VIRAL LOAD AND LYMPHOCYTE SUBPOPULATIONS IN NEWLY DIAGNOSED PATIENTS WITH CHRONIC HEPATITIS B

Anastasiya Mihaylova[1], Marta Baleva[1], Atanaska Georgieva[1], Valentina Atanasova[1], Diana Petrova[2], Snejina Mihailova[1], Georgi Popov[3], Elissaveta Naumova[1]

[1]Department of Clinical Immunology, University Hospital „Alexandrovska", Medical University of Sofia, [2]Clinic of Propaedeutics of Internal Diseases, University Hospital „Alexandrovska", Medical University of Sofia, [3]Clinic of Infectious Diseases, Military Medical Academy of Sofia

ABSTRACT

INTRODUCTION: The immune response in the Hepatitis B virus (HBV) represents a key factor in the infection outcome. However, the relation between the viral replication and the host immune reactivity is still a matter of investigation.

AIM: To investigate whether the cellular immune response of newly diagnosed and treatment naïve chronic hepatitis B (CHB) patients may be influenced by the replicative status of HBV.

MATERIALS AND METHODS: A total of 45 (17 female and 28 male) newly diagnosed untreated CHB patients aged 42.48±13.19 years (19÷71 years) were enrolled in this study. The patients were divided into two groups according to the viral load: >0÷≤10^4 copies/ml (n=25) and >10^4÷<10^8 copies/ml (n=17). Flow cytometric immunophenotyping was performed for evaluation of the cellular immunity. Serum HBV DNA load was assessed by quantitative real-time polymerase chain reaction.

RESULTS: Similar alterations were observed in both patients' groups in comparison to the healthy controls. It could be summarized as it follows: decreased total T cells (CD3+) due to low helper-inducer (CD3+CD4+) and suppressor-cytotoxic (CD3+CD8+) subpopulations; reduced effector cytotoxic (CD8+CD11b-; CD8+CD28+) and activated (CD3+HLA-DR+, CD8+CD38+) T-cell subsets; increased CD57+CD8- cells; elevated percentage of B lymphocytes. No significant differences in the studied immune parameters were detected between both patients' groups except the significantly elevated CD4/CD8 ratio in individuals with higher in comparison to those with lower HBV DNA levels.

CONCLUSION: Alterations in the cellular immune response of CHB patients were observed resulting mainly in significantly decreased T-cell subpopulations, particularly those with effector cell immune phenotype regardless of the viral load.

Keywords: *chronic hepatitis B, viral load, cellular immunity, immune cell subsets*

Address for correspondence:
Anastasiya Mihaylova
Department of Clinical Immunology,
University Hospital „Alexandrovska",
Medical University of Sofia
1 Sv. Georgi Sofiyski Str.
1431 Sofia, Bulgaria
e-mail: mihaylova_ap@abv.bg

Received: August 13, 2015
Accepted: September 15, 2015

INTRODUCTION

Hepatitis B virus (HBV) infection is a major global public health problem. Of the approximately 2 billion people who have been infected worldwide, more than 350-400 million remain infected chronically and become carriers of the virus (1). The prevalence of HBV infection varies markedly in different geographic areas of the world, as well as in dif-

ferent population subgroups (2). Overall, about 5% of the population in Bulgaria is infected with HBV, in some districts reaching up to 7% (3), which places our country among the regions of the world with moderate prevalence (2-7%) of the virus but among those with higher rates in Europe.

The patient's immune response is one of the major factors influencing the viral eradication or chronification of the liver damage. The chronic course of HBV infection depends also on the host genetic factors and the virus itself – genotype and viral load.

The aim of our study was to investigate whether the cellular immune response of the newly diagnosed and treatment naïve chronic hepatitis B (CHB) patients may be influenced by the replicative status of HBV.

MATERIALS AND METHODS

Study subjects

We investigated 45 (17 female and 28 male) treatment naïve patients with chronic viral hepatitis B aged 42.48±13.19 years (19÷71 years). All patients were referred from the Department of Gastroenterology at the Clinic of Propaedeutics of Internal Diseases, University Hospital "Alexandrovska", Sofia, after clinical and laboratory evaluation according to predefined inclusion and exclusion criteria. The diagnosis of chronic hepatitis B was based on the recommendations of the American Association for Study of Liver Diseases. The results were compared to 29 age- and sex-matched healthy volunteers with normal liver function and negative serological markers for viral hepatitis. Prior to the evaluation, written informed consent for participation in the study was obtained from all patients and control subjects.

Cellular immunity

For the assessment of the cellular immunity, phenotyping of peripheral blood cells was performed using different combinations of monoclonal antibodies. The analysis was done with FACSCanto flowcytometer and FACSDiva software (Becton Dickinson, USA).

The following immune cells were investigated: total T (CD3+) cells; helper-inducer (CD3+CD4+) and suppressor-cytotoxic (CD3+CD8+) T-cell subsets; B (CD19+) and NK (CD3-CD16+56+) cells; T-lymphocytes at different differentiation stages and functional characteristics: effector (cytotoxic and/or memory T cells - CD8+CD28+), terminally differentiated cellular subpopulations with memory/effector function (CD8+CD28-, CD8+CD57+), cellular populations with cytotoxic (CD8+CD11b-) and suppressor (CD8+CD11b+) potential; activated T-cells (CD3+HLA-DR+, CD8+HLA-DR+ and CD8+CD38+); T-lymphocytes with NK activity [NKT – CD3+CD(16+56+)].

Viral load

The quantitative evaluation of viremia was performed using TaqMan quantitative RT-PCR (Applied Biosystem Real Time PCR primers and probe; Applied Biosystem 7300 RT-PCR system). The method is based on the detection of fluorescent signal generated by hydrolysis of a TagMan probe during target DNA amplification. For the generation of a reference curve four standards (Clonit HBV DNA complete genome) were used. The high sensitivity of the method (98%) allowed determining low hepatitis B viral load (≤100 copies/ml).

Statistical analysis

The comparative analysis of the cellular populations in different groups was performed using parametric (Student's t-test) and non-paramertic (Mann-Whitney) methods. The correlation analysis (viral load/cellular populations) was done by Spearman's rank correlations. The statistical analysis was performed using SPSS v.16. Values of $P<0.05$ were considered statistically significant.

RESULTS

According to the viral load the investigated HBV patients were divided into two groups: group 1 – viral load $>0 \div \leq 10^4$ copies/ml (25 patients) and group 2 – viral load $> 10^4 \div \leq 10^8$ copies/ml (17 patients). No patients with extremely high viremia ($>10^8$ copies/ml) were diagnosed during the study. The results of immunophenotyping are presented in Table 1.

Our data (Table 1) showed similar alterations in both groups of patients as compared to healthy controls which could be summarized as it follows:

1. Low values of total T-cells (CD3+) due to decreased helper-inducer (CD3+CD4+) and suppressor-cytotoxic (CD3+CD8+) subpopulations;

Scripta Scientifica Medica, vol. 47, No 3, 2015, pp. 65-70

Table 1. Cellular populations in patients with chronic hepatitis B depending on the viral load (copies/ml) and in healthy control

Cellular populations	Patients		Healthy		P	
(%/absolute count*)	>0÷≤10^4 group 1 (n=25)	>10^4÷ ≤10^8 group 2 (n=17)	(n=29)	Group 1/ Healthy	Group 2/ Healthy	Group 1/ Group 2
Total T cells(CD3+)	67.35±8.0 955±305	67.29±6.28 1201±436	74.51±6.31 1512±439	0.03 0.000	0.001 0.040	0.946 0.137
Activated T cells (CD3+HLA-DR+)	6.6±4.96 96±59	5.41±3.84 91±82	9.00±4.74 166±65	0.1 0.008	0.021 0.005	0.586 0.389
Helper-inducer T cells (CD3+CD4+)	37.18±6.63 519±164	41.76±7.03 749±287	46.08±6.64 965±313	0.000 0.000	0.047 0.04	0.057 0.01
Suppressor-cytotoxic T cells (CD3+CD8+)	26.12±5.23 366±137	23.18±7.58 416±207	29.76±5.97 624±167	0.018 0.000	0.002 0.001	0.057 0.591
CD4/CD8	1.47±0.35	2.12±1.41	1.65±0.48	0.061	0.566	0.031
B cells (CD19+)	13.3±5.03 203±142	12.82±3.54 237±105	9.24±2.81 192±82	0.001 0.911	0.001 0.158	0.919 0.217
NK cells (CD3-CD16+56+)	15.3±5.16 229±146	15.59±6.0 269±9.5	14.00±6.41 289±149	0.322 0.138	0.447 0.906	0.786 0.202
NKT cells (CD3+CD16+56+)	10.65±5.71 139±60	7.65±5.02 121±80	6.56±4.68 137±113	0.014 0.345	0.377 0.834	0.114 0.285
CD57+CD8+ cells	15.94±5.65 248±128	12.65±7.11 209±139	12.74±6.20 267±151	0.101 0.768	0.725 0.181	0.079 0.345
CD8+CD11+ cells	15.3±6.38 223±112	11.82±4.42 217±120	10.71±4.09 220±104	0.022 1.0	0.518 0.941	0.114 0.935
CD8+CD11b- cells	14.25±3.3 203±82	15.0±5.0 270±134	19.30±6.10 388±138	0.011 0.000	0.062 0.037	0.838 0.161
CD8+CD28+ cells	13.88±3.62 191±66	11.82±3.57 228±110	17.34±3.80 340±115	0.021 0.000	0.000 0.023	0.182 0.325
CD8+CD28- cells	15.86±5.3 241±123	14.91±7.35 241±152	12.58±5.89 245±141	0.128 0.983	0.448 1.0	0.423 1.0
CD8+CD38+ cells	15.2±6.75 218±150	15.59±6.46 251±111	21.70±7.04 425±123	0.026 0.002	0.007 0.001	1.0 0.254
CD8+HLA-DR+ cells	5.06±2.86 73±42	5.06±3.77 77±61	6.78±4.14 132±70	0.2 0.02	0.149 0.014	0.708 1.0

** cells/ml*

2. Reduced T-cell subsets with effector cytotoxic activity (CD8+CD11b-; CD8+CD28+);
3. Decreased activated (CD3+HLA-DR+, CD8+CD38+) T-cells;
4. Elevated percentage of B cells.
5. Additionally, individuals with lower viral load had increased percentages of NKT [CD3+(CD16+56+)] and CD8+CD11b+ cells in comparison to healthy controls.

As a whole, no significant differences in the studied immune parameters were observed between both patients' groups with the exception of significantly higher values of CD4+ T cells and a trend of more pronounced reduction of CD8+ T-cell subsets in individuals with higher as compared to those with lower rate of viral replication. These disturbances lead to a markedly higher (p=0.031) CD4/CD8 ratio in patients with viral load >10^4 copies/ml in comparison to those with DNA HBV levels ≤ 10^4 copies/ml.

As another approach to test whether the viral replication rate could influence the cellular immunity of patients with chronic HBV infection, we have performed a correlation analysis between the level of viremia and the values of the investigated cell subsets. No statistically significant correlations were found between the HBV replication rate and the cellular immune alterations.

DISCUSSION

In the majority of cases the HBV infection is self-limiting due to the fact that effective immune responses from innate and adaptive immune systems can lead to complete clearance of the virus or at least to suppression of viral replication. Studies have revealed that T-cellular immune reactivity is essential for the disease pathogenesis. Powerful CD8 immune response has been proven in patients with spontaneous eradication of HBV, and in those with chronic disease evolution this reactivity is much weaker or absent (4,5,6). The effector cell dysfunction is one of the main factors for the chronic evolution of infection. The chronic course of the disease is associated with multiple defects in T-cellular immunity correlating with the viral replication (7). The higher HBV DNA levels inhibit both CD4+ and CD8+ T-cells (8) and these alterations increase the risk for liver dysfunction, disease chronification, liver cirrhosis and hepatocellular carcinoma. It should be taken into consideration that the interpretation of viral load data and comparison of results have been complicated by the inconsistency among the various units of measure for HBV DNA and the different criteria for grading the level of viral replication. A study in India (9) divided the HBV patients into two groups according to their viral loads (lower - <2000 IU/ml and higher - >2000 IU/ml) and found decreased total T-cell counts in both patients' groups as compared to healthy individuals. Additionally, subjects with higher viral replication demonstrated a decrease of cytotoxic T-cells in comparison to controls, whereas such differences were not observed in individuals with lower HBV DNA levels. Using another approach for the classification of viral load Xibing et al. (10) found elevated numbers of specific cytotoxic T-lymphocytes and NK cells, and decreased non-specific cytotoxic T-cell subsets in the group with lower (HBV DNA 10^4-10^5 copies/ml) compared to this with higher (10^6-10^7 copies/ml) rate of viral replication. You et al. (11,12) described negative correlation between the levels of HBV DNA on one hand, and CD4+ cells and CD4/CD8 ratio – on the other hand. On the contrary, their data revealed positive correlation of CD8+ cells with viral load.

The results of our study showed no differences between the two groups of CHB patients (with low $\leq 10^4$ copies/ml and high $>10^4$-10^8 copies/ml viral DNA levels) concerning the studied cellular immune parameters. In both patients' groups we observed marked T-cell deficiency. These findings are consistent with the results of Mukherjee et al. (9), but do not comply with those of others authors (11,12). The detailed analysis of T-lymphocytes according to differentiation stages and functional characteristics revealed decreased cell subpopulations with putative cytotoxic potential (CD8+CD11b- and CD8+CD28+) in both patients' groups compared to healthy controls. These results correlate with the reported reduced nonspecific cytotoxic T-cells in chronic hepatitis B (9). It should be pointed out that Sun et al. (13) established increased percentages of CD8+CD28- subset in the CD8+ population in CHB correlating with the levels of viral DNA. In line with the above mentioned observations Li et al. (14) described decreased CD8+CD28+/CD8+CD28- T-cells ratio in CHB patients compared to healthy controls due to elevated values of CD8+CD28- cell subset and associated with HBV loads. In contrast to the abovementioned data, no alterations of CD8+CD28- cell subsets in CHB were found by us. A possible explanation of the observed discrepancies could be attributed to the different approaches of cell subset analysis – in the lymphocytes in our study or within the T-cell population in the other investigations. It is interesting to note the decreased levels of T-cells expressing different activation markers (CD3+HLA-DR+, CD8+CD38+) found in our HBV positive patients (regardless of viral loads) in comparison to healthy controls. Cao et al. (15) established significant positive association between the CD8+CD38+ T-cell proportions and serum HBV DNA, while Nikolova et al. (16) reported the lack of such correlation. It should be also noted that Ye et al. (17) observed lower proportions of activated CD38+ T cells in patients with non-recovered acute-on-chronic liver failure (ACLFs) (characterized by higher HBV DNA levels)

Scripta Scientifica Medica, vol. 47, No 3, 2015, pp. 65-70

and recovered ACLFs (showing lower viral load) in comparison to healthy controls. Based on their data the authors suggest that a decrease in activated $CD8^+$ T-cells may be related to poor outcomes in patients with severe hepatitis. Another observation in this topic is the described by Tan et al. (18) a negative correlation between serum HBV DNA levels and activated CD4+HLA-DR+ cells.

Relatively few studies have evaluated the role of B and NK cells in chronic hepatitis B and their relation to viral load. The increased proportions of B-cells observed by us in untreated HBV positive individuals compared to healthy controls are in accordance with the findings of Sun et al. (19). Lower NK cell levels in chronic HBV in comparison to controls (20) and a more pronounced decrease in patients with higher viral load have been reported in previous studies on immune cells and viral replication (10). On the contrary, no relationship between viral load and NK cell numbers, as well as differences between patients and normal individuals were observed in our study which is consistent with the data of Mukherjee et al. (9). These contradicting findings could be partially explained by the kinetics of innate and adaptive immune responses in viral infections. Since NK cells are the first-line of immune defense a rise of their levels, concomitant with viral replication, could be observed in the early stage of HBV infection, while with the development of adaptive immune response their number may decline to normal values. The elevated proportion of T-cells with NK-like activity [CD3+CD(16+56)+] found in our study in patients with lower viral load could be interpreted as an indirect evidence of the inverse correlation between the serum HBV DNA levels and NKT cells, described by other investigators (10,21).

In conclusion, the hepatitis B virus has no direct cytopathogenic effect. The liver damage and involvement of other organs are consequences of the host immune response against the virus. The variable results for immune reactivity observed in different investigations could be explained with the fact that chronic hepatitis B represents a dynamic disease state. However, it should be pointed that the T-cell immune reactivity has a crucial role in HBV elimination. This process is mediated by CD4+ and CD8+ T-cell subpopulations. Our results did not show significant differences in T-, B- and NK-cell response of patients with different viral load. Nevertheless, we found alterations in some of these parameters in individuals with chronic HBV infection (regardless of viral load) as compared to healthy controls. Our observations show that the cellular immune disturbances in CHB patients are associated with significantly decreased T-cell populations, particularly those with effector cell immune phenotype.

REFERENCES

1. World Health Organization, "Hepatitis B," 2012, World Health Organization Fact Sheet, Available from: http://www.who.int/mediacentre/ factsheets/ fs204/en.
2. Lavanch D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat. 2004;11:97–107.
3. Kojuharova M. Prevention of viral hepatitis B in Bulgaria. Department of epidemiology and communicable disease surveillance. National Center of Infectious and Parasitic Diseases, 2011.
4. Maini MK, Boni C, Lee CK et al. The role of virus-specific CD8 cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med. 2000;191:1269-1280.
5. Bertoletti A and Maini MK. Protection or damage: a dual role for the virus specific cytotoxic T lymphocyte response in hepatitis B and C infection? Cur Opin Microbiol. 2000;3:387-392.
6. Bertoletti A and Gehring A. Immune response and tolerance during hepatitis B virus infection. Hepatol Res. 2007;37,Suppl 3:S331-S338.
7. Bertoletti A and Gehring A. The immune response during hepatitis B infection. J Gen Virol. 2006;87(6):1439-1449.
8. Boni C, Penna A, Ogg GS, Bertoletti A, Pilli M, Cavallo C, et al. Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiviness in chronic hepatis B: new perspectives for immune therapy. Hepatology. 2001;33:963-971.
9. Mukherjee R, Reddy PB, Arava J, Rao P, Mitnala S, Gupta R, Reddy D. Relationship between serum HBsAg level, HBV DNA level, and peripheral immune cells in patients with chronic hepatitis B infection. Hepatic Medicine: Evidence and Research. 2010;2:157-162.
10. Xibing G, Xiaojuan Y, Zhonghua L, Juanhua W. Alteration in cellular immunity after chronic hepati-

tis B deteriorated into severe hepatitis and its significance. Hepat Mon. 2011;11(10):810-815.

11. You J, Sriplung H, Geater A, Chongsuvivatwong V, Zhuang L, Chen HY, et al. Effect of viral load on T-lymphocyte failure in patients with T-lymphocyte B. World J Gastroenterol. 2008;14 (7):1112-1119.

12. You J, Zhuang L, Zhang YF, Chen HY, Sriplung H, Geater A, et al. Peripheral T-lymphocyte subpopulations in different clinical stages of chronic HBV infection correlate with HBV load. World J Gastroenterol. 2009;15(27):3382-3393.

13. Sun XH, Liu QL, Li M, Gao YQ. [The study of CD4+ and CD8+ T subsets in chronic hepatitis B patients]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi (Article in Chinese). 2011;27(5):545-547, 550.

14. Li X, Kong H, Tian L, Zhu Q, Wang Y, Dong Y, et al. Changes of costimulatory molecule CD28 on circulating CD8+ T cells correlate with disease pathogenesis of chronic hepatitis B. Biomed Res Int. 2014:423181. doi: 10.1155/2014/423181.

15. Cao W, Qiu ZF, Li TS. Parallel decline of CD8+CD38+ lymphocytes and viremia in treated hepatitis B patients. World J Gastroenterol. 2011;17(17):2191-2198.

16. Nikolova M, Petrova M, Muhtarova M, Nikolovska D, Krastev Z, Taskov H. Circulating CD8+ T cell subsets and CD39+ T regulatory cells in patients with HBeAg-negative chronic hepatitis B. Probl Infect Parasit Dis. 2001;39(1):34-39.

17. Ye Y, Liu J, Lai Q, Zhao Q, Peng L, Xie C, et al. Decreases in activated CD8+ T cells in patients with severe hepatitis B are related to outcomes. Dig Dis Sci. 2015;60(1):136-145.

18. Tan G, Zhao W, Liu X, Wang J, Wu Y. Immunophenotypic profile of intrahepatic and circulating lymphocytes in chronic hepatitis B patients. Hepatogastroenterology. 2012;59(117):1516-1521.

19. Sun H, Lv J, Tu Z, Hu X, Yan H, Pan Y, et al. Antiviral treatment improves disrupted peripheral B lymphocyte homeostasis in chronic hepatitis B virus-infected patients. Exp Biol Med (Maywood). 2013;238(11):1275-1283.

20. Zhao PW, Jia FY, Shan YX, Ji HF, Feng JY, Niu JQ, Ayana DA, Jiang YF. Downregulation and altered function of natural killer cells in hepatitis B virus patients treated with entecavir. Clin Exp Pharmacol Physiol. 2013;40(3):190-196.

21. Diao H, He J, Zheng Q, Chen J, Cui G, Wei Y, et al. A possible role for NKT-like cells in patients with chronic hepatitis B during telbivudine treatment. Immunol Lett. 2014;160:66-71.