The Korean Journal of Internal Medicine
Vol. 2, No. 2, July, 1987

Immunological Studies in Patients with HBsAg-positive Chronic Active Hepatitis

—Spontaneous Lymphocyte Transformation and Natural Killer Cell Activity—

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The present study was to address the nature of cells which are responsible for enhanced spontaneous lymphocyte transformation (SLT) observed in patients with HBsAg-positive chronic active hepatitis (CAH). The subjects consisted of 34 cases with HBsAg-positive CAH (group I), 31 HBsAg carrier (group II), and 27 normal persons (group III) who had no serological evidence of hepatitis B virus (HBV) infection. SLT values and the number of cells bearing HLA-DR antigens in group I (1021.45±276.40 cpm, 36.94±4.90%) were significantly ($p<0.01$) elevated as compared to group II (103.74±30.44 cpm, 13.26±4.72%) and III (118.92±30.84 cpm, 14.93±5.10%), but there was no difference of the number of the IL-2 receptor-bearing cells among each groups. Though natural killer (NK) cell activity in both group I (65.42±15.77%) and II (59.14±14.89%) were significantly enhanced as compared to group III (46.25±20.20%), there was no difference in between group I and II. These findings indicate that the cells bearing HLA-DR antigen, but not NK cells. are responsible for the enhanced SLT in patients with CAH.

Key Words: *Spontaneous lymphocyte transformation, HLA-DR antigens, IL-2 receptor, Natural killer (NK) cells*

INTRODUCTION

The prominent intrahepatic mononuclear inflammatory cell infiltrate characteristic of chronic viral B hepatitis suggests that the associated hepatocellular injury may be mediated by cell-mediated autoimmune mechanisms[1]. This autoimmune hypothesis seems to be supported by recent reports describing the presence of relevant cytotoxic effector lymphocytes capable of killing autologous hepatocytes[2~4] and observations of defective immunoregulatory suppressor cell function[5~7] in the peripheral blood mononuclear cell (PBMNC) population of patients with chronic hepatitis B virus (HBV) infection.

However, since those assays concerning T cell-mediated cytotoxic reaction requiring major histocompatibility complex (MHC)-restriction[8] and specific T suppressor cell function are not possible in an vivo system, and because the precise nature of target antigens aganist which effector cells are reacting is not known, the pathogenetic mechnaisms of hepatocellular damage of those patients have not yet been clarified. Moreover, since most of those assays are rather complicated in vitro tests, it is difficult to use as immune monitors in routine clinical laboratories, in assessing the activity and prognosis of chronic hepatitis.

Spontaneous lymphocyte transformation (SLT)

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assay has been used as an immune monitor in assessing the activity and prognosis of chronic active hepatitis (CAH)[9~10]. SLT measures an in vitro blastogenic property of circulating lymphocytes without addition of mitogens and/or antigens and represent therefore a measure of the blastogenic activity of lymphoctyes which were already stimulated in vivo[11]. We observed in the previous study that SLT in patients with HBsAg-positive CAH was significantly enhanced and postulated that SLT test might be a useful in vitro assay for the understanding of pathogenetic mechanisms as well as for better assessment of the activity of HBsAg-positive CAH. The present study was designed to investigate the nature of the lymphocytes which are responsible for enhanced SLT observed in those cases, by using mouse monoclonal antibodies to human HLA-DR antigens and the interleukin-2 (IL-2) receptor (Tac). In addition, we also examined natural killer (NK) cell activity of PBMNC of those patients.

MATERIALS AND METHODS

1. Subjects

Ninty-two subjects entered into this study. These included 34 patients with HBsAg-positivie CAH (group I) diagnosed by clinical[12] and histological observation according to criteria of De-Groote et al[13], and 31 chronic HBsAg carriers (group II) with normal serum transaminases level during observation period of at least 6 months. Twenty-seven normal control persons without clinical evidence of liver diseases and HBV infection (group III) were included as a control group. The ranges of serum transaminases and HBV serology results were summarized in Table 1. None of these subjects was receiving corticosteroid or other immunosuppressive therapy. Patients with alcoholic liver disease, advanced liver cirrhosis, primary hepatocellular carcinoma, organ transplantation or with known lymphoproliferative diseases were excluded from this study.

2. Isolation of Perepheral Blood Mononuclear Cells (PBMNC)

PBMNC were isolated using a method described elsewhere[14]. Briefly, blood samples form each subject were collected by venipuncture into a heparinized (10 U/ml blood) syringe. PBMNC were seperated by centrifugation on a Ficoll-Hypaque gradient[15], and washed three times with phosphate-buffered saline (PBS, 0.15M, PH 7.2) without calcium and magnesium, counted and resuspended at a cell density of 10^6/ml in RPMI 1640 (Flow) medium supplemented with 5% fetal bovine serum (FBS, Gibco Lab., New York, NY, U. S.A.).

3. Spontaneous Lymphocyte Transformation (SLT)

SLT asay of PBMNC was carried out according to the method described by Vessella et al.[11]. and Crowther et al.[16]. with minor modifications. Four × 10^5 PBMNC in 0.25 ml of RPMI 1640 supplemented with 20% heat-inactivated pooled human AB plasma were cultured in a microtitration plate (round-bottomed, Costar, 205 Broadway/Camgridge, MA, U.S.A.) at 37℃ in humidified atmosphere containing 5% CO_2. At the start of cultivation, the cells were labelled with 1 uCi/well of ^{3}H-thymidine (Tritiated thymidine, SA: 20 Ci/mM, The Radiochemical Centre, Amersham) and harvested 18 hr later using a cell harvestor (Cell harvestor 530, Flow). The cell pellets on the filer

Table 1. Laboratory Findings in Patient Groups

Patient group	Age (yr)	SGOT (IU/L)* (5.0 – 45.0)	SGPT (IU/L)* (5.0 – 40.0)	HBsAg/HBcAb/HBsAb
I (n=34) HBsAg (+) - CAH	37.5 ± 6.8	124.7 ± 40.4	164.98 ± 50.5	+ / + / –
II (n=31) HBsAg- carrier	39.4 ± 5.6	19.7 ± 3.9	20.32 ± 4.9	+ / + / –
III (n=27) All HBV markers (–)	40.2 ± 6.4	20.4 ± 4.8	19.32 ± 4.8	– / – / –

– CAH in group was diagnosed by liver biopsy findings
* : Normal values.

paper were dissolved for 4 hr at room temperature in 2ml of Insta-gel (Packard). The radioactivity was counted by a Packard Tri-carb Liquid Scintillation Spectrometer (Model 4530). All cutures were carried out in triplicate under sterile condition.

4. Determination of HLA-DR Antigens and the IL-2 Receptor-bearing Cells

Cells bearing HLA-DR antigens and IL-2 receptor (Tac) were determined by indirect immunofluorescent staining by using mouse anti-human monoclonal antibdies (MAb). Two MAbs are used; mouse anti-human HLA-DR MAb (Ortho Pharmaceutical Corp., Raritan, NJ, U.S.A.) reacting with a varity of human cells including activated T cells, B cells, and macrogphage[17] bearing HLA-DR antigens, and mouse anti-human IL-2 receptor MAb (T cell Sciences, Inc. Cambridge, MA, U.S.A.) reacting with activiated T lymphocytes[18]. A volumer of 0.1 ml of PBMNC suspension (2×10^6/ml) was mixed with 5 ul of each reconstituted MAb (50 ug/ml) and incubated at 4℃ for 30 min. After incubation the cells were washed three times in cold PBS and resuspended in 0.1 ml PBS. They were stained at 4℃ for 30 min with fluorescein-conjugated goat anti-mouse IgG (GAM/IgG-Fc/FITC, Ortho Pharmaceutical Corp., Raritan, NJ, U.S.A.) and then washed three times in cold PBS and resuspended in a drop of PBS. Membrane immunofluorescence was evaluated with AO immunofluorescent microscope equipped with an excitation filter, a barrier filter, and a incident illumination. Three to four hudred cells were counted for the calculation of percentage of the positively stained cells.

5. In Vitro Cytotoxic Assay

Nk cell activity of PBMNC from each subject aganinst the human erythroleukemic cell line K562 was carried out by using a 4hr ^{51}Cr release assay[19]. Aliquots containing 1.5×10^6 target cells (K562) were labelled with 100 uCi of sodium chromate solution (Korea advanced energy research institute, Seoul, Korea.) for 90 min o.5 ml of RPMI 1640 supplemented with 10% FBS. After three washings, 10^4 cells in 0.1 ml of medium were added into U-bottomed microtiter plates (Costar, 205 Broadway/Cambridge, MA, U.S.A.). In each assay, the effector cells were added to a constant number of target cells (10^4) at final effector: target cell ratio of 40:1. After incubation for 4hr at 37℃ in a humidified atmosphere containing 5% CO_2 in air, supernatant were harvested and counted in a gamma counter. Per cent specific ^{51}Cr release was calculated as follows:

$$\frac{\text{cpm test release} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100.$$

Spontaneous and maximum releases are cpm release from target cells inculbated in medium alone and in 1 N HCl-added medium, respectively. The range of spontaneous release from K562 was 7-14% of the total isotope count. Maximum release determined by 1 N HCl was about 95%.

RESULTS

1. SLT Assay

The SLT values of patients with HBsAg-positive CAH (group I) was significantly ($p<0.001$) elevated when compared to HBsAg carrier (group II) or healthy controls (group III) who had no serological evidences of HBV infection. However, there are no appreciable differences of SLT values between HBsAg carriers (group II) and control persons (group III).

2. Determination of HLA-DR Antigens and the IL-2 Receptor-bearing Cells

As summarized in Table 2, the number of cells bearing HLA-DR antigens in HBsAg-positive CAH (group I) with enhanced SLT reaction was

Table 2. SLT*, HAL-DR Antigens and Tac-Assay in Patients Groups

Groups and number of cases	SLT (cpm ± SD)	HLA-DR antigens (+) cells (%)	Tac (+) ** cells (%)
I (n=34)	1021.45 ± 276.40 [a]	36.94 ± 4.90 [d]	< 0.5
II (n=31)	103.74 ± 30.44 [b]	13.26 ± 4.72 [e]	< 0.5
III (n=27)	118.92 ± 30.84 [c]	14.43 ± 5.10 [f]	< 0.5

* : SLT : Spontaneous lymphocyte transformation ** : Tac : Interleukin-2 (IL-2) receptor
p-value between ; – (a) and (b) : < 0.01, (a) and (c) : < 0.01, – (d) and (e) : < 0.01, (d) and (f) : < 0.01

Table 3. NK Activity in Chronic Liver Diseases

Groups	SGPT (IU/L)	NK activity (Percent ± SD)
Group I (n=13) HBsAg (+)-CAH	142.42 ± 49.32	65.42 ± 15.77[a)]
Group II (n=11) HBV carrier	20.32 ± 4.90	59.04 ± 14.89[b)]
Group III (n=12) All HBV markers (−)	19.32 ± 4.80	46.25 ± 20.20 [c)]

P value between: (a) and (b) : NS
(b) and (c) : < 0.05
(a) and (c) : < 0.05

significaclty ($p<0.01$) increased as compared to those of HBsAg carriers and normal controls. However, no such differences were observed in between group II and III patients. Neither CAH patients nor HBsAg carriers showed an increased number of cells bearing IL-2 receptor defined by anti-Tac MAb when compared to normal contorls.

3. NK Cell Activity

Table 2 shows comparison of NK cell activity in PBMNC from patients with HBsAg-postivie CAH, HBsAg carriers, and normal healthy controls. NK activity of CAH patients and carriers was 65.42± 15.77% and 59.04±14.89%, respectively. The difference in between these two groups was not statistically significant. On the other hand, NK activity observed in normal healthy controls was significantly ($p<0.05$) lower than those of CAH patients and carriers.

DISCUSSION

We previously reported that SLT of PBMNC in patients with HBsAg-positive CAH was significantly enhanced as compared to the cases of inactive CAH and HBsAg carriers[10]. Since enhanced SLT of PBMNC usually reflects the presence of proliferating cell population(s) in the peripheral blood and because the alterations in serum transaminases during relapsing phase of CAH lagged one to two weeks behind changes in SLT response, we postulated that abnormal SLT responses observed in those patients might be related to a primary pathogenetic determinant in on-going liver injury.

The current study was undertaken to address two issues of interest related to SLT responses observed in chronic hepatitis B viral infection; first, whether the elevated SLT value is associated with an increased number in PBMNC of activated T lymphocytes bearing HLA-DR antigens and/or the IL-2 receptor; second, whether NK cell activity might be correlated to the enhanced SLT. The results of the present study, while confirming our previous results, did not show a direct correlation between SLT values and the number of activated T cells bearing HLA-DR antigens and/or the IL-2 receptor, as tested by single and double immunofluorescent staining techniques. Instead, the enhanced SLT response was correlated with an increased number in PBMNC of cells bearing HLA-DR antigens. Although these findings, the presence of increased number of HLA-DR antigens-bearing cells but the virtual absence of IL-2 receptor-bearing cells, is consistent with our previous result that mixed lymphocyte culture (MLC) reaction in those patients were significantly enhanced in both syngeneic and allogeneic co-cultures, the immunological meaning of the data available herein are indeed open to several interpretations. Firstly, the increased number of HLA-DR antigens-bearing cells together with enhanced SLT responses in PBMNC of those patiens might be caused by an increased blastogenic transformation of B lymphocytes. This impression was indeed reported by Budillon et al[20]. who observed, in PBMNC of patients with HBsAg-posive CAH, a significant correlation between the decreased level of T active cells and increased blastogenic transformation of B lymphocytes as tested by spontaneous thymidine incorporation. Since B cells also express HLA-DR antigens[17,21], one may speculate that the increased number of cells bearing HLA-DR antigens observed in the present study may be caused by enhanced blastogenic transformation of B lymphocytes. This impression, in conjunction with other recent report[7] describing impaired suppressor cell function and subsequently increased spontaneous IgG production by B lymphocytes, provides a substantial evidences in favor of B lymphocyte series in enhancing the SLT responses. It should be, however, pointed out that alteration of SLT during relapsing and remission phase of CAH precede the changes of serum tranaminases and therefore it may probably be related to a promary pathogenetic determinatn in liver cell injury. Moreover, our recent preliminary observation shows that a substantial number of cells bearing HLA-DR antigens were still remained

even after removal of B lymphocytes. Some recent studies[22,23] report that the mononulear cells which infiltrated in areas of piecemeal necrosis and spotty necrosis are not killer (K) cells which are responsible for antibody dependent cellular cytotoxidity (ADCC) but are predominantly CD4- and CD8-positive T cells. These findings are in fact not consistent with the hypothesis stating that enhanced blastogeneic transformation of B cells and subsequently increased production of various antibodies may be an important pathogenetic determinant of CAH.

Secondly, in spite of the virtual absence of T cells bearing IL-2 receptor observed in patients of HBsAg-positive CAH, the possiblility should still be considered that the cells bearing HLA-DR antigens may be activated T cells. It has been known that the IL-2 receptor absent in normal resting T cells appears when T cells are stimulated by antigens[18,24~26]. Expression on T cells of the IL-2 receptor is much dependent in circulation blood on stimulating antigens and its expression on activated T cells in vivo is much more transient than HLA-DR antigens[27]. Although it might be possible that in vivo activated T cells in pateitns with HBsAg-poitive CAH may lose the IL-2 receptor earlier than HLA-Dr antigens and there have been a number of reports describing the presence of T cells bearing HLA-DR antigens in patients with HBsAg-positive CAH[7,28], we at this stage do not have a clear explanation on the presence of cells which devoid of the IL-2 receptor in those cases. On the basis of our preliminary results stated previously, however, the remained cell population bearing HLA-DR antigens after reomval of B cells are likely to be T cells. Much more studies including simultaneous examination of T cell markers for the circulating PBMNC as well as for infiltrated cells in areas of liver cell inflammation, however, should be done for better assessment of T cell function playing in pathogenetic mechanisms of CAH.

NK cells have been implicated in resistance aganist viral infections[29,30]. Since enhanced cytolytic activity is usually demonstrated after activation of these cells by a variety of stimuli, including viral infections, interferons[31], and IL-2[32,33], it is quite possible to consider that activation and subsequently enhanced NK activity could be observed in patients with chronic HBV infection. We observed indeed an enhanced NK activity in both the patiehts with CAH and HBsAg carriers as compared to normal controls. However, NK activity in CAH patients with an enhanced SLT response was not different from those of HBsAg carriers and therfore NK cells may not play a role in hepatocellular injury observed in CAH. Although similar observation was also reported by other workers[34], more convincing evidence supportive to this hypothesis was recently reported by Van den Oord et al[23] who clearly demonstrated that mononulear cells infiltrated in areas of piece meal necrosis were not NK or K cells. All these findings indicate that NK cells do not play a role in eliminating HBV-infected cells and for actual hepatocellular damage observed in patients with CAH.

In conclusion, although we do not know yet the precise nature of HLA-DR antigen-bearing T cells in our cases, we speculate that the high SLT values together with the increased number of HLA-DR antigen-bearing cells in PBMNC of patients with HBsAg-positive CAH may be an in vitro measurement of the level of activated T cells generated in vivo by the presence of unknown neoantigen (s) against which host lymphocytes are reacting. These activated lymphocytes can be recirculated and further differentiated into cytotoxic effector cells which are infiltrated in areas of piece meal necrosis leading to hepatocellular injury.

REFERENCES

1. Eddleston ALWF, Williams R: *Inadequate antibody response to HBsAg or suppressor T cell defect in development of chronic active hepatitis. Lancet ii:1543, 1974*
2. Geubel AP, Keller RH, Summerskill WHT, Dickson ER, Tomasi TB, Shorter RG: *Lymphocyte cytotoxidity and inhibition studied with autologous liver cells: observations in chronic active liver disease and the primary biliary cirrhosis syndrome. Gastroenterology 71:450, 1976*
3. Wands JR, Isselbacher KJ: *Lymphocyte cytotoxidity to autologous liver cells in chronic active hepatitis. Proc Natl Acad Sci 72:1301, 1975*
4. Paronetto F, Vernace S: *Immunological studies in patients with chronic active hepatitis. Cytotoxic activity of lymphocytes to autologous liver cells grown in tissue culture. Clin Exp Immunol 19:99, 1975*
5. Nonomura A, Tanio M, Kurumaya H, Ohta G, Kato Y, Kobayasi K: *Disoredered immunoregulatory functions in patients with chronic active hepatitis. Clin Exp Immunol 47:595, 1982*
6. Chisari FV, Castle KL, Xavier C, Anderson DS: *Functional properties of lymphocytes subpopulation in hepatitis B virus infection. J Immunol 126: 38, 1981*

7. Nouri-Aria K, Alexander GJM, Portman B, Vergani D, Eddleston ALWF, Williams R: *In vitro study of IgG production and Concanavalin A induced suppressor cell function in acute and chronic hepatitis B virus infection. Clin Exp Immunol 64: 50, 1986*
8. Zinkernagel RM, Doherty PC: *MHC-restricted cytotoxic T cells: studies on the biologic role of polymorphic major transplantation antigens determining T cell restriction, specificity, function and responsiveness. Adv Immunol 27:51, 1979*
9. Maeker-Alzer G, Schumacher K, Gross R: *Detection of lymphocytes with increased DNA synthesis in the peripheral blood of patients with active chronic hepatitis. Kin Wschr 52:190, 1974*
10. Park BC, Koo JY, DeGroote J: *Immunological aspects in patients with chronic active hepatitis. Cellular immune responses. J Korean Medical Science 1:15, 1986*
11. Wright R: *Type B hepatitis: progression to chronic hepatitis. Clinic in Gastroenterology 9:97, 1980*
12. Sherlock S: *Diseases of the liver and biliary system. 6th ed p 275, Oxford, Blackwell-Mosby, 1981*
13. Degroote J, Desmet VJ, Gedigk P, Korb G, Popper H, Poulsen H, Scheuer PJ, Schmidt M, Thaler H, Wepler W: *A classification of chronic hepatitis. Lancet ii: 626, 1968*
14. Park B, Sobis H, Delacourt M, Vandeputte M: *In vitro and in vivo effects of rat amniotic fluid on cell-mediated and humoral immunity in rats. Europ J Cancer 15:965, 1979*
15. Boyum O: *Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest Suppl 97:77, 1968*
16. Crowther D, Fairley GH, Sewell RL: *Significance of the changes in circulating lymphoid cells in Hodgkin's disease. Br Med J 2:473, 1969*
17. Radka SF, Charron DJ, Brodsky FM: *Review: Class II molecules of the major histocompatobility complex considered as differentiation markers. Human Immunol 16:390, 1986*
18. Waldman TA: *The structure, function, and expression of interleukin-2 receptors on normal and malignant lymphocytes. Science 232:727, 1986*
19. Uchida A and Yanagawa E: *Natural killer cell activity and autologous tumor killing activity in cancer patients: overlapping involvement of effector cells as determined two-target conjugate cytotoxidity assay. JNCI 73:1093, 1984*
20. Budillon G, Scala G, Donofrio C, Cassano S, Dertis F: *Diminished active T rosette levels and increased spontaneous B lymphocyte blastogenesis in hepatitis B virus positive chronic active hepatitis. Clin Exp Immunol 52:472, 1983*
21. Stites DP, Stobo JD, Wells JV: *Basic and clinical immunolgy, 6th ed p 76, Norwalk, Connecticut/ Los Altos, CA, Appleton and Lange, 1987*
22. Alexander G and Williams R: *Editorial. Characterization of the mononuclear cell infiltrate in piecemeal necrosis. Lab Invest 50:247, 1984*
23. Van den Oord JV, Devos R, Desmet VJ: *In situ distribution of major histocompatibility complex products and viral antigens in chronic hepatitis B virus infection: Evidence that HBc-containing hepatocytes may express HLA-DR antigens. Hepatology 6:981, 1986*
24. Hemler ME, Brenner MB, Mclean JM, Strominger JL: *Antigenic stimulation regulates the level of expression of interleukin-2 receptor on human T cells. Proc Natl Acad Sci 81:2172, 1984*
25. Diamantstein T, Osawa H: *The interleukin-2 receptor, its physiology and a new approach to a selective immunosuppressive therapy by anti-interleukin-2 receptor monoclonal antibodies. Immunological Review 92:5, 1986*
26. Shimizu A, Kondo S, Sabe H, Ishida N, Hondo T: *Structure and function of the interleukin-2 receptor: Affinity conversion model. Immunological review 92: 103, 1986*
27. Yachie A, Miyawaki T, Uwadana N, Ohzeki S, Tanaguchi N: *Sequential expression of T cell activation (Tac) antigen and Ia determinants on circulating human T cells after immunization with tetanus toxoid. J immunol 131:731, 1983*
28. Pignata C, Vajro P, Troncone R, Monaco G, Ciriaco M: *Increased circulating Ia bearing T cells in HBsAg-Positive chronic active hepatitis. New Engl J Med 307:501, 1982*
29. Herberman RB, Oraldo JR: *Natural killer cells: their role in defence against disease. Science 214:24, 1981*
30. Trichieri G, Santoli D: *Antiviral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility by target cell to lysis. J Exp Med 147, 1983*
31. Herberman RB Ortaldo JR, Bernard GD: *Augumentation by interferon of human natural and antibody-dependent cell-mediated cytotoxidity. Nature 227:221, 1979*
32. Domzig W, Stadler BM, Herberman RB: *Interleukin-2 dependence of human natural killer cell activity. J Immunol 130:1970, 1983*
33. Henney CS, Kuribayasi DE, Gillis S: *Interleukin-2 auguments natural killercell activity. Nature 291: 335, 1981*
34. Dienstag JL, Savarese AM, Bhan AK: *Increased natural killer cell activity in chronic hepatitis B virus infection. Hepatology 2:107S, 1982*