was found between this method and the Architect HBsAg quantitative assay (Abbot) [22].

E. Statistical analysis

The data of experiments were analyzed using SPSS version 21.0 for Windows (IBM, Chicago, IL, USA). Descriptive data such as "mean ± standard deviation, and frequency" were performed. A series of independent t-tests and Pearson correlation coefficient (r) were applied to the data as inferential tests. A two-tailed P-value of ≤ 0.05 was deemed statistically significant results.

III. Results

Out of 50 HBV–infected patients enrolled in the study, 31 were male (n=31). Some study subjects were positive for HBeAg (n= 10) and the rest were negative (n=40). Table (1) shows the percentage of PBL subsets in patients and healthy controls to display whether any particular immunophenotypic profile could be correlated with disease outcomes. As shown in this table, there were no significant differences found in the percentage of Total T cells (CD3+), B cells and NK cells in patients as compared with controls. In contrast, the patients have a significant reduction in the percentage of CD4+ cells (P= 0.05). The same reduction was seen in CD8+ (cytotoxic T cells) and CD4+/CD8+ ratio as compared with healthy donors (P<0.01). Moreover, there was a highly significant increase in serum aminotransferase (ALT and AST) (P< 0.01) in comparison with healthy controls. Interestingly, in table (2), a significant correlation was observed between HBsAg level and the percentage of T cells (r= 0.366) in (figure 2) and NK cells (r=-0.462, P<0.05) in(figure 3), while no significant correlation was shown with B cells, T helper, T cytotoxic, CD4+/ CD8+ ratio and lymphocyte absolute count (r=0.04, r=0.226, r=0.091 , r= 0.23, and r=-0.017) respectively.

TABLE I
COMPARISON OF PBLS BETWEEN PATIENTS AND HEALTHY

Parameters	Patients (mean±SD)(n=50)	control (mean±SD)(n=25)	t-statistics (df)	P value
T cells (mean±SD)	63.32±8.103	64.15±7.534	0.407 (68)	0.694
B cells (mean±SD)	17.42±5.761	17.15±5.019	-0.195 68)	0.855
NK cells (mean±SD)	19.64±7.244	20.80±6.678	.641(68)	0.538
Th cells(mean±SD)	44.34±14.518	50.80±5.146	1.935	0.053*
Tc cells(mean±SD)	20.82±20.522	42.85±5.958	4.703(68)	0.001*
Th:Tc ratio(mean±SD)	1.2245±.31201	4.9810±3.96725	-4.211(68)	0.001*
Lymphocyte absolute count cells/uL	37.95	34.52	451.000	0.524
ALT(IU/L)	41.32	24.38	209.000	0.001*
AST(IU/L)	39.95	20.95	277.500	0.004*

Note:*: statistically significant (independent t- test). Abbreviations: SD: standard deviation, NK:Natural killer cells Th: helper T cells, Tc: cytotoxic T cells, CHB: Chronic hepatitis B, ALT: alanine aminotransferase, AST: aspartate aminotransferase

TABLE II
CORRELATION BETWEEN PERCENTAGES OF PBL SUBSETS AND HBSAG LEVEL IN CHB PATIENTS

Parameter	r value	p value
T cells	0.366	0.009*
B cells	0.046	0.749
NK cells	-0.462	0.001*
Th cells	0.226	0.114
Tc cells	0.091	0.531
Th: Tc ratio	0.23	0.873
Lymphocyte absolute number cells/ul	-0.017	0.905

Note: *statistically significant (P<0.05).Abbreviations: r value: correlation coefficient, T cells:Total T cells, B cells: Total B cells, NK: Total natural killer cells, Th: helper T cells, Tc: CytotoxicT cells, Th: Tc : T helper / T cytotoxic cell ratio.

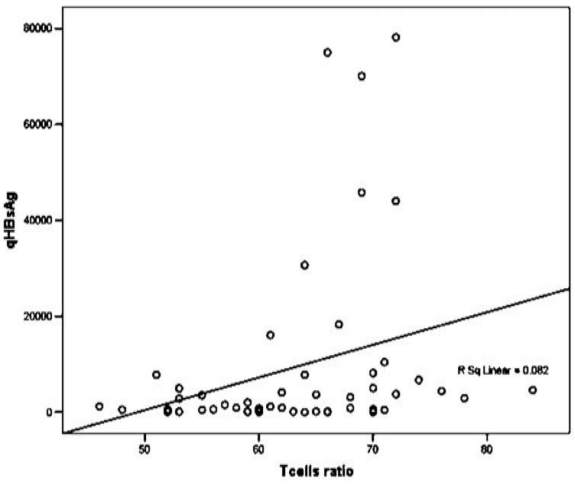

Fig. 2

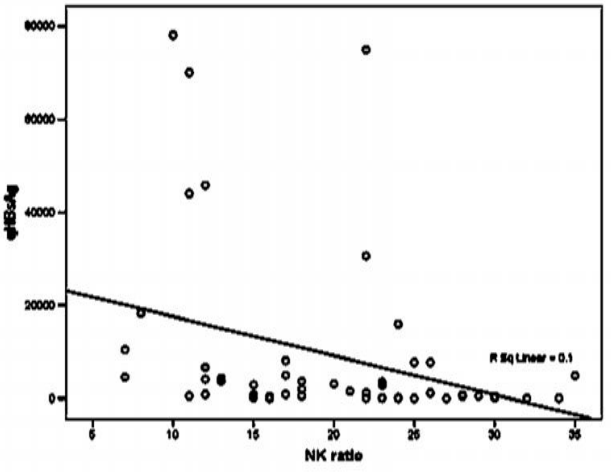

Fig. 3

Fig. 2, 3 show the positive and negative correlation between HBsAg levels (IU/mL) and total T cells(r= 0.366) & NK(r=-0.462) cells in patients with CHB. in statistical significant P <0.05.

IV. DISCUSSION

High percentage of patients infected with HBV are able to clear the virus from their body as a result of a combination of cellular and humoral immune responses, only a low percentage of them, despite the presence these immunological mechanisms of HBV elimination, could not eradicate the virus and thus become chronically infected. The key feature of persistent HBV infection is the impairment of cellular immune responses [23] that is related to the composition of resident immune cells in the liver and the production of excessive viral antigens [4]. Even though many studies have discovered the role of alteration in immune responses during HBV infection, many questions remain unanswered like the relationship between changes in the immune cells population and levels of the viral antigens like HBs, HBe, and HBc. Accordingly, it is very important to continue attempting to answer these questions in order to develop new strategies of therapeutic aspects. In our study, we primarily attempted to observe and analyse the changes of PBL subsets in chronic hepatitis B, and secondly, to discover the correlation between PBL subsets and HBsAg level in these patients. Our aim is to seek for the relationship between PBL derangement and disease progression.

The adaptive immune response during chronic HBV infection is dependent on antigen presenting cells (APCs) namely Kupffer cells and in particular DC (denderic cells), which are important cells for the presentation and maturation of HBV–specific T cells that are the main effectors of HBV clearance. APCs present foreign antigen to T cells (CD4+ and CD8+) and produce the cytokines IL-12 and TNF-α which induce IFN-γ production and the proliferation of T cells CD8+. In addition, IL-12 induces T cells CD4+ differentiation into T helper cells Type 1 (TH1) subset [17, 24].

The outcome of HBV infection is usually influenced by the

kind of cell-mediated response which is expressed in the early phase of infection. Therefore, in the chronic phase, the HBV–specific T cell responses are weak as reflected in the PBL population [25], especially during periods of high viral antigen load [26].

In the present study, our results showed there was no suppression of total peripheral T cells population, B cells, and NK cells in chronic HBV patients in comparison with healthy controls despite the fact that HBV infection induces both humoral and cell-mediated immune responses [27]. Our data, to some extent, differ from those reported in some earlier studies that showed a decrease in percentage of T cells population (CD3+) [8, 28], but similar results to ours have been observed by others [29] showing no significant alterations in the levels of T, B and NK cells. Regarding the

importance of the cytotoxic T cells mediated response for elimination and suppression of HBV replication [30], selective reduction of cytotoxic T cells (CD8+), but not helper T cells (CD4+) is an evidence that these patients have a higher viral load (> 2000IU/ml) and is indicative of persistent HBV infection[31]. However, our results identified only minor reduction in percentages of CD4+ and CD8+ cells as compared with healthy controls. These results may indicate the lacking of CD4+ T cells role to impair CD8+ T cell activity and antibody production [32, 33].

The CD4+/CD8+ ratio is a reflection of immune system health. Several investigators have reported an increasing or a decreasing ratio [34] and sometimes no significant variation in this ratio [29]. The present results showed that CD4+/CD8+ ratio was lower in patients than in normal controls. This result is in concordance with that reported by YinYing et al. (32) who confirmed the existence of a decline in CD4+ /CD8+ in chronic patients. The CD4+/CD8+ ratio is known to reflect the state of the immune response [35], therefore, its up- regulation reflects a strong immune response in patients, conversely its reduction indicates weak immune function and impaired immune regulation. [8] Which is attributed to either liver damage or to increased viral replication [12]. Thus, impairment of immunoregulation may be a big effector in the failure of HBV clearance and the progression to chronic HBV infection [36,37].

Our study confirmed that there is an elevation in ALT and AST levels in CHB patients. Our results are in agreement with Hyodo, et al, who also detected a similar increased ALT level in CHB but no difference between HBeAg negative and positive patients [38]. However, ALT and AST levels did not correspond to the population size of T lymphocytes. Cooper et al [39] observed that there were a high number of T lymphocytes in CHB patients with either normal or elevated aminotransferase levels, while another study could not find a correlation between the population size of CD4+ and CD8+ cells on one hand and ALT levels on the other (30). Thus, in chronic liver diseases, it can be mentioned that the AST and ALT levels, do not positively correspond to the extent of the inflammatory reaction occurring in the liver. Our observation did not reveal a significant difference in T lymphocyte population size between patients who are positive or negative for the HBeAg. However, this result is in concordance with the findings of some researchers [33].

Several recent reports illustrated that HBsAg quantification has a very useful role in the clinical management of chronic HBV, being able to conclude the good response to antiviral therapy as well as to help in optimizing the clinical classification of these patients. Even though, changeable amount of HBsAg have been predicated to reflect different degrees of immune control [40] however, HBsAg level effects on immune response remain controversial and have not been investigated clearly.

In the current study, we attempted to evaluate the association between the status of peripheral immunocompetent cells in chronic HBV infection and serum HBsAg load to estimate the potential relationship between the antiviral immune profile and the sero-virological parameters of HBV infection. Overall, the data indicated that there is a significant negative correlation between HBsAg quantity and the number of NK cells, while a positive correlation was noted with the total number of peripheral T lymphocyte population. However, our data to some extent differed from earlier studies that showed a suppressive effect of HBsAg on the adaptive cell-mediated immune response [30, 40]. Many studies have been described that HBsAg level could be involved in the regulation of the immune response [32] through direct or indirect suppression of the T cells, and there are few reports referring to the direct suppression of T, B, NK, and NKT cells by increasing HBsAg load in chronic hepatitis B virus infection [32]. In 2005, Chen et al [41] showed that the number of NK cells was decreased with the increased expression of HBsAg antigen. These findings are probably in agreement with ours. However, the amount of HBsAg in peripheral blood might influence the HBV- specific CTL response.

V. Conclusion

By analyzing the lymphocyte subsets (T, B, and NK) of peripheral blood in chronic hepatitis B in antiviral drug-naïve patients, the changes in immune system response in these patients is indirectly explored. It was found no significant alteration in peripheral T, B, and NK cells populations, while significant changes were found in CD4+, CD8+, and CD4+/CD8+ ratio in Chronic HBV. Moreover, we observed the important correlation between HBsAg load and immune cells response. It is interesting to note that HBsAg level might conceal the activity of various kinds of immune cells contributing to innate and adaptive immunity. The understanding of these interactions between HBsAg and peripheral blood cells with the alteration in absolute numbers and percentages of these cells could be utilized as a useful biomarker of the persistence and progression of HBV infection. One of limitations in this study is the small sample size which could be remedied by further studies.

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