[14] Iuchi H, Horiike N, Masumoto T, et al. Specially designed needle-guided for intercostals puncture of hepatocellular carcinoma seen on computed tomography and not visualized on ultrasonography. Hepatol Res 2003;25:319–28.
[15] Rossi S, Buscarini E, Garbagnati F, et al. Percutaneous treatment of small hepatic tumors by expandable RF needle electrode. Am J Roentgenol 1998;170:1015–22.
[16] Goldberg SN, Solbiati L, Hahn PF, et al. Large-volume tissue ablation with radiofrequency by using a clusterd, internally cooled electrode technique laboratory and clinical experience in liver metastasis. Radiology 1998;209:371–9.
[17] Horiike N, Iuchi H, Ninomiya T, et al. Influencing factors for recurrence of hepatocellular carcinoma treated with radiofrequency ablation. Oncol Rep 2002;9:1059–62.
[18] Vilana R, Llovet JM, Bianchi L, et al. Contrast-enhanced power Doppler sonography and helical computed tomography for assessment vascularity of small hepatocellular carcinomas before and after percutaneous ablation. J Clin Ultrasound 2003;31:119–28.
[19] Choi D, Lim HK, Kim SH, et al. Assesment of therapeutic response in hapatocellular carcinoma treated with percutaneous radio frequency ablation: comparison of multiphase helical computed tomography and power Doppler ultrasonography with a microbubble contrast agent. J Ultrasound Med 2002;21:391–401.
[20] Fiore F, Vallone P, Ricchi P, et al. Levovist-enhanced Doppler sonography versus spiral computed tomography to evaluate response to percutaneous ethanol injection in hepatocellular carcinoma. J Clin Ultrasound 2000;31:164–8.

J Gastroenterol 2005; 40:52–56
DOI 10.1007/s00535-004-1493-8

Journal of Gastroenterology

Soluble CD163 in patients with liver diseases: very high levels of soluble CD163 in patients with fulminant hepatic failure

Atsushi Hiraoka[1], Norio Horiike[1], Sk. Md. Fazle Akbar[1], Kojiro Michitaka[2], Takami Matsuyama[3], and Morikazu Onji[1]

[1]The Third Department of Internal Medicine, Ehime University School of Medicine, Ehime 791-0295, Japan
[2]Endoscopy Center, Ehime University School of Medicine, Ehime, Japan
[3]Department of Immunology, Kagoshima University School of Medicine, Kagoshima, Japan

Background. The levels of several cytokines and chemokines are elevated in various liver diseases, especially in fulminant hepatic failure (FHF). Activated macrophages may have a role in the production of these immune modulators. CD163 is a member of a scavenger receptor family and is expressed mainly on activated macrophages, and a soluble form of CD163 (sCD163) is released from activated macrophages. The aim of this study was to assess sCD163 levels in patients with FHF and to evaluate their clinical significance. ***Methods.*** The levels of sCD163 in the sera were measured in 21 patients with FHF, 17 patients with acute hepatitis (AH), 22 patients with chronic hepatitis (CH), and 14 normal healthy controls (NC), by an enzyme-linked immunosorbent assay. The levels of sCD163 were observed serially in patients with FHF and AH. ***Results.*** The levels of sCD163 in the sera from patients with FHF were significantly higher than those in patients with AH and CH and the NC group ($P < 0.0001$). There was a good correlation between serum levels of sCD163 and prothrombin time ($r = -0.677$; $P < 0.0001$). A kinetic study revealed that the levels of sCD163 decreased in patients with AH and in survivors of FHF, whereas the levels of sCD163 progressively increased in nonsurvivors of FHF. ***Conclusions.*** This study shows that the products of activated macrophages may be involved in the pathogenesis of FHF. This study also inspires optimism that sCD163 may possess prognostic importance in FHF.

Key words: soluble CD163, fulminant hepatic failure, acute hepatitis, macrophage

Introduction

Patients with hepatitis are characterized by hepatic inflammation and the destruction of hepatocytes. Viral antigen-specific cytotoxic T lymphocytes, polyclonal cytokines, immune modulators, and products of oxidative stress have been shown to induce damage and destruction of hepatocytes in these patients.[1] Accumulation of macrophages is a characteristic feature of different types of hepatitis, although the degree of accumulation varies among different types of hepatitis. Severe accumulation of macrophages is usually seen in the liver tissues from patients with fulminant hepatic failure (FHF) and acute hepatitis (AH). Although increased esterase activity is a marker of activated macrophages, it is not easy to get liver biopsy specimens and to quantify the levels of enzyme activity in clinical situations. Recently, it has been shown that CD163 is expressed on activated macrophages.[2,3] It is also expressed on blood monocytes, but the levels of expression are much higher on macrophages. This indicates that increased expression of CD163 may be related to the progressive maturation of monocytic cells to macrophages. CD163 is a 130-kDa transmembrane protein that is a member of the scavenger receptor cysteine rich (SRCR) family; it contains monocyte lineage differentiation antigen containing nine copies of the SRCR domain.[4,5]

While the specific function of CD163 remains unclear, CD163 was classified as a signal receptor scavenging hemoglobin-haptoglobin complexes from plasma.[6] CD163 has been shown to be present in a natural soluble form (sCD163) in plasma and serum.[7] Because of the constant shedding of CD163 from activated macrophages,[7] sCD163 expression may be a good marker of a progressive inflammatory milieu; Matsushita et al.[3] have reported elevated levels of sCD163 in rheumatoid arthritis; however, there is a paucity of information regarding the expression of sCD163 in liver diseases.

Received: February 17, 2004 / Accepted: May 10, 2004
Reprint requests to: N. Horiike

Table 1. Clinical characteristics of patients with hepatitis

	FHF	AH	CH
Number	21	17	22
Age (years)	47.6 ± 17.1	43.3 ± 17.4	49.0 ± 8.4
Sex (male:female)	8:13	13:4	14:8
Total bilirubin (mg/dl)	15.4 ± 9.3*	8.4 ± 7.1**	0.7 ± 0.6
Prothrombin time (%)	14.9 ± 9.1*	67.5 ± 25.5**	94.5 ± 13.2
Aspartate aminotransferase (IU/l)	1845.3 ± 4222.0	1681.0 ± 2131.3	75.1 ± 63.2
Alanine aminotransferase (IU/l)	1343.5 ± 2198.6***	2224.8 ± 2506.0**	145.5 ± 239.7
Outcome (survivors:nonsurvivors)	8:13	17:0	22:0
Etiology			
Hepatitis A virus	—	1	—
Hepatitis B virus	12	8	—
Hepatitis C virus	2	2	22
Drug-induced	2	6	—
Non-A, non-B, non-C	5	—	—

* $P < 0.01$ vs AH, CH; ** $P < 0.01$ vs CH; *** $P < 0.05$ vs CH
FHF, fulminant hepatic failure, AH, acute hepatitis, CH, chronic hepatitis

With the postulation that activated macrophages might have a role during FHF, we measured sCD163 in the sera from patients with FHF, and in patients with AH and chronic hepatitis (CH). The levels of sCD163 were highly elevated in patients with FHF compared to those with CH and AH, indicating the marked activation of macrophages in FHF. Next, we checked the levels of sCD163 in the sera from patients with FHF at different times in the disease course to assess whether sCD163 could have any clinical importance in FHF.

Patients and methods

The study population comprised 21 patients with FHF, 17 patients with AH, 22 patients with CH, and 14 normal healthy controls (NC). All patients were admitted to our hospital between 1984 and 2002. The diagnosis of liver diseases was done according to subjective symptoms and from the data of liver function tests. FHF was defined as severe liver dysfunction with grade II or greater hepatic encephalopathy and with prothrombin time (PT) being 40% or less[8] on admission to our hospital. None of the patients with FHF or AH had a previous history of liver diseases. Patients with FHF did not show features of sepsis. The clinical profiles of the patients with FHF, AH, and CH are shown in Table 1. The mean age of the patients with FHF was 47.6 years (range, 8 to 73 years); 13 were female and 8 were male. The etiology of FHF was variable. Twelve and 2 patients were positive for hepatitis B surface antigen (HBsAg) and antibody to hepatitis C virus (HCV), respectively. Two patients with drug-induced hepatitis were also enrolled among the FHF patients. The etiology of FHF was unknown in 5 patients (non-A, non-B, non-C).

AH was defined in patients in whom hepatic encephalopathy did not develop during the course of the disease. The mean age of the AH patients was 43.3 years (range, 20 to 76 years). Thirteen of these patients were male and 4 were female. IgM type antibody to hepatitis A virus (HAV) was detected in one patient; eight patients were positive for HBsAg, and two were positive for antibody to HCV. Six patients were diagnosed as having drug-induced hepatitis. As shown in Table 1, there were no significant differences regarding age between the patient groups. The PT was significantly lower in patients with FHF ($P < 0.0001$), as expected. Informed consent was obtained from patients or their relatives after an explaination of the nature of the study. Sera were collected from all patients and were stored at −20°C until use.

Estimation of markers of hepatitis viruses

The presence of IgM type antibody to HAV, HBsAg, and antibody to HCV was determined using enzyme immunoassay kits (AxSYM HAVAB-M; Dainabot, Tokyo, Japan; AxSYM HBsAg; Dainabot, Tokyo, Japan; and Imcheck-FHCV, Kokusai-Shiyaku, Kobe, Japan, respectively), exactly according to the instructions of the manufacturer.

Assay of sCD163 in sera

The levels of sCD163 were examined by a sandwich enzyme-linked immunosorbent assay (ELISA) method. A Nunc maxi-sorp immunoplate (Nalge Nunk Interna-

tional, Tokyo, Japan) was coated with 100 µl of 2 µg/ml Mac 2-158 (MAB180P; Maine Biotechnology Services, ME, USA)[7] diluted in ELISA coating buffer (0.1 M NaHCO3, 0.5 M NaCl) at 4°C overnight. After incubation, the plates were washed with 200 µl phosphate-buffered saline (PBS) plus 0.05% Tween-20 four times. Nonspecific binding was blocked by using 200 µl of a blocking buffer containing PBS plus 10% fetal bovine serum (FBS) for 30 min. After three washings, 100 µl of patient's sera or control sera (dilution, 1:25) diluted in blocking buffer was added to the plates and the plates were incubated for 2 h at room temperature. After four washings with PBS plus Tween-20, 100 µl of biotinylated R20 (0.5 µg/ml diluted in blocking buffer, MAB172P; Maine Biotechnology Services)[3] was added, and the plates were incubated at room temperature for 1 h. After another round of washing, 150 µl of streptavidin horseradish peroxidase (HRP-Streptavidin Conjugate; Zymed Laboratories, South San Francisco, CA, USA), diluted 1:1000 in blocking buffer, was added, and the plates were incubated at room temperature for 30 min. After washing, color development was done with 200 µl of 2,2-azino-bis3-ethylbenz-thiazoline-6-sulfonic acide (Zymed Laboratories) solution. The enzymatic reaction product was read by an ELISA reader (Immuno Mini NJ-2300; Nalge Nunc International) at 405 nm. The levels of sCD163 were estimated from the standard curve prepared by plotting the optical density (OD) values of the standard serum against the OD values of the samples.

Statistical analysis

All statistical analysis was carried out on a personal computer with StatView program version 5.0 (SAS Institute, Berkeley, CA, USA). The difference between mean values of groups was tested using Students's T-test, one-way analysis of variance (ANOVA) and Scheffe's F-test. P values of less than 0.05 were considered statistically significant. Correlation coefficients between variables were calculated by use of Fisher's R to Z transformation.

Results

The levels of sCD163 in the sera from patients with different types of hepatitis and the NC group are shown in Fig. 1. The mean levels of sCD163 were significantly higher in patients with FHF (808.6 ± 433.0 ng/ml; n = 21) compared to those with AH (248.5 ± 168.5 ng/ml; n = 17) and CH (148.3 ± 51.4 ng/ml; n = 22) and the NC group (59.5 ± 27.6 ng/ml; n = 14) ($P < 0.0001$).

In order to identify the factors that influenced the serum levels of sCD163 in patients with liver diseases, we evaluated the correlation between parameters of liver function tests and serum sCD163. A strong correlation was seen between the levels of serum sCD163 and PT ($r = -0.677$; $P < 0.0001$). However, the levels of aspartate aminotransferase and alanine aminotransferase did not show any good correlation with serum sCD163 (Table 2).

The levels of sCD163 in the sera in individual patients with AH and FHF are shown in Fig. 2. The levels of

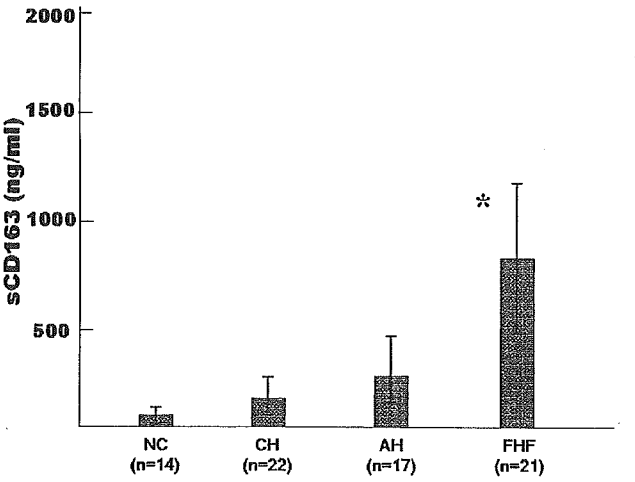

Fig. 1. Increased levels of soluble CD163 (*sCD163*) in patients with CH, AH, and FHF. *CH*, chronic hepatitis; *AH*, acute hepatitis; *FHF*, fulminant hepatic failure; *NC*, normal control. *$P < 0.0001$ compared to AH, CH, and NC

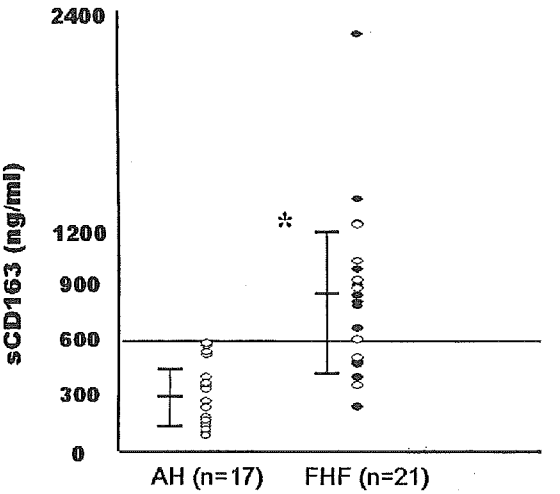

Fig. 2. The levels of sCD163 in individual patients with AH and FHF on admission. All patients with AH had sCD163 serum levels of less than 600 ng/ml; however, most of the patients with FHF had sCD163 serum levels of more than 600 ng/ml on admission. *$P < 0.0001$ compared to AH. *Open symbols*, survivors; *closed symbols*, nonsurvivors

Table 2. Relationship between the levels of sCD163 in the sera and parameters of liver function tests

	Number of patients	*r*	*P*
Prothrombin time (%)	60	−0.677	<0.0001
Total bilirubin (mg/dl)	60	0.278	0.031
Aspartate aminotransferase (IU/l)	60	0.19	0.14
Alanine aminotransferase (IU/l)	60	0.12	0.37

Serum levels of total bilirubin, prothrombin time, and aspartate aminotransferase and alanine aminotransferase levels were measured in a total of 60 patients (FHF, $n = 21$; AH, $n = 17$; CH, $n = 22$) on admission. Correlation coefficients between variables were calculated by use of Fisher's R to Z transformation

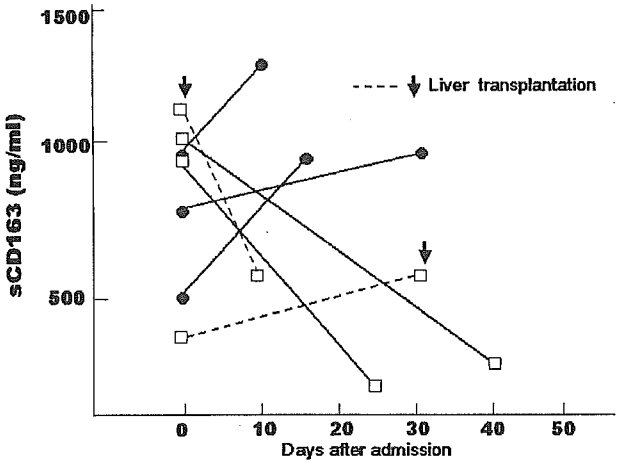

Fig. 3. The levels of sCD163 in the sera were decreased in survivors of FHF (*open squares*). In contrast, the levels of sCD163 increased progressively in nonsurvivors of FHF (*closed circles*)

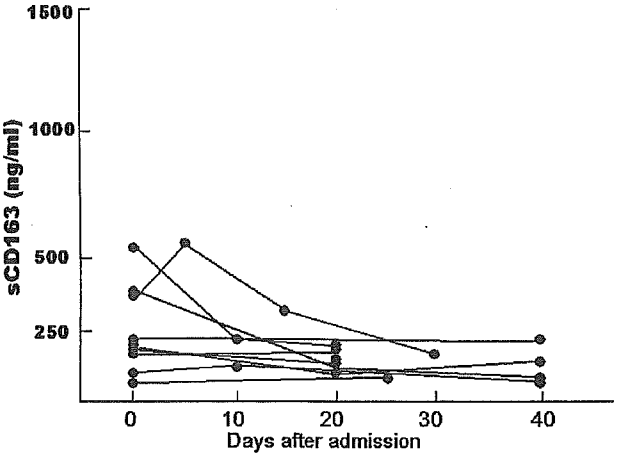

Fig. 4. Kinetics of sCD163 in patients with AH. In most of the patients with AH, the levels of sCD163 fell with time

sCD163 on admission were highly variable among patients. The levels of serum sCD163 were below 600 ng/ml in all patients with AH. However, in 14 of the 21 patients with FHF, the levels of serum sCD163 were more than 600 ng/ml.

The levels of sCD163 in the sera from patients with FHF (Fig. 3; $n = 7$) and patients with AH (Fig. 4; $n = 9$) were serially observed. As shown in Fig. 3, of four patients with FHF who survived, the levels of sCD163 fell in three patients. In one patient with FHF, the level of sCD163 was increased slightly on day 30 after admission. This patient was on a "downhill" course, but survived, with a liver transplantation done on the thirtieth day after admission. On the other hand, the three patients with FHF who did not survive showed progressive elevation of sCD163 in the sera (Fig. 3). In contrast, in most of the patients with AH, the levels of sCD163 in the sera decreased with time (Fig. 4).

Discussion

The extent of hepatitis varies considerably among patients with liver diseases. Patients with FHF develop massive liver damage, which is progressive in nature. These patients can only be saved by intensive therapies, including liver transplantation. On the other hand, although patients with AH exhibit features of severe liver damage, the pathological lesion is self-limiting and most of the patients recover after a single episode of hepatitis. The factors that regulate the extent of liver damage are not well understood. However, virus-specific cytotoxic lymphocytes, polyclonal immune modulators, and oxidative stress play important roles in this regard. In fact, several studies have shown a role of lymphocytes in the induction and progression of hepatitis.

In the present study, we have shown that macrophage-related factors may have a dominant role in determining the severity of hepatitis in patients with FHF. The levels of sCD163, which is released from

activated macrophages, were significantly higher in the sera from patients with FHF compared with levels in patients with AH and CH and the NC group.

In the clinical situation, it is very important to assess whether a patient presenting with features of acute liver damage would recover after an acute attack of hepatitis or would eventually develop FHF. Shiota et al.[9] have reported the prognostic importance of hepatocyte growth factor (HGF) by estimating the serum levels of HGF on admission and after recovery in five patients with FHF.

In this study, we showed that the levels of sCD163 were significantly higher in patients with FHF compared to those in AH patients at diagnosis, suggesting that sCD163 may have a role in the progression to FHF. Interestingly, on admission, the levels of sCD163 in the sera were more than 600 ng/ml in most patients with FHF, but not in any patient with AH. However, the importance of levels of sCD163 in FHF should be confirmed by conducting a controlled study in future.

The kinetic study of serum sCD163 in patients with FHF and AH showed that nonsurvivors of FHF showed progressive increases of sCD163, in spite of receiving intensive therapy (high-dose corticosteroid, plasma exchange, steroid pulse therapy, and hemodiafiltration). However, the levels of serum sCD163 decreased in FHF patients who survived. It might be hard to draw a firm conclusion from the finding of this study, because it was retrospective, and the number of subjects was not large enough to draw a firm conclusion. The utility of sCD163 as a marker of the prognosis of FHF should be confirmed in a prospective study, and such a clinical evaluation has been started in our laboratory.

The mechanism underlying the increase of sCD163 in FHF, especially the progressive increase of sCD163 in nonsurvivors of FHF, is not known. It is understandable that sCD163 may be directly produced by activated macrophages in the liver of FHF patients, because macrophages play a dominant role in the pathogenesis of severe inflammation in FHF.[10] It is possible that sCD163 may be produced because of the stimulation of activated macrophages by some intermediate inflammatory mediators that are produced in FHF. It was reported that lipopolysacharides induced sCD163 from the surface of isolated monocytes.[11] The direct effect of sCD163 on hepatocytes has not been explored. CD163 was recently recognized as a specific hemoglobin-haptoglobin receptor. Hemoglobin oxide is toxic and has a proinflammatory effect. sCD163 may be involved in the production of several inflammatory cytokines or chemokines that may alter the hepatic inflammatory milieu in FHF. Whatever the mechanism or mode of action may be, sCD163 appears to represent more severe forms of FHF, and further study may reveal its clinical significance.

Acknowledgments. The expert technical assistance of Satomi Yamanaka is greatly appreciated.

References

1. Tsutsui H, Adachi K, Seki E, Nakanishi K. Cytokine-induced inflammatory liver injuries. Curr Mol Med 2003;3:545–59.
2. Sanchez C, Domenech N, Vazquez J, Alonso F, Ezquerra A, Dominguez J. The Procine 2A10 antigen is homologous to human CD163 and related to macrophage differentiation. J Immunol 1999;162;5230–7.
3. Matsushita N, Kashiwagi M, Wait R, Nagayoshi R, Nakamura M, Matsuda T, et al. Elevated levels of soluble CD163 in sera and fluids from rheumatoid arthritis patients and inhibition of the shedding of CD163 by TIMP-3. Clin Exp Immunol 2002;130:156–61.
4. Law SK, Micklem KJ, Shaw JM, Zang XP, Dong Y, Willis AC, et al. A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. Eur J Immunol 1993;23:2320–5.
5. Hogger P, Dreier J, Droste A, Buck F, Sorg C. Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). J Immunol 1998;161:1883–90.
6. Graversen JH, Madsen M, Moestrup SK. CD163: a signal receptor scavenging haptoglobin-hemoglobin complexes from plasma. Int J Biochem Cell Biol 2002;34:309–14.
7. Sulahian TH, Hintz KA, Wardwell K, Guyre PM. Development of an ELISA to measure soluble CD163 in biological fluids. J Immunol Methods 2001;252:25–31.
8. Takahashi Y, Shimizu M. Aetiology and prognosis of fulminant viral hepatitis in Japan: a multicentre study. The Study Group of Fulminant Hepatitis. J Gastroenterol Hepatol 1991;6:159–64.
9. Shiota G, Okano J, Kawasaki H, Kawamoto T, Nakamura T. Serum hepatocyte growth factor levels in liver diseases: clinical implications. Hepatology 1995;21:106–12.
10. Muto Y, Nouria-Aria KT, Meager A, Alexander GJ, Eddleston AL, Williams R. Enhanced tumor necrosis factor and interleukin-1 in fulminant hepatic failure. Lancet 1988;II:72–4.
11. Hintz KA, Rassias AJ, Wardwell K, Moss ML, Morganelli PM, Pioli PA, et al. Endotoxin induces rapid metalloproteinase-mediated shedding followed by up-regulation of the monocyte hemoglobin scavenger receptor CD163. J Leukoc Biol 2002;72:711–7.

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2005.02676.x

Impaired functional capacities of liver dendritic cells from murine hepatitis B virus (HBV) carriers: relevance to low HBV-specific immune responses

A. Hasebe, S. M. F. Akbar, S. Furukawa, N. Horiike and M. Onji
Third Department of Internal Medicine, Ehime University School of Medicine, Japan

Summary

The chronic hepatitis B virus (HBV) carrier exhibits ongoing replication of HBV and expresses abundant amounts of HBV-related antigens in the liver. However, HBV-specific immune responses are either absent or narrowly focused in these subjects. With the postulation that impaired functional abilities of liver dendritic cells (DCs) might be responsible for this, we assessed the functions of liver DCs in HBV transgenic mice (HBV-TM), an animal model of the HBV carrier state. Liver DCs were isolated from normal C57BL/6 mice and HBV-TM without the use of cytokines or growth factors. Lymphoproliferative assays were conducted to evaluate the ability of liver DCs to induce the proliferation of allogenic T lymphocytes and hepatitis B surface antigen (HBsAg)-enriched T lymphocytes. Liver DCs were stimulated with viral and bacterial products to assess their cytokine-producing capacities. In comparison to liver DCs from normal C57BL/6 mice, liver DCs from HBV-TM exhibited significantly decreased T cell proliferation-inducing capacities in allogenic mixed leucocyte reaction ($P < 0{\cdot}05$) and HBsAg-enriched T lymphocytes proliferation assays ($P < 0{\cdot}05$). Liver DCs from HBV-TM produced significantly lower levels of interleukin-12p70, tumour necrosis factor-alpha, interferon-gamma, and interleukin-6 ($P < 0{\cdot}05$) compared to liver DCs from normal C57BL/6 mice. This study provides evidence that liver DCs from HBV-TM had impaired ability to induce both innate and adaptive immune responses. This might account for a weak and almost undetectable HBV-specific immune response in chronic HBV carriers. This inspires hope that up-regulation of the functions of liver DCs *in situ* may have therapeutic implications in chronic HBV carriers.

Keywords: antigen processing, cytokines/interleukins, dendritic cells, hepatitis B virus, liver immunology/disease

Accepted for publication ?????? 2004
Correspondence: Morikazu Onji, Third Department of Internal Medicine, Ehime University School of Medicine, Shigenobu-Cho, Ehime 791–0295, Japan.
E-mail: akbar@m.ehime-u.ac.jp

Introduction

About 350 million people in the world are chronically infected with the hepatitis B virus (HBV). Considerable numbers of chronic HBV carriers will develop progressive liver diseases such as liver cirrhosis and hepatocellular carcinoma. Also, all chronic HBV carriers are permanent sources of HBV infection and are able to transmit the HBV to uninfected healthy people [1,2]. The exact mechanism underlying the pathogenesis of the HBV carrier state is not well understood; however, the immune responses of the host are presumed to play a critical role in this regard. Patients who control HBV after an acute infection are characterized by having a clearly detectable HBV-specific CD4 and CD8 response for a wide range of different epitopes within the HBV core, polymerase and envelope proteins [3]. In contrast, circulating HBV-specific T cells are very difficult to find in chronic HBV carriers, and when they are present they are often few and specific for single epitopes [4].

As the interaction between the virus and the host determines the magnitude of the virus-specific immune response, defective processing and presentation of the HBV by antigen-presenting dendritic cells (DCs) [5,6] might be responsible for the impaired HBV-specific immune responses in chronic HBV carriers. DCs originate in the bone marrow and migrate through the blood to different non-lymphoid

tissues, where the phenotypes and functions of DCs undergo modifications. This is evident from the phenotypic and functional diversities of DCs in different tissues such as the liver, lung, small intestine, large intestine, heart, kidney and skin [7,8]. DCs in these non-lymphoid tissues recognize, capture and process viruses in their endosomal compartments, and express the antigenic peptides of the virus on their surface. From this scenario, it is evident that improper handling and processing of HBV by liver DCs might be responsible for defective HBV-specific immune responses in chronic HBV carriers. Currently, there is no study describing the functions of liver DCs in human and murine HBV carriers.

Some studies have reported on the functions of peripheral blood and spleen DCs in humans and murine chronic HBV carriers, respectively [9–11]. Although these studies represent the pioneer research on HBV/DC interactions in chronic HBV infections, they have major and fundamental limitations. In fact, blood and spleen DCs are not related directly to the recognition, capturing and processing of HBV in chronic HBV carrier [12].

This study was conducted to determine if the functions of liver DCs were impaired in murine HBV carriers. It is very difficult to isolate adequate numbers of liver DCs from normal mice for functional studies because there are very few DCs in the liver. Accordingly, some investigators have expanded liver DCs by administering fms-like tyrosine kinase-3 ligand *in vivo* [13]. Others have cultured precursor or progenitor populations of liver DCs from normal mice with cytokines, growth factors and collagenase *in vitro* to obtain adequate numbers of liver DCs [14,15]. However, most of these agents induce either maturation or activation of liver DCs, therefore altering the functions of liver DCs and potentially compromising our study.

Hence, we isolated fresh liver DCs from HBV transgenic mice (HBV-TM) and normal C57BL/6 mice without the use of growth factors, cytokines or collagenase. This was achieved by killing 10–15 normal mice or HBV-TM for each experiment. After confirming the phenotype and functions of liver DCs, we compared the T cell proliferation-inducing capacities of liver DCs between HBV-TM and normal C57BL/6 mice. Our study revealed that the liver DCs from HBV-TM were inefficient in inducing proliferation of both allogenic T cells and antigen-specific T cells. The mechanism underlying the impaired T cell proliferation-inducing capacity of liver DCs of HBV-TM was studied from their capacity to produce proinflammatory cytokines in response to different bacterial and viral products.

Methods

Mice

The HBV-TM (official designation, 1.2HB-BS10) were prepared by microinjecting the complete genome of HBV plus 619 base pairs of HBV DNA into the fertilized eggs of C57BL/6 mice. The HBV-TM expressed mRNAs of 3·5, 2·1, and 0·8 kilo base pairs of HBV in the liver and high levels of the hepatitis B surface antigen (HBsAg) in the sera [16].

We purchased normal C57BL/6 mice (C57BL/6 J Jel, H-2^b) and normal C3H/He mice (C3H/HeN Jcl, H-2^k) from CLEA Japan Inc. (Tokyo, Japan). HBV-TM and normal mice (C57BL/6 and C3H/He) were maintained separately in the animal house at Ehime University School of Medicine, Ehime, Japan, under controlled conditions (22°C, 55% humidity and 12-h day/night rhythm), and were provided with an unlimited supply of standard laboratory feed and water. All animals received humane care and the study protocols conformed to institutional guidelines.

Detection of HBsAg and HBV DNA

The levels of HBsAg and HBV DNA in the sera of HBV-TM were estimated using the chemiluminescence enzyme immunoassay method (Tokyo Institute of Immunology, Tokyo, Japan) and the real time polymerase chain reaction method (Special Reference Laboratory, Osaka, Japan), respectively.

Isolation of liver DCs

Liver DCs were isolated from mouse liver using a methodology described by Lu *et al.*, with some modifications [14]. The mice were anaesthetized with pentobarbital sodium (Dai Nippon Pharmaceutical Co., Ltd, Osaka, Japan). After opening the abdomen, a 24-gauge intravenous canulla (Vasculon™, Becton Dickinson, Helsborge, Sweden) was inserted into the portal vein and the liver was perfused with 5 ml of phosphate-buffered saline. The inferior vena cava was cut to ensure the free outflow of blood. We removed the liver and disrupted the perfused liver using the flat portion of a plunger from a 10-ml syringe. The cell suspensions were then passed through a sterile 75-μm pore size steel mesh (Morimoto Yakuhin Co, Matsuyama, Japan), pelleted and resuspended in 35% Percoll solution (Percoll™, Amersham Bioscience Co, Tokyo, Japan) containing 100 μ/ml of heparin. After centrifugation at 450 *g* for 15 min at room temperature, non-parenchymal cells (NPCs) of the liver were collected.

In order to isolate liver DCs, NPCs were fractionated based on CD11c expression with immunomagnetic beads (MicroBeads Miltenyi Biotech, Bergisch Gladbach, Germany) according the manufacturer's instructions, after blocking Fc receptors with monoclonal antibody 2.4G2 (BD Biosciences Pharmingen, San Jose, CA, USA).

Preparation and isolation of HBsAg-enriched T lymphocytes

In order to prepare HBsAg-enriched lymphocytes, normal C57BL/6 mice were injected with 10 μg of recombinant

HBsAg in aluminium hydroxide, twice at 2-week intervals, as described previously [17]. Allogenic T lymphocytes and HBsAg-enriched T lymphocytes were isolated from C3H/He mice and HBsAg-immunized C57BL/6 mice by magnetic cell sorting using auto-MACS (Miltenyi Biotec). Single cell suspension of the spleen was incubated with immunomagnetic beads coated with monoclonal antibodies to mouse CD90 (Thy 1·2) (Miltenyi Biotec). These cells were then washed twice through a MACS column. The positively selected cells were collected as T lymphocytes.

Flow cytometry

After blocking the Fc receptor and then staining the liver DCs with fluorescence isothiocyanate-conjugated, phycoerythrin-conjugated or peridinin chlorophyll protein-conjugated antibody, flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences). Liver DCs were stained for DC (CD11c, HL3), lineage markers (CD3, 17A2; CD8α, 53–6·7, CD11b, M1/70, B220, RA3–6B2), major histocompatibility complex (MHC) class I (AF6-88·5), MHC class II (AF6-120·1) and co-stimulatory molecules (CD80, 16–10A1; CD86, GL1). Analysis of the flow cytometry data was conducted using CellQuest software (BD Biosciences).

Proliferation-inducing capacities of liver DCs

T cells and DCs were cultured in 96-well U-bottomed plates (Corning, Tokyo, Japan) for 4 days, as described [17]. During the last 12 h, [^{3}H]-thymidine, 1·0 μCi/ml (Amersham Biosciences UK Limited, Buckinghamshire, UK) was added to the culture. The levels of incorporation of [^{3}H]-thymidine were determined using a liquid scintillation counter (Beckman LS 6500, Beckman Instruments, Inc., Fullerton, CA, USA) and the blastogenesis levels were expressed as counts per minute (cpm).

Cytokines production by liver DCs

To assess cytokine production, 4×10^5 immunomagnetic bead-purified CD11c$^+$ liver DCs were cultured in 200 μl of medium in a 96-well U-bottomed Corning tissue culture plate. Lipopolysaccharides, 5 μg (Sigma, St Louis, MO, USA), *Staphylococcus aureus*, Cowan strain-1 (SACS-1, 0·01%, Pansorbin, no. 507861: Calbiochem, La Jolla, CA, USA) and herpes simplex virus-1 (HSV-1, 1×10^5 plaque-forming units, kindly provided by Dr Masaki Yasukawa, Japan) were added to the cultures. After 24 h, the culture supernatants were harvested and the levels of interleukin (IL)-6, IL-10, IL-12p70, tumour necrosis factor (TNF)-α and interferon (IFN)-γ (BD Pharmingen) were calculated using a commercial kit for the cytometric bead array, as described [18]. The standard negative control containing 0 pg/ml of different cytokines and the standard positive control containing 5000 pg/ml of different cytokines were provided with the kit. The mean fluorescence intensities of the standard negative control, different dilutions of standard positive controls and culture supernatants were measured using flow cytometry (FACSCalibur flow cytometer). A standard curve was prepared from the mean fluorescence intensities of the negative control and various dilutions of the positive controls using Cytometric Bead Array Software (BD Biosciences). The levels of cytokines in the culture supernatants were calibrated from the mean fluorescence intensities of the samples by Cytometric Bead Array Software (BD Biosciences) using a Macintosh computer (SAS Institute, Cary, NC, USA).

Statistical analysis

The data are shown as mean ± standard deviation. Means were compared using Student's *t*-test. For differences determined by *F*-test, the *t*-test was adjusted for unequal variances (Mann–Whitney *U*-test). $P < 0{\cdot}05$ was considered to be statistically significant. Statistical calculations were performed using STATVIEW (version 5·0) statistical computer program.

Results

Features of isolated liver DCs

In this study, DCs were isolated from mouse liver using perfusion, density centrifugation and magnetic cell sorting without using cytokines or growth factors. Phenotypic analyses revealed that more than 95% of the liver DCs expressed CD11c (Fig. 1a). Different subtypes of liver DCs were also detected based on their expression of CD8α and CD11b (Fig. 1b). About one-fourth of the liver DCs expressed the B220 antigen, indicating that these liver DCs represented plasmacytoid DCs (Fig. 1c). Functional analysis revealed that liver DCs stimulated allogenic T cells from C3H/He mice in a dose-dependent manner (Fig. 1d). Histopathological analysis revealed no features of hepatitis in HBV-TM (data not shown).

Phenotypes of liver DCs from normal C57BL/6 mice and HBV-TM

The expression levels of MHC class I, MHC class II, CD80 and CD86 on liver DCs from normal C57BL/6 mice and HBV-TM did not exhibit any significant difference. The data shown in Fig. 2 are the representative flow cytometric profiles of five separate experiments of liver DCs from age- and sex-matched normal C57BL/6 mice and HBV-TM.

Low T cell proliferation-inducing capacity of liver DCs from HBV-TM

The T cell proliferation-inducing capacities of liver DCs were assessed in an allogenic mixed lymphocyte reaction (MLR), where allogenic T lymphocytes from C3H/He mice were cul-

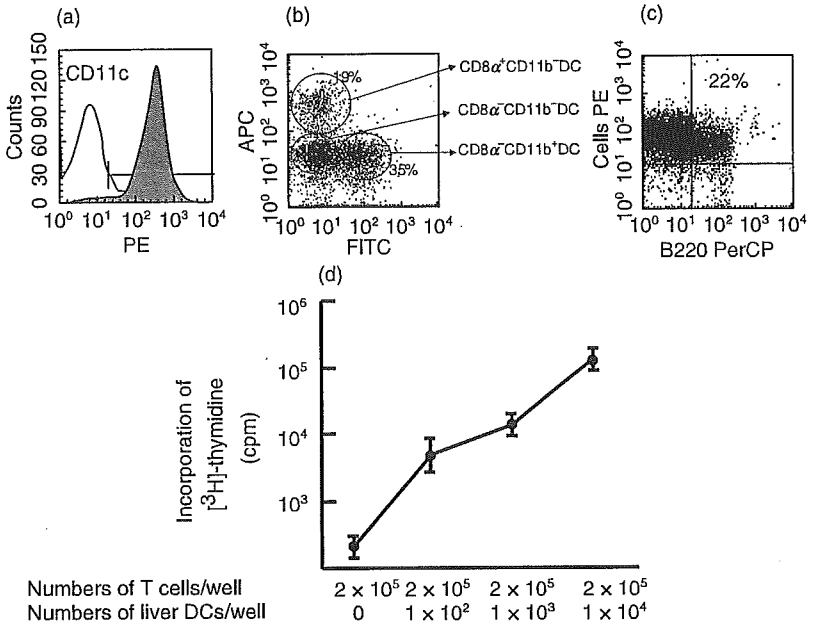

Fig. 1. Phenotypic and functional characterization of liver DCs. Liver DCs phenotypes were assessed in five separate experiments. The flow cytometric profile of one representative staining pattern was shown. (a) Flow cytometric analysis revealed that >95% of the liver DCs were expressing CD11c. (b) Liver DCs were subdivided into three subsets based on the expression of CD8α and CD11b. (c) Approximately one-fourth of the liver DCs expressed B220, a marker of plasmacytoid DCs. (d) The T cell proliferation-inducing capacities of liver DCs from five separate experiments are shown. Liver DCs from C57BL/6 mice stimulated allogenic T cells from C3H/He mice in a dose-dependent manner.

tured with liver DCs from normal C57BL/6 mice or HBV-TM. To evaluate the ability of liver DCs to induce proliferation of HBsAg-enriched lymphocytes, HBsAg-enriched T lymphocytes were cultured with liver DCs either without or with HBsAg. The blastogenesis levels in lymphocyte proliferative assays were measured in cpm. The blastogenesis levels in allogenic MLR containing liver DCs from normal C57BL/6 mice and liver DCs from HBV-TM were 101 299 ± 13 293 ($n = 5$), and 14 689 ± 6308 ($n = 5$) cpm, respectively ($P < 0{\cdot}01$) (Table 1).

The ability of liver DCs from HBV-TM and normal C57BL/6 mice to induce proliferation of HBsAg-enriched lymphocytes was evaluated in lymphoproliferative assays. In comparison to liver DCs from normal C57BL/6 mice, liver DCs from HBV-TM had significantly decreased capacity to stimulate HBsAg-enriched lymphocytes (Fig. 3). Although the blastogenesis levels increased along with increased amounts of HBsAg in culture, liver DCs from HBV-TM induced significantly lower levels of proliferation of HBsAg-enriched lymphocytes at all doses of HBsAg (data not shown).

Decreased levels of cytokines in the supernatants of allogenic MLR containing liver DCs from HBV-TM

The T cell proliferation-inducing capacities of liver DCs from HBV-TM were significantly lower than those of liver DCs from normal C57BL/6 mice. However, the expression of surface antigens was almost the same on liver DCs from HBV-TM when compared to normal C57BL/6 mice. To determine the underlying causes, we estimated the levels of various cytokines in the supernatants of allogenic MLR. As shown in Table 2, the levels of IL-12p70, TNF-α, IFN-γ and IL-10 were significantly lower in the supernatants of allogenic MLR cultures containing liver DCs from HBV-TM compared to cultures containing liver DCs from normal C57BL/6 mice. However, the levels of IL-6 in allogenic MLR supernatants containing liver DCs from normal C57BL/6 mice and HBV-TM are almost comparable (Table 2).

Decreased cytokine-producing ability of liver DCs from HBV-TM in response to bacterial and viral products

The levels of various cytokines in allogenic MLR culture supernatants containing liver DCs from HBV-TM were low. However, this does not reflect the cytokine-producing ability of only liver DCs because both T cells and DCs produce cytokines in allogenic MLR. To study the cytokine-producing ability of the pure population of liver DCs, we cultured liver DCs from normal C57BL/6 mice and HBV-TM with different viral and bacterial products, such as lipopolysaccharides, SACS and HSV-1. The levels of various cytokines produced by liver DCs varied considerably based on the

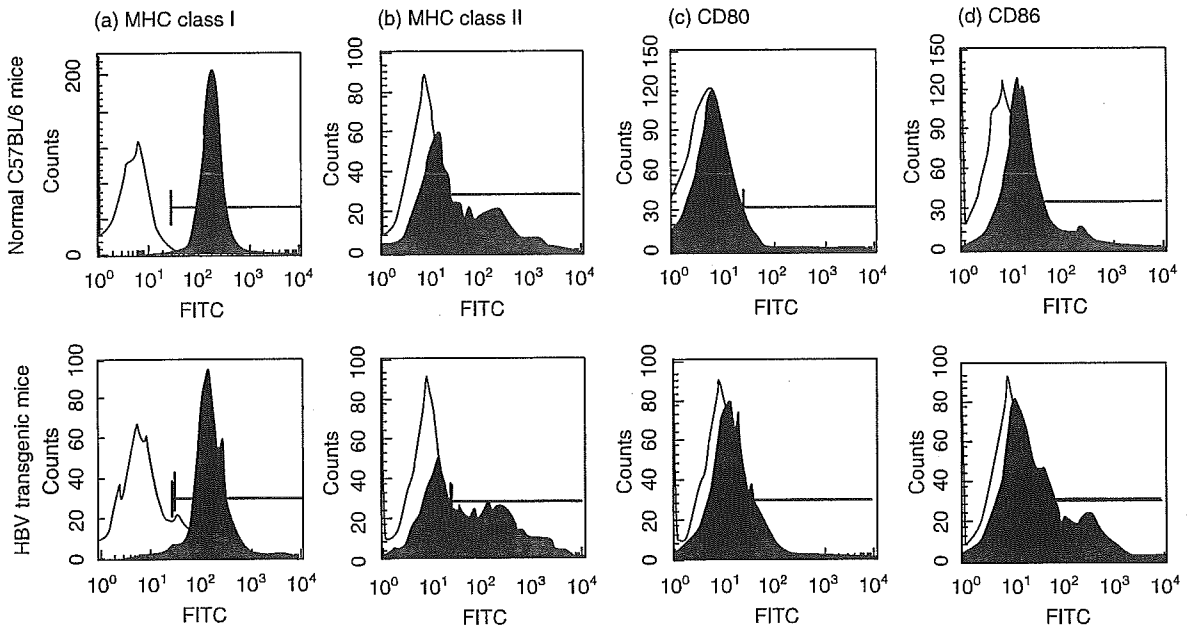

Fig. 2. There was no significant difference in the expression of MHC class I, MHC class II, CD80, and CD86 on liver DCs from normal C57BL/6 mice and HBV-TM. Liver DCs were isolated from the mouse liver without use of cytokines or growth factors. Five separate experiments were conducted to evaluate the expression of MHC class I, MHC class II, CD80 and CD86 on liver DCs using direct flow cytometry. The data of one representative experiment are shown.

Table 1. Low T cell proliferation-inducing ability of liver DCs from HBV transgenic mice.

Mouse	T cell proliferation-inducing capacity (cpm)	Mean ± standard deviation (cpm)
Normal C57BL/6 mice		
Experiment 1	78 873	
Experiment 2	101 001	
Experiment 3	105 306	101 299 ± 13 293
Experiment 4	113 169	
Experiment 5	108 144	
HBV-transgenic mice		
Experiment 1	12 828	
Experiment 2	7 097	
Experiment 3	20 419	14 689 ± 6308*
Experiment 4	21 950	
Experiment 5	11 151	

Liver DCs from normal C57BL/6 mice and HBV transgenic mice were cultured with allogenic T cells from C3H/He mice. The blastogenesis levels were assessed by incorporating [^{3}H]-thymidine in T lymphocytes from 12 microtitre wells and measured as counts per minutes (cpm). Mean and standard deviation of cpm of five separate experiments are shown. The mean cpm in cultures containing only 2×10^5 T lymphocytes or only 1×10^4 liver DCs were less than 500 and 100 cpm, respectively. *$P = 0{\cdot}009$, compared to the mean cpm of cultures containing liver DCs from normal C57BL/6 mice.

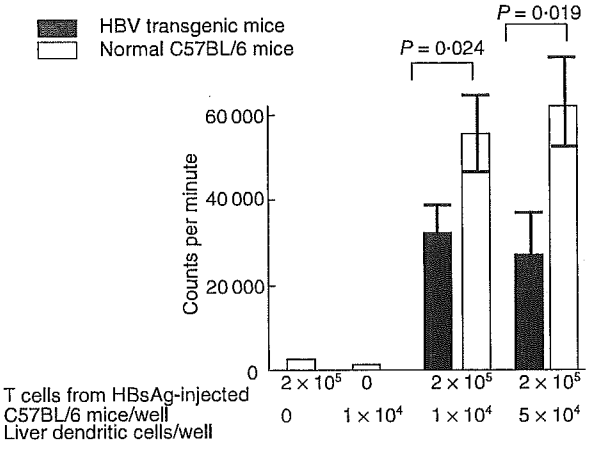

Fig. 3. Decreased ability of liver DCs from HBV-TM to induce proliferation of HBsAg-enriched lymphocytes. HBsAg-enriched lymphocytes (2×10^5) were produced by injecting HBsAg in adjuvant to normal C57BL/6 mice, as described in the Methods section. HBsAg-enriched lymphocytes were cultured with liver DCs from normal C57BL/6 mice and HBV-TM (1×10^4 and 5×10^4) for 4 days in the presence of HBsAg (50 ng/ml), as described in the Methods section. The incorporation of [^{3}H]-thymidine during the last 12 h of the culture was assayed and expressed as counts per minute (cpm). The mean levels of cpm of five separate experiments are shown. The mean cpm in cultures containing only 2×10^5 T lymphocytes or only 1×10^4 liver DCs were less than 500 and 100 cpm, respectively. HBsAg-enriched lymphocytes did not proliferate if HBsAg was not added to the cultures.

Table 2. Decreased levels of cytokines in the supernatants of allogenic MLR cultures containing liver DCs from HBV transgenic mice.

	IL-12p70	TNF-α	IFN-γ	IL-6	IL-10
Normal C57BL/6 mice	101 ± 43	422 ± 98	2825 ± 608	63 ± 32	1494 ± 820
HBV transgenic mice	13 ± 7*	167 ± 45*	1018 ± 293*	71 ± 41	227 ± 56*

Liver DCs were isolated from normal C57BL/6 and HBV transgenic mice. Liver DCs (1×10^4) and allogenic T cells (2×10^5) were cultured for 4 days. The levels of cytokines in the culture supernatants were measured using the cytometric bead array method and are expressed as pg/ml. Data are shown as mean ± standard deviation of duplicate cultures of five separate experiments. *$P < 0.05$ compared to normal C57BL/6 mice.

nature of the stimulants. However, liver DCs from HBV-TM produced lower levels of various cytokines, except IL-10, when compared to those produced by liver DCs from normal C57BL/6 mice (Fig. 4).

Discussion

The purpose of this study was to assess the functional ability of liver DCs in HBV-TM and to determine the role, if any, of

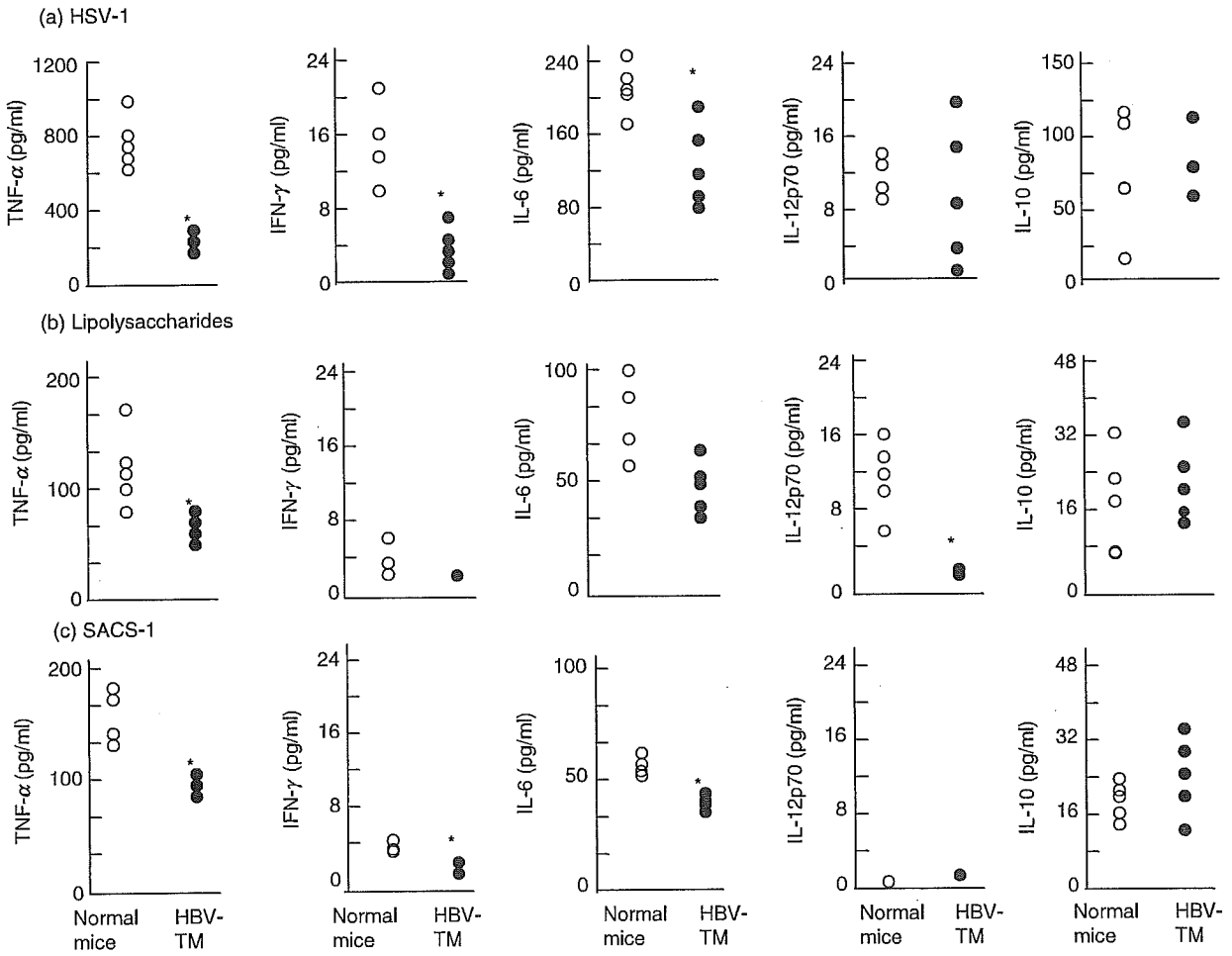

Fig. 4. Decreased production of proinflammatory cytokines from liver DCs of HBV-TM due to stimulation with viral and bacterial products. Liver DCs from normal (open circles) and HBV-TM (filled circles) were stimulated with HSV-1 (1×10^5 plaque forming unit) (a), lipopolysaccharides (5 μg) (b) and SACS (0·01%) (c) for 24 h. The levels of cytokines in the culture supernatants were measured using the cytometric bead array method and expressed as pg/ml. *$P < 0.05$ compared to the levels of cytokines produced by liver DCs from normal C57BL/6 mice.

liver DCs in impaired HBV-specific immune responses in HBV-TM. There were some distinctive features to this study. First, we used HBV-TM, an animal model of the HBV carrier state. HBV-TM have been used widely for studying immunopathogenesis of the chronic HBV carrier state when it could not be studied directly in human subjects [19,20]. These HBV-TM express HBV-related mRNAs (3·5, 2·1 and 0·08 kb) and antigens (HBsAg and hepatitis B core antigen) in the liver [16]. Furthermore, liver DCs expressed HBV DNA. Therefore, liver DCs of HBV-TM reside in a tissue microenvironment, where HBV is replicating continuously. We isolated fresh liver DCs, which were not expanded *in vivo* or cultured *in vitro* by cytokines or other immune modulators because these mediators induce maturation and activation of liver DCs [12–14]. In addition, all functional assays were conducted just after isolation of the liver DCs. This study was conducted using liver DCs which mostly retained their *in situ* phenotypes and functions.

There was no significant difference in the frequencies of liver DCs between normal C57BL/6 mice and HBV-TM. Also, the viability of liver DCs did not differ between these mice (data not shown). However, in comparison to liver DCs from normal C57BL/6 mice, the T cell proliferation-inducing capacities of liver DCs from HBV-TM were significantly lower. This was evident in allogenic MLR and HBsAg-enriched lymphoproliferative assays. Furthermore, liver DCs from HBV-TM produced significantly lower amounts of IL-12p70, IFN-γ and TNF-α in response to viral and bacterial products.

Human liver DCs secrete significant amounts of IL-10 and very little IL-12p70 [21]. Moreover, Hyodo *et al.* have reported that hepatitis B core antigen stimulates IL-10 secretion from peripheral blood T cells and monocytes from patients with chronic hepatitis B [22]. We also found high levels of IL-10 and low levels of IL-12 in cultures containing liver DCs from normal C57BL/6 mice and HBV-TM. However, IL-10 production by liver DCs due to stimulation with HSV-1, lipopolysaccharides and SACS was not significantly different between normal C57BL/6 mice and HBV-TM. It would be interesting to evaluate IL-10 production from hepatitis B core antigen-stimulated DCs.

To the best of our knowledge, this is the first study about liver DCs in HBV carriers, although there are some studies about spleen and blood DCs in HBV carriers [9–11]. Although those studies provided some insights into DC/HBV interactions, blood and spleen DCs are not involved in the recognition, capture and processing of viruses. In fact, these DCs represent the overall status of DCs under pathological conditions and their functions show extreme heterogeneity among infected subjects. This has been supported by studies regarding DCs in chronic hepatitis C virus infection. Bain *et al.* have shown a decrease in the allostimulatory ability and lower IL-12 production by blood DCs in patients with chronic hepatitis C [23], but Longman *et al.* did not find any functional defects of blood DCs in these patients [24].

However, the most important question is why the functions of liver DCs were impaired in HBV-TM. This is an unresolved but important issue regarding the DC/virus interaction. Possibly, the HBV was not properly recognized by liver DCs in the HBV-TM in spite of having abundant amounts of HBV and their antigens around liver DCs. Liver DCs from HBV-TM produced lower levels of proinflammatory cytokines. Ridge *et al.* have shown that proinflammatory cytokines provide the inflammatory mucosal milieu, which are required for recognition of viruses as 'danger' and 'harmful entities' by DCs [25]. In addition, the HBV may interfere with the antigen capturing and processing abilities of liver DCs. Some viruses also block the maturation of DCs. Further studies are required to clarify the underlying mechanism which impairs the functional capabilities of liver DCs in HBV-TM.

To our knowledge, there is no report about the phenotypes and functions of liver DCs in other transgenic mouse models. We checked the functions of liver DCs in HBV-TM; however, it is important to characterize liver DCs in patients with chronic hepatitis B in order to transfer what is learned in the laboratory to the patient's bedside. We have reported previously about the localization of CD83-positive liver DCs in patients with chronic hepatitis B [26]. At this time, there is no established methodology of isolating liver DCs from human livers for a functional study, although it might be possible to isolate liver DCs from a transplanted liver. Furthermore, it might be possible to check the cytokine-producing ability of liver DCs by isolating the liver-infiltrating cells and then checking their expression of intracellular cytokines.

Characterization of liver DCs in chronic HBV infection is also important in the context of DC-based therapy for chronic HBV carriers. We have shown that the administration of antigen-pulsed spleen DCs induced antigen-specific immune responses in HBV-TM, but could not reduce HBV replication [27–29]. There is little information regarding the utility of antigen-pulsed tissue DCs for treatment purposes. The therapeutic utility of HBsAg-pulsed liver DCs in HBV-TM should be assessed. Furthermore, it would be interesting to see if the use of antigen-pulsed spleen DCs alters the functions of liver DCs in HBV-TM.

In conclusion, we isolated liver DCs from murine liver without using cytokines or growth factors. To the best of our knowledge, this is the first study showing that the functions of liver DCs were impaired in HBV-TM. This study also provides the first direct evidence that liver DCs might be responsible for impaired HBV-specific immune responses of HBV-TM. At the same time, this study inspires hope that DC-based therapy may be developed for chronic HBV carriers by up-regulating the functions of liver DCs.

Acknowledgements

We would like to thank San Francisco Edit (http://www.sfedit.net) for their assistance in editing this manuscript.

References

1 Hilleman MR. Critical overview and outlook: pathogenesis, prevention, and treatment of hepatitis and hepatocarcinoma caused by hepatitis B virus. Vaccine 2003; **21**:4626–49.

2 Lok AS. Hepatitis B infection: pathogenesis and management. J Hepatol 2000; **32** (Suppl.):89–97.

3 Rehermann B, Fowler P, Sidney J *et al.* The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 1995; **181**:1047–58.

4 Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 1995; **13**:29–60.

5 Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 1991; **9**:271–96.

6 Morel PA, Feili-Hariri M, Coates PT, Thomson AW. Dendritic cells, T cell tolerance and therapy of adverse immune reaction. Clin Exp Immunol 2003; **133**:1–10.

7 Onji M. Dendritic cells in clinics. Tokyo: Springer, 2004.

8 Steinman RM. Some interfaces of dendritic cell biology. APMIS 2003; **111**:675–97.

9 Arima S, Akbar SMF, Michitaka K *et al.* Impaired function of antigen-presenting dendritic cells in patients with chronic hepatitis B. Localization of HBV DNA and HBV RNA in blood DC by *in situ* hybridization. Int J Mol Med 2003; **11**:169–74.

10 Beckebaum S, Cicinnati VR, Dworacki G *et al.* Reduction in the circulating pDC1/pDC2 ratio and impaired function of *ex vivo*-generated DC1 in chronic hepatitis B infection. Clin Immunol 2002; **104**:138–50.

11 Akbar SMF, Onji M, Inaba K, Yamamura K, Ohta Y. Low responsiveness of hepatitis B virus-transgenic mice in antibody response to T-cell-dependent antigen: defect in antigen-presenting activity of dendritic cell. Immunology 1993; **78**:468–75.

12 Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nat Med 2004; **10**:80.

13 Steptoe RJ, Fu F, Li W *et al.* Augmentation of dendritic cells in murine organ donors by Flt3 ligand alters the balance between transplant tolerance and immunity. J Immunol 1997; **159**:5483–91.

14 Lu L, Maureen L, Drakes ML, Thomson AW. Isolation and propagation of mouse liver-derived dendritic cells. In: Robinson, SP, Stagg, AJ, eds. Dendritic cell protocols. Totowa, NJ: Humana Press, 2001:85–95.

15 Lian Zx Okada T, He XS *et al.* Heterogeneity of dendritic cells in the mouse liver: identification and characterization of four distinct populations. J Immunol 2003; **170**:2323–30.

16 Araki K, Miyazaki J, Hino O, Tomita N, Chisaka O, Matsubara K, Yamamura K. Expression and replication of hepatitis B virus genome in transgenic mice. Proc Natl Acad Sci USA 1989; **86**:207–11.

17 Akbar SMF, Abe M, Masumoto T, Horiike N, Onji M. Mechanism of action of vaccine therapy in murine hepatitis B virus carriers: vaccine-induced activation of antigen presenting dendritic cells. J Hepatol 1999; **30**:755–64.

18 Kobayashi Y, Murakami H, Akbar SM, Matsui H, Onji M. A novel and effective approach of developing aggressive experimental autoimmune gastritis in neonatal thymectomized BALB/c mouse by polyinosinic : polycytidylic acid. Clin Exp Immunol 2004; **136**:423–31.

19 Chisari FV. Hepatitis B virus transgenic mice. models of viral immunobiology and pathogenesis. Curr Top Microbiol Immunol 1996; **206**:149–73.

20 Akbar SMF, Onji M. Hepatitis B virus (HBV)-transgenic mice as an investigative tool to study immunopathology during HBV infection. Int J Exp Pathol 1998; **33**:909–13.

21 Goddard S, Youster J, Morgan E, Adams DH. Interleukin-10 secretion differentiates dendritic cells from human liver and skin. Am J Pathol 2004; **164**:511–9.

22 Hyodo N, Nakamura I, Imawari M. Hepatitis B core antigen stimulates interleukin-10 secretion by both T cells and monocytes from peripheral blood of patients with chronic hepatitis B virus infection. Clin Exp Immunol 2004; **135**:462–6.

23 Bain C, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology 2001; **120**:512–24.

24 Longman RS, Talal AH, Jacobson IM, Albert ML, Rice CM. Presence of functional dendritic cells in patients chronically infected with hepatitis C virus. Blood 2004; **103**:1026–9.

25 Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. Science 1996; **271**:1723–6.

26 Tanimoto K, Akbar SMF, Michitaka K, Horiike N, Onji M. Antigen-presenting cells at the liver tissue in patients with chronic viral liver diseases: CD83-positive mature dendritic cells at the vicinity of focal and confluent necrosis. Hepatol Res 2001; **21**:117–25.

27 Akbar SMF, Horiike N, Onji M. Prognostic importance of antigen presenting dendritic cells during vaccine therapy in murine hepatitis B virus carriers. Immunology 1999; **96**:98–108.

28 Akbar SMF, Furukawa S, Horiike N, Onji M. Vaccine therapy for hepatitis B virus carrier. Curr Drug Targets Infect Disord 2004; **4**:93–101.

29 Akbar SMF, Furukawa S, Hasebe A, Horiike N, Michitaka K, Onji M. Production and efficacy of a dendritic cell-based therapeutic vaccine for murine chronic hepatitis B virus carrier. Int J Mol Med 2004; **14**:295–9.

ELSEVIER

Journal of Clinical Virology 32 (2005) 156–161

JOURNAL OF CLINICAL VIROLOGY

www.elsevier.com/locate/jcv

In vivo immunization by vaccine therapy following virus suppression by lamivudine: a novel approach for treating patients with chronic hepatitis B

Norio Horiike[a], Sk. Md. Fazle Akbar[a], Kojiro Michitaka[b], Kouji Joukou[c], Kazuhisa Yamamoto[d], Naohiko Kojima[e], Yoichi Hiasa[a], Masanori Abe[a], Morikazu Onji[a,*]

[a] *Third Department of Internal Medicine, Ehime University School of Medicine, Shigenobu-Cho, Ehime 791 0295, Japan*
[b] *Endoscopy Center, Ehime University School of Medicine, Ehime, Japan*
[c] *Internal Medicine, Matsuyama Red Cross Hospital, Ehime, Japan*
[d] *Internal Medicine, Matsuyama Shimin Hospital, Ehime, Japan*
[e] *Internal Medicine, Ohzu Central Hospital, Ehime, Japan*

Received 1 December 2003; accepted 22 July 2004

Abstract

Background: Both the hepatitis B virus (HBV) and the immune response of the hosts to HBV play important roles in the pathogenesis of chronic hepatitis B (CHB). Lamivudine is a potent antiviral agent with *minimal* immune modulator capacity. Moreover, lamivudine causes severe side effects like breakthrough of HBV DNA and breakthrough hepatitis in patients with CHB. On the other hand, vaccine therapy, a recently-developed immune therapy, exhibits potent immune modulatory potentials and almost no side effects, but possesses little antiviral capacity in patients with CHB.
Objectives: The aim of this clinical trial is to evaluate the efficacy of a combination therapy of lamivudine and vaccine in patients with CHB.
Study design: Seventy-two patients with CHB (hepatitis B e antigen (HBeAg)-positive, 40; antibody to HBeAg (anti-HBe)-positive, 32). All patients received lamivudine at a dose of 100 mg daily for 12 months. Fifteen patients ($HBeAg^+$, 9; $anti\text{-}HBe^+$, 6) receiving oral lamivudine were also given a vaccine containing 20 μg of hepatitis B surface antigen, intradermally, once every 2 weeks for 12 times (combination therapy).
Results: Twelve months after the start of therapy, serum HBV DNA became negative in 9 of 9 (100%) $HBeAg^+$ CHB patients receiving combination therapy and in 15 of 31 (48%) $HBeAg^+$ CHB patients receiving lamivudine monotherapy ($P < 0.05$). The rate of seroconversion from HBeAg to anti-HBe was also significantly higher in patients receiving combination therapy (56% versus lamivudine monotherapy, 16%, $P < 0.05$). Of the 57 patients receiving lamivudine monotherapy, breakthrough of HBV DNA was found in 10 and breakthrough hepatitis was found in 4; however, these were not seen in any patient receiving combination therapy.
Conclusions: Combination therapy represents a better therapeutic regimen with few complications in patients with CHB.

Keywords: Chronic hepatitis B; Lamivudine; Vaccine therapy; Combination therapy

1. Introduction

Chronic hepatitis B (CHB) is associated with significant morbidity and mortality and characterized by several episodes of exacerbation and remission of hepatitis; a considerable numbers of patients with CHB eventually experience serious complications such as liver cirrhosis and hepatocellular carcinoma (Chisari, 1995; Mast and Alter, 1993). During the last two decades, patients with CHB have mainly been

Abbreviations: CHB, chronic hepatitis B; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; anti-HBe, antibody to HBeAg; HBsAg, hepatitis B surface antigen; LGE, log genomic equivalent

* Corresponding author. Tel.: +81 89 960 5308; fax: +81 89 960 5310.
E-mail address: akbar@m.ehime-u.ac.jp (M. Onji).

1386-6532/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jcv.2004.07.004

treated with interferon-alpha. However, the use of interferon-alpha is costly and they have shown limited therapeutic efficacy and considerable side effects in patients with CHB (Lok, 2002; Lok et al., 1993). Currently, lamivudine, an oral nucleoside analogue that potentially inhibit the replication of hepatitis B virus (HBV) by interfering with HBV reverse transcriptase (rt) activity, is used to treat patients with CHB. Lamivudine therapy has resulted in marked decreases in HBV DNA and alanine aminotransferase (ALT), the seroconversion of hepatitis B e antigen (HBeAg) to antibody to HBeAg (anti-HBe), and histological improvement in patients with CHB (Dienstag et al., 1999; Lai et al., 1998). However, the emergence of lamivudine-resistant HBV strains in patients on long-term lamivudine therapy has been reported. Such resistant viruses exhibit a characteristic mutation of the 204th amino acid methionine of reverse transcriptase domain of DNA polymerase gene to isoleucine or valine (rtM204I/V) (Condreay, 1998). The emergence of these mutations of HBV DNA results in the re-elevation of HBV DNA (DNA breakthrough) and ALT (breakthrough hepatitis) (Stuyver et al., 2001).

Several investigators are presently looking for other therapies, including immune therapies, for patients with CHB. Vaccine therapy, in which vaccine containing hepatitis B surface antigen (HBsAg) or other HBV-related proteins is injected, is now used as an immune therapy for patients with CHB (Pol et al., 1994, 1998). Although few double-blind, placebo-controlled trials of vaccine therapy have been conducted during the last 10 years, it is now apparent that vaccine monotherapy does not possess the potent antiviral activity that interferon-alpha and lamivudine do. However, vaccine monotherapy is capable of inducing sustained normalization of ALT and seroconversion to anti-HBe in some patients with CHB (Couillin et al., 1999; Michel et al., 2001; Horiike et al., 2002). Furthermore, in contrast to antiviral therapies, vaccine therapy is cheaper and is devoid of any side effects in patients with CHB.

Studies on the pathogenesis of CHB provide some insights regarding the causes underlying the poor efficacy of monotherapy with antiviral agents (interferon-alpha and lamivudine) or immune modulators (vaccine therapy) in patients with CHB. The pathogenesis of HBV is largely immune-mediated; the virus itself is not cytopathic (Chisari, 1995). Accordingly, drugs or agents with both antiviral and immune modulatory activities are needed for treating patients with CHB. Unfortunately, the antiviral therapy and immune therapy that are presently used individually to treat patients with CHB are not endowed with both adequate antiviral and immune modulatory activities.

We postulated that patients with CHB might benefit from being treated with a combination of both antiviral drugs and immune modulatory agents. In this study, we treated a group of patients with CHB with only lamivudine (they were known as the lamivudine monotherapy group). Another group of patients with CHB was treated with both lamivudine and vaccine (they were known as the combination therapy group). The titer of HBV DNA, the parameters of the liver function test, the rate of seroconversion to anti-HBe, the occurrence of breakthrough of HBV DNA, and the occurrence of breakthrough hepatitis were checked once a month for 12 consecutive months in these patients.

2. Materials and methods

2.1. Patients

Seventy-two patients with CHB were enrolled in the study. The clinical profiles of the patients are shown in Table 1. All patients were suffering from chronic liver diseases and regularly *attended* the hospitals for periodic monitoring and therapy. The diagnosis of CHB was made from the clinical symptoms and the biochemical data of the liver function test (Desmet et al., 1994). All patients were tested and found to be positive for HBsAg and HBV DNA in the sera. They also exhibited elevated levels of serum ALT. In 40 patients with CHB, the final diagnosis was confirmed by the histological evaluation of the liver biopsy specimens. Forty patients were positive for HBeAg and the remaining 32 were positive for anti-HBe in the sera. There was no significant difference regarding age, sex, and the levels of ALT or HBV DNA between patients receiving lamivudine monotherapy and those receiving combination therapy. None of these patients had a history of other liver diseases such as autoimmune diseases, alcoholic liver diseases, or metabolic diseases. Concomitant infection and super-infection with hepatitis A virus, hepatitis C virus, cytomegalovirus, Epstein–Barr virus, or herpes simplex virus were ruled out serologically or genomically. The nature and possible consequences of this clinical trial were explained to all patients with CHB. All patients with CHB (n = 72) gave written consent for receiving lamivudine therapy. In addition, 15 patients with CHB also gave written consent to receive vaccine therapy in addition to lamivudine therapy. The study protocol was approved by the Ethical Committee of Ehime University, Japan.

2.2. Estimation of HBV markers

The presence of HBsAg in the sera were determined using chemilumiscent immunoassay kits (Architect™ HBsAg, Tokyo, Japan). The levels of HBeAg and anti-HBe were determined using enzyme immunoassay kits (AxSYM™ HBeAg Assay and AxSYM™ HBeAg Assay, Dainabot). The HBV DNA in the sera was assayed with a commercial kit (DNA probe 'Chugai-HBV', Chugai Diagnostic Science Co. Ltd., Tokyo, Japan) and the levels were expressed as log genomic equivalent (LGE/ml) (limit of HBV DNA detection >3.7 LGE/ml). The HBV sequencing at the precore and core promoter regions was performed by direct sequencing of the polymerase chain reaction product, as described elsewhere

Table 1
The clinical profiles of patients

	Combined therapy[a]		Lamivudine monotherapy		Total	
	HBeAg+	HBeAb+	HBeAg+	HBeAb+	Combined	Monotherapy
Numbers	9	6	31	26	15	57
Age (years)	38.2 ± 91	35.0 ± 12.1	45.4 ± 12.7	45.6 ± 12.4	36.9 ± 10.1 45.5 ± 12.6	
Sex (male:female)	6:3	4:2	25:6	19:7	10:5	44:13
ALT (IU/L)	133 ± 49	272 ± 328	206 ± 221	169 ± 237	193 ± 142	189 ± 230
Liver histology[a]						
Fibrosis						
F1	2	1	3	1	3	4
F2	1	1	5	2	2	7
F3	6	4	7	7	10	14
Activity						
A1	3	1	7	4	4	11
A2	3	3	7	4	6	11
A3	3	2	1	2	5	3
HBV DNA (LGE/ml)	7.3 ± 1.3	4.8 ± 3.4	7.3 ± 1.0	6.0 ± 1.3	6.3 ± 2.2	6.7 ± 1.4
Titer of HBV						
High (>7.5 LGE/ml)	4	2	12	10	6	22
Low (<7.5 LGE/ml)	5	4	19	16	9	35

The grading of fibrosis and activity of hepatitis in the liver specimen were done according to international criteria, as described by Desmet et al. (1994).
[a] Patients with combined therapy received both lamivudine and vaccine; patients with lamivudine monotherapy received only lamivudine, as described in Section 2. ALT, alanine aminotransferase.

(Kawai et al., 2003). The samples were collected just before the start of therapy. The alteration of G to A at nucleotide (nt) 1896 in the precore region was defined as the precore mutation. The alteration of A to G at nt 1762 or that of G to A at nt 1764 was defined as a core promoter mutation.

2.3. Lamivudine monotherapy and combination therapy in patients with chronic hepatitis B

The protocols for lamivudine monotherapy and combination therapy (lamivudine with vaccine therapy) are shown in Fig. 1. All 72 patients with CHB were provided with lamivudine at an oral dose of 100 mg once daily for 12 months. Out of these 72 patients, 57 patients received only lamivudine for 12 months and these patients belonged to the lamivudine monotherapy group (Fig. 1A). As shown in Fig. 1B, the other 15 patients were enrolled in the combination group. They received lamivudine for 12 months according to the protocol of the lamivudine monotherapy group. They also received 12 intradermal injections with vaccine, once every 2 weeks. The first injection of vaccine was given 3 months after they started taking lamivudine. Commercial vaccine containing 20 μg of HBsAg (Tokyo Mitsubishi, Tokyo, Japan) was used. The therapeutic evaluation of lamivudine monotherapy and combination therapy was performed 12 months after the start of lamivudine therapy. Sera were collected from each patient once a month for 12 consecutive months and assayed for the parameters of the liver function test as well as HBeAg, anti-HBe, HBV DNA, the incidence of breakthrough of HBV DNA, and the incidence of breakthrough hepatitis.

2.4. Statistical analysis

The data were expressed as mean ± standard deviation. The statistical analyses were done with unpaired or paired *t*-tests and the chi-square test where indicated. *P*-values less than 0.05 were considered to indicate statistical significance. Statistical analysis was performed using the Stat View™ (version 4.5) statistical program on a Macintosh computer.

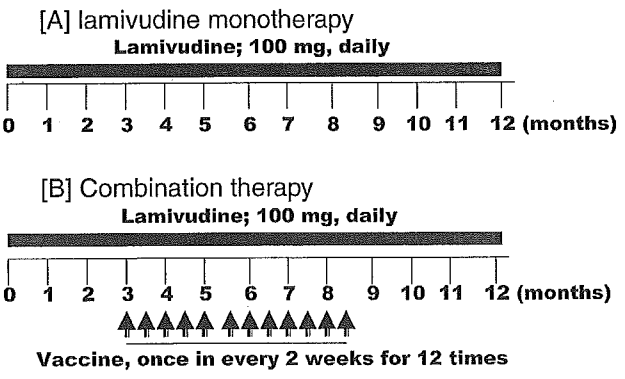

Fig. 1. The protocols for lamivudine monotherapy and combination therapy are shown. (A) Patients in the lamivudine monotherapy group received lamivudine orally at a dose of 100 mg daily for 12 months. (B) The patients in the combination group also received lamivudine orally at a dose of 100 mg daily for 12 months. In addition, they were given injections of vaccine once every 2 weeks for 6 months (12 injections total). The first injection of vaccine was given 3 months after the start of lamivudine therapy.

Table 2
Clearance of HBV DNA from the sera of anti-HBe$^+$ patients with chronic hepatitis B

Therapy	Number	Seronegativity of HBV DNA	
		6 months (%)[b]	12 months (%)
Lamivudine monotherapy[a]	26	26 (100)	24 (92)[c]
Combination therapy	6	6 (100)	6 (100)

[a] Patients receiving lamivudine monotherapy and those receiving combination therapy received lamivudine orally at a dose of 100 mg once daily for 12 months. The patients receiving combination therapy also received 12 vaccines, one every 2 weeks, as described in Section 2.

[b] The duration is counted from the start of lamivudine therapy.

[c] Two patients in the lamivudine monotherapy group who became negative for HBV DNA 6 months after the start of therapy expressed HBV DNA in the sera due to breakthrough of HBV.

3. Results

3.1. Both combination therapy and lamivudine monotherapy induced HBV DNA negativity in anti-HBe$^+$ patients with CHB

All patients with CHB enrolled in the study completed the therapy. As expected, all patients positive for the anti-HBe antibody who received lamivudine monotherapy or combination therapy became negative for HBV DNA in the sera 6 months after the start of therapy (Table 2). When the final therapeutic evaluation was performed 12 months after the start of therapy, two patients in the lamivudine monotherapy group were found to have breakthrough of HBV DNA; however, this was not seen in any patient who had received combination therapy.

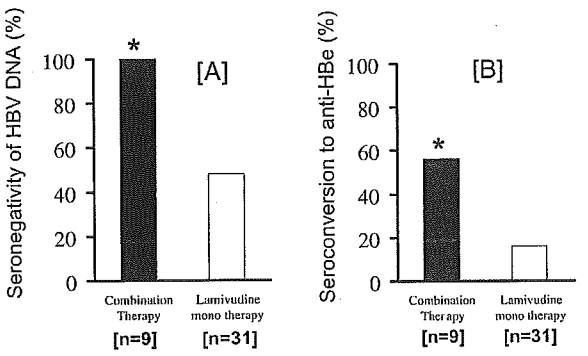

Fig. 2. Patients in the lamivudine monotherapy group and those in the combination group received lamivudine orally at a dose 100 mg once daily for 12 months. The patients in the combination group received additional therapy with injections of vaccine once every 2 weeks for 6 months as described in Fig. 1. The therapeutic evaluation was performed 12 months after the start of lamivudine therapy. (A) All nine HBeAg$^+$ patients who received combination therapy (100%) and 15 of 31 patients who received lamivudine monotherapy (48%) became negative for HBV DNA in the sera ($P < 0.05$). (B) The rate of seroconversion from HBeAg$^+$ to anti-HBe$^+$ was also significantly higher in patients receiving combination therapy.

Table 3
Influence of initial viral load on seroconversion to anti-HBe in HBeAg$^+$ patients with chronic hepatitis B who received combination therapy

Factors	HBeAg$^+$ patients seroconverted to anti-HBe	HBeAg$^+$ patients not seroconverted to anti-HBe
Numbers	5	4
Age (years)	34.4 ± 8.0	43.0 ± 8.9
Sex (male:female)	4:1	2:2
ALT (IU/L)	122 ± 11	146 ± 70
Liver histology[a]		
Fibrosis		
F1	1	1
F2	0	1
F3	4	2
Activity		
A1	2	1
A2	1	2
A3	2	1
HBV DNA (LGE/ml)	5.9 ± 1.0	8.4 ± 04[a]
Titer of HBV		
High (>7.5 LGE/ml)	0	4[a]
Low (<7.5 LGE/ml)	5	0
HBV mutations		
Precore	0	0
Core promoter	3	4

Patients who received combination therapy received lamivudine orally at a dose 100 mg once daily for 12 months. They also received 12 injections with vaccine, one every 2 weeks as described in Section 2. Vaccination was started 3 months after the start of lamivudine administration.

[a] $P < 0.05$ compared with the patients who did not seroconvert to anti-HBe.

3.2. Potent therapeutic effect of combination therapy in HBeAg$^+$ CHB patients

The potent antiviral activity of combination therapy was evident in HBeAg$^+$ patients with CHB. Twelve months after the therapy began, 9 of 9 (100%) HBeAg$^+$ patients with CHB who received combination therapy and 15 of 31 patients (48%) HBeAg$^+$ patients with CHB who received lamivudine monotherapy became negative for HBV DNA in the sera ($P < 0.05$) (Fig. 2A). Combination therapy also produced a favorable effect on seroconversion from HBeAg to anti-HBe. Twelve months after the therapy began, the rate of seroconversion was significantly higher in patients receiving combination therapy (56%) than that in patients receiving lamivudine monotherapy (16%) ($P < 0.05$) (Fig. 2B).

3.3. Effect of increased viral load on the seroconversion of patients receiving combination therapy

Five of the nine HBeAg$^+$ patients with CHB who received combination therapy seroconverted to anti-HBe. To determine the factors that influenced the seroconversion to anti-HBe, we compared the clinical profiles and virological markers between the patients who received combination

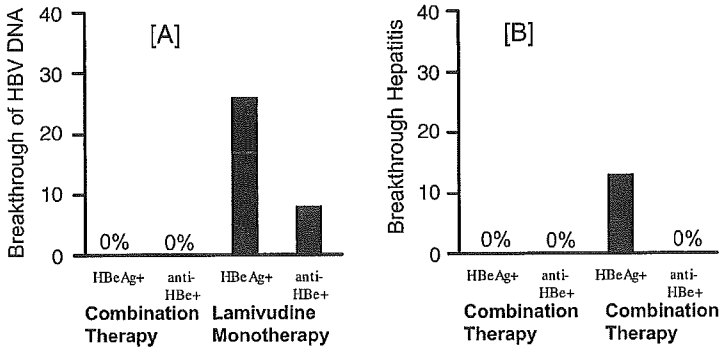

Fig. 3. The incidences of breakthrough of HBV DNA and breakthrough hepatitis from lamivudine monotherapy and combination therapy are shown. Patients receiving combination therapy showed neither breakthrough of HBV DNA (A) nor breakthrough hepatitis (B). On the other hand, patients receiving lamivudine monotherapy showed both breakthrough of HBV DNA and breakthrough hepatitis.

therapy with and without seroconversion (Table 3). The initial viral load of the patients with CHB was significantly higher in those patients who did not seroconvert compared to those who seroconverted to anti-HBe ($P < 0.05$). Patients with CHB with low fibrosis score appears to had better seroconversion to anti-HBe compared to patients with high fibrosis score (Table 3). However, this should be confirmed in a future study.

3.4. Combination therapy is capable of reducing the incidences of breakthrough of HBV DNA and breakthrough hepatitis in patients with CHB

The occurrence of complications like the breakthrough of HBV DNA and breakthrough hepatitis in patients with CHB who were treated with lamivudine is shown in Fig. 3. Eight of 31 $HBeAg^+$ patients with CHB (26%) and two of 26 anti-HBe^+ patients with CHB (8%) who received lamivudine monotherapy exhibited the breakthrough of HBV DNA. Breakthrough hepatitis was seen in four of 31 $HBeAg^+$ patients with CHB (13%) who received lamivudine monotherapy. However, neither breakthrough of HBV DNA nor breakthrough hepatitis was seen in any patient with CHB who received combination therapy (Fig. 3).

4. Discussion

Despite the presence of an effective vaccine since 1982, HBV still ranks among the highest causes of mortality from infectious diseases worldwide (Alter, 2003). Evidence increasingly indicates that an active antiviral treatment for patients with progressive disease associated with active HBV replication might lead to significant reductions in morbidity and mortality. The ultimate therapeutic goal for these patients has also been reconsidered because it is now certain that complete eradication of HBV from patients with CHB is unachievable due to the presence of covalently closed circular DNA in the liver and extrahepatic sites (Lok, 2000). The therapy for patients with CHB is now directed at a reduction in the replication of HBV, the normalization of ALT, and the seroconversion to anti-HBe. All of these would limit the progression of liver diseases and minimize the damage to hepatocytes.

In this context, two new therapeutic agents-lamivudine, an antiviral agent, and vaccine therapy, an immune therapy, have inspired considerable optimism for patients with CHB. Lamivudine is a potent suppressor of viral replication that is inexpensive and can be given orally (Dienstag et al., 1999). On the other hand, vaccine therapy exhibits moderate antiviral activity and considerable immune modulatory capacity (Pol et al., 1994) in patients with CHB. However, lamivudine monotherapy is associated with occurrences of breakthrough of HBV DNA and breakthrough hepatitis in a considerable number of patients with CHB (Schalm, 1997). On the other hand, vaccine therapy does not possess strong antiviral potential and is mainly effective in patients with low levels of HBV replication.

On the basis of this information, we conducted a 12-month therapeutic trial of lamivudine monotherapy and a combination therapy of lamivudine and vaccine in patients with CHB. Vaccine therapy was given 3 months after the start of lamivudine therapy because the levels of HBV DNA in the sera reduced considerably during this time. Data from this study show that a combination therapy of lamivudine and vaccine is better than lamivudine monotherapy in patients with CHB regarding HBV DNA negativity and seroconversion to anti-HBe (Fig. 2). Most strikingly, none of the patients with combination therapy showed the occurrences of breakthrough of HBV DNA and breakthrough hepatitis, although some patients receiving lamivudine monotherapy showed both of these complications (Fig. 3).

Although the data on the combination therapy is highly promising, it would be premature to draw a firm conclusion regarding the efficacy of this therapy from this study because only 15 patients were enrolled in combination therapy group. A multi-center trial of combination therapy with large numbers of patients with different ethnic and clinical backgrounds is needed. However, a recent study indicates that the therapeutic strategy that we have proposed here might

be very effective. Sprengers et al. (2003) have shown that in vivo immunization following virus suppression might be a novel therapeutic approach for patients with CHB. They used interferon-alpha to induce immunity in two patients with CHB.

We are currently trying to determine why combination therapy with lamivudine and vaccine showed such a potent therapeutic effect and why there was no breakthrough of HBV DNA or breakthrough hepatitis in patients with CHB who received combination therapy. However, lamivudine by virtue of its potent antiviral effect probably secured a favorable environment for the action of vaccine therapy in patients with CHB (Couillin et al., 1999). Vaccines may act directly to down-regulate the occurrence of mutation of HBV DNA in patients with CHB. In fact, vaccine therapy induces many cytokines and activates many cells of the immune system including antigen-presenting dendritic cells (Horiike et al., 2002). Further study of the means of action of combination therapy is needed to understand the therapeutic effects of this therapy and the cellular events underlying the down-regulation of breakthrough of HBV DNA mutation in patients with CHB who are treated with lamivudine.

In conclusion, we have shown that a combination therapy consisting of lamivudine and vaccine would be a better therapeutic choice for treating patients with CHB than either therapy alone. The combination therapy produced no visible side effects. Patients with CHB who received combination therapy did not show any breakthrough of HBV DNA or breakthrough hepatitis during the follow-up period.

References

Alter MJ. Epidemiology and prevention of hepatitis B. Semin Liver Dis 2003;22:39–46.

Chisari FV. Hepatitis B virus immunopathogenesis. Annu Rev Immunol 1995;13:29–60.

Condreay LD. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. Hepatology 1998;27:1670–7.

Couillin I, Pol S, Mancini M, Driss F, Brechot C, Tiollais P, Michel ML. Specific vaccine therapy in chronic hepatitis B: induction of T cell proliferative response specific for envelope antigens. J Infect Dis 1999;180:15–26.

Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading, and staging. Hepatology 1994;19:1513–20.

Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256–63.

Horiike N, Akbar SMF, Ninomiya T, Abe M, Michitaka K, Onji M. Activation and maturation of antigen-presenting dendritic cells during vaccine therapy in patients with chronic hepatitis due to hepatitis B virus. Hepatol Res 2002;23:38–47.

Kawai K, Horiike N, Michitaka K, Onji M. The effects of hepatitis B virus core promoter mutations on hepatitis B core antigen distribution in hepatocytes as detected by laser-assisted microdissection. J Hepatol 2003;48:635–41.

Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. N Engl J Med 1998;339:61–8.

Lok AS. Hepatitis B infection: pathogenesis and management. J Hepatol 2000;32(Suppl.):89–97.

Lok AS, Chung HT, Liu VW, Ma OC. Long-term follow-up of chronic hepatitis B patients treated with interferon alpha. Gastroenterology 1993;105:1833–5.

Mast EE, Alter MJ. Epidemiology of viral hepatitis. Sem Virol 1993;4:273–83.

Michel M-L, Pol S, Brechot C, Tiollais P. Immunotherapy of chronic hepatitis B by anti HBV vaccine: from present to future. Vaccine 2001;19:2395–9.

Pol S, Diss F, Michel ML, Nalpas B, Berthelot P, Brechot C. Specific vaccine therapy in chronic hepatitis B infection. Lancet 1994;344:342.

Pol S, Couillin I, Michel ML, Driss F, Nalpas B, Carnot F, Berthelot P, Brechot C. Immunotherapy of chronic hepatitis B by anti HBV vaccine. Acta Gastroenterol Belg 1998;61:228–33.

Schalm SW. Clinical implications of lamivudine resistance by HBV. Lancet 1997;9044:3–4.

Sprengers D, Janseen HLA, Kwekkbaom J, Niesters HGM, de Man RA, Schalm SW. In vivo immunization following virus suppression: a novel approach for inducing immune control in chronic hepatitis B. J Viral Hepat 2003;10:7–9.

Stuyver LJ, Locarnini SA, Lok A, Richman DD, Carman WF, Dienstag JL, Schinazi RF. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. Hepatology 2001;33:751–7.