Table 1. Clinical profiles of patients with chronic hepatitis C

Case	Age (years)	Sex	Histology[a]	HCV Serogroup	HCV Baseline level[b] 10^3 copies/ml	Serum ALT (IU/l)	Therapy	Outcome
1	39	Male	F4/A1	2	5.9	97	Mono IFN	SVR
2	34	Female	F3/A3	2	530	256	Riba + IFN	SVR
3	69	Female	F3/A2	1	>850	250	Mono IFN	NR
4	62	Male	F2/A2	2	16	35	Mono IFN	SVR
5	40	Female	F1/A1	1	340	28	Riba + IFN	TR
6	46	Male	F2/A2	2	400	208	Mono IFN	TR
7	67	Male	F1/A2	1	>850	40	Riba + IFN	TR
8	54	Male	F4/A2	1	68	81	Riba + IFN	TR

HCV, hepatitis C virus; ALT, alanine aminotransferase; Mono IFN, interferon-α monotherapy (24 weeks); Riba + IFN, combination therapy of ribavirin and interferon-α (24 weeks); SVR, sustained viral response; NR, nonresponse; TR, transient response
[a]Liver histology is classified according to the Inuyama criteria
[b]HCV viral load was detemined by amplikor HCV

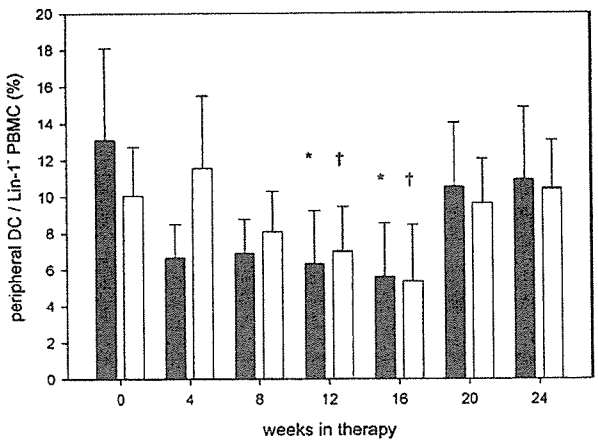

Fig. 2. Kinetics of DC subsets during antiviral therapy and a comparison by viral response. Frequencies of mDCs (*black bars*) and pDCs (*white bars*) were determined sequentially at weeks 0, 4, 8, 12, 16 20, and 24 of therapy. The DC populations are shown as percentages of Lin-1-negative peripheral blood mononuclear cells (PBMCs) (see also Fig. 1). Data are means ± SD of seven patients. Both subsets showed a significant decrease at weeks 12 and 16 (* for mDC and † for pDC)

treatment. A transient increase in t*24/c129-positive cells was observed at week 4 (21.33 ± 11.36 cells/10^5 mononuclear cells, mean ± SD) from the baseline level (10.88 ± 4.72), while t*24/ns1296-positive cells decreased from 21.37 ± 12.86 (the baseline level) to 5.96 ± 1.99 at week 12 and remained below the baseline thereafter.

Time-dependent changes of tetramer-positive cells and DC subsets in each subject are summarized in Fig. 3. We separated the responses into sustained viral response (SVR) and transient response (TR) groups to investigate the direct relationship between immunological data and clinical outcome. However, these two groups did not differ significantly with respect to either DC population or tetramer frequencies.

IFN monotherapy versus combination therapy

Three patients received IFN-α monotherapy, and the others received ribavirin and IFN-α combination therapy. We compared the kinetics of DC subsets or tetramer-positive cells (HCV-specific T cells) and biochemical markers such as ALT and white blood cells to determine whether ribavirin affected their response. However, no significant relationship was found with ribavirin usage.

Discussion

This study aimed to investigate the changes in DC subsets and their relationship with HCV-specific CTLs in peripheral blood, since functional impairment of DCs is still under discussion, whereas researchers agree on its contribution to weak antiviral immunity.[21] Functional impairment of pDCs in CHC patients has been recently reported;[22–25] moreover, other studies have discovered the restoration of a mixed lymphocyte reaction and IL-12 production after antiviral therapy by taking advantage of directly isolated DC subsets,[26] while still other studies have reported functional DCs.[27,28]

Although we found a reduced mDC population in untreated CHC, this trend was not seen among the seven followed-up patients, perhaps because of the small number of cases. No significant differences were observed in the frequency of Lin^- cells between the two groups, in agreement with Wertheimer et al.[29] In addition, frequencies of peripheral mDCs and pDCs showed a similar kinetics during IFN therapy. These two types of DCs conceivably arise from different lineages and are

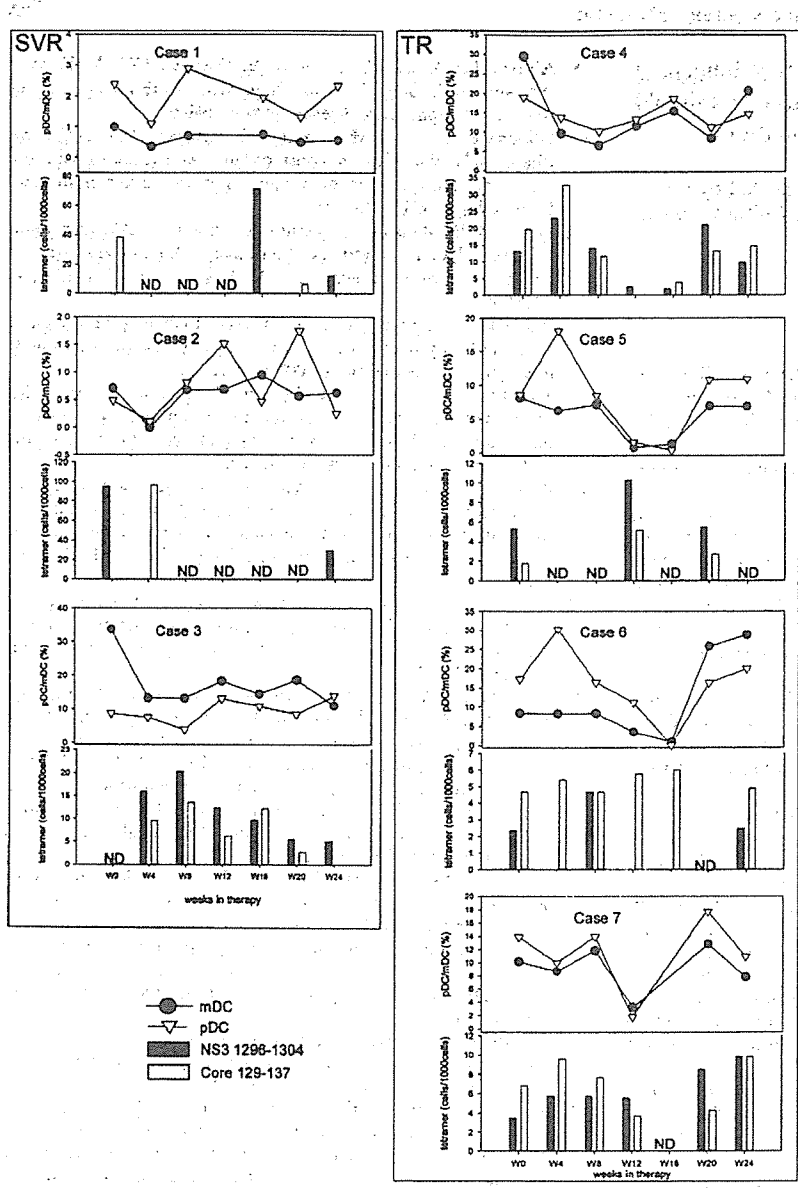

Fig. 3. Changes in frequencies of tetramer-positive cells and DC subsets in individual patients. Frequencies of tetramer-positive cells for t*24/c129 (*black bars*), t*24/ns1294 (*white bars*), mDCs (*filled circles*) and pDCs (*open triangles*) are shown at each time point: cases 1–3, sustained viral response; cases 4–7, transient response. ND, not detected. The DC populations are shown as percentages of Lin-1-negative PBMCs, while frequencies of tetramers are shown as positive cells per 10^5 PBMCs.

distinguished from each other by surface molecules and cytokine production.[30,31] Accumulating evidence suggests that these two distinct populations are closely regulated by each other.[13,32] Type I IFN plays a role in the activation and migration of pDCs,[33] and mature pDCs produce much IFN in response to bacterial and viral infections.[34,35] On the other hand, some reports suggest the negative function of IFN toward DC maturation in lymphocytic choriomeningitis virus infection in mouse[36] or in humans receiving IFN (and ribavirin) therapy.[37] These findings suggest that IFN-α (and ribavirin) treatment might affect peripheral DC populations.

Two HCV-derived CTL epitopes were used to evaluate CTL frequencies in HLA-A24-positive patients, the most common HLA-A allele in Japanese.[38] Frequencies of HCV-specific CTLs have been reported to decline during antiviral therapy in cases of both acute hepatitis C[39] and CHC.[8] Our results support these finding that frequencies of HCV-specific CTLs are not enhanced by antiviral therapy. However, comprehensive analysis of the CTL response using more HLA-A24-restricted epitopes[40] should clarify the meaning of these findings.

Collectively, our results, even though from a limited study, show unique dynamics of DC subsets and no enhancement of specific CTLs during antiviral therapy,

despite efficient viral reduction. How IFN influences DCs in HCV-infected hosts in relation to CTLs should be further examined and discussed in the future.

Acknowledgment. This study was partly supported by Grant #12877084 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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World J Gastroenterol 2006 July 21; 12(27): 4310-4317
World Journal of Gastroenterology ISSN 1007-9327

VIRAL HEPATITIS

Mechanism of T cell hyporesponsiveness to HBcAg is associated with regulatory T cells in chronic hepatitis B

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Received: 2006-01-13 Accepted: 2006-02-18

Abstract

AIM: To study the mechanisms of hyporesponsiveness of HBV-specific $CD4^+$ T cells by testing T_H1 and T_H2 commitment and regulatory T cells.

METHODS: Nine patients with chronic hepatitis B were enrolled. Peripheral blood mononuclear cells were stimulated with HBcAg or HBsAg to evaluate their potential to commit to T_H1 and T_H2 differentiation. HBcAg-specific activity of regulatory T cells was evaluated by staining with antibodies to CD4, CD25, CTLA-4 and interleukin-10. The role of regulatory T cells was further assessed by treatment with anti-interleukin-10 antibody and depletion of $CD4^+CD25^+$ cells.

RESULTS: Level of mRNAs for T-bet, IL-12R β2 and IL-4 was significantly lower in the patients than in healthy subjects with HBcAg stimulation. Although populations of $CD4^+CD25^{high}CTLA\text{-}4^+$ T cells were not different between the patients and healthy subjects, IL-10 secreting cells were found in $CD4^+$ cells and $CD4^+CD25^+$ cells in the patients in response to HBcAg, and they were not found in cells which were stimulated with HBsAg. Addition of anti-IL-10 antibody recovered the amount of HBcAg-specific T_H1 antibody compared with control antibody ($P < 0.01$, 0.34% ± 0.12% *vs* 0.15% ± 0.04%). Deletion of $CD4^+CD25^+$ T cells increased the amount of HBcAg-specific T_H1 antibody when compared with lymphocytes reconstituted using regulatory T cells ($P < 0.01$, 0.03% ± 0.02% *vs* 0.18% ± 0.05%).

CONCLUSION: The results indicate that the mechanism of T cell hyporesponsiveness to HBcAg includes activation of HBcAg-induced regulatory T cells in contrast to an increase in T_H2-committed cells in response to HBsAg.

Key words: Hepatitis B virus; Regulatory T cells; IL-10; FOXP3; T_H1

Kondo Y, Kobayashi K, Ueno Y, Shiina M, Niitsuma H, Kanno N, Kobayashi T, Shimosegawa T. Mechanism of T cell hyporesponsiveness to HBcAg is associated with regulatory T cells in chronic hepatitis B. *World J Gastroenterol* 2006; 12(27): 4310-4317

http://www.wjgnet.com/1007-9327/12/4310.asp

INTRODUCTION

Hepatitis B virus (HBV) is a noncytopathic DNA virus which causes chronic hepatitis and hepatocellular carcinoma as well as acute hepatitis[1]. HBV now affects more than 300 million people worldwide[2] and in approximately 5% of adults and 95% of neonates who become infected with HBV, persistent infection develops.

It has been shown that cytotoxic T lymphocytes (CTLs) play a central role in the control of virus infection[3]. In addition, $CD4^+$ T cells provide help for both CTLs and B-cell responses[4]. Hyporesponsiveness of HBV-specific T cells in peripheral blood has been shown in patients with chronic HBV infection[5]. Recently, lamivudine treatment in chronic hepatitis B has been reported to restore both $CD4^+$ T cells and CTL hyporesponsiveness following the decline of serum levels of HBV DNA and HBAg[6,7]. However, previous reports have indicated that HBV-specific T cells restored by lamivudine treatment are insufficient to completely suppress HBV replication[8,9]. In our previous study, we observed a defect in recovery of HBcAg-specific T_H1 cells despite restoration of CTLs, although they showed limited functions[10,11]. Since type 1 helper T (T_H1) cells are believed essential for immunity against intracellular pathogens[12], more detailed study of HBV-specific $CD4^+$ cells is needed in order to understand the mechanisms of persistent infection in CHB.

Increasing evidence has suggested that both cytokine

balance including interferon-γ (IFN-γ) and interleukin-4 (IL-4) and direct signaling through the T cell receptor is important for TH1 and TH2 commitment[13]. The critical transcription factors for commitment of T cells to the TH1 or TH2 pathway are T-bet or GATA-3 respectively[14-16]. Whether various antigens derived from the HBV genome affect expression of these factors is unknown. It is important to understand how cytokine balance and antigen types could affect TH1/TH2 commitment in chronic hepatitis B.

There have also been reports about the possible induction of anergy by regulatory T cells (T_{reg} cells), that constitutively express CD25 (the IL-2 receptor alpha-chain) in the physiological state[17-19]. In humans, this T_{reg} population, as defined by $CD4^+CD25^+CTLA\text{-}4^+$ expression, constitutes 5% to 10% of peripheral $CD4^+$ T cells and has a broad repertoire that recognizes various self and nonself antigens. It has been indicated that T_{reg} cells have several different mechanisms for suppressing various kinds of immune cells[20,21]. The important mechanisms are cell to cell contact and secretion of cytokines including IL-10 and transforming growth factor-beta (TGF-beta)[22-26]. Antigens derived from HBV might induce T_{reg} cells to escape from immunological pressure as reported in persistent infection of EB virus, hepatitis C virus and HIV-1[24,26,27].

In this study we examined the mechanisms of hyporesponsiveness of HBV-specific $CD4^+$ T cells by evaluating the TH1/TH2 commitment and activity of T_{reg} cells.

MATERIALS AND METHODS

Study design

Nine patients with chronic hepatitis B (CHB) were enrolled in this study (Table 1). The patients had more than 5.0 log genome equivalent (LGE /mL; Chugai Pharmaceutical Co., Tokyo, Japan) of serum HBV DNA and had elevated alanine aminotransferase (ALT) values (normal range < 40 IU/L) for more than 6 mo prior to the study. Six patients were seropositive for HBeAg and three patients were seropositive for anti-HBe. All the patients were negative for antibodies to hepatitis C virus (HCV) and did not have liver diseases due to other causes, such as alcohol, drug, congestive heart failure and autoimmune disease. For control subjects, ten healthy HBsAg-vaccinated subjects were included.

Permission for the study was obtained from the Ethical committee at Tohoku University School of Medicine. Written informed consent was obtained from all the subjects enrolled in this study. The study comprised 6 mo of monitoring before obtaining peripheral blood with assessments at 1, 2, 4, and 6 mo. At each assessment, patients were evaluated for serum HBV DNA, HBeAg, anti-HBe, blood chemistry and hematology. HBsAg, anti-HBs, total and IgM anti-HBc, HBeAg, anti-HBe, and anti-HCV were determined by commercial enzyme immunoassay kits (Abbott Laboratories, Chicago, IL). Serum levels of HBV DNA were measured by transcription mediated amplification-hybridization protection assay (lower limit of detection: 3.7 LGE/mL).

Table 1 Summary of clinical characteristics of patients with chronic hepatitis B enrolled in the study

Case	Age (yr)	Gender	ALT (IU/L)	HBeAg (Cutoff index)	Anti-HBe (Inhibition %)	HBV DNA (LGE/mL)	HBV Genotype
1	55	M	78	67	< 0.5	5.8	C
2	36	M	183	100	< 0.5	7.6	ND
3	31	M	50	66.9	< 0.5	7.6	C
4	42	M	141	100	< 0.5	6.8	C
5	27	M	77	75.7	< 0.5	7.6	C
6	42	F	42	93.8	< 0.5	7.0	C
7	32	M	70	< 0.5	100	6.2	C
8	29	M	81	< 0.5	86.9	5.3	C
9	58	M	117	0.7	100	7.3	C

The values for serum levels of ALT, HBeAg, anti-HBe, HBV DNA and HBV genotypes were determined at the time of blood sampling. Abbreviations: M, male; F, female; LGE/mL, log genome equivalent /mL; ND, not determined.

Reagents

IL-10 and IFN-γ secretion assay kits were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Monoclonal antibodies to human CTLA-4 (APC-labeled), CD4 (PerCP-labeled), CD3 (FITC-labeled), CD25 (FITC-labeled), IL-10 (No Azide / Low Endotoxin) and isotype-matched control antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA). HBsAg and HBcAg were obtained from Biodesign International (Saco, MA).

Cell culture

Peripheral blood mononuclear cells (PBMCs) isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation were resuspended in RPMI 1640 supplemented with 8% human AB serum (Nabi, Miami, FL; complete medium) and were cultured in a 96-well plate at a concentration of 1×10^7 cells/mL in complete medium in the presence of HBsAg (29 μg/mL) or HBcAg (10 μg/mL) for 24 h. Thereafter, $CD4^+$ cells (4×10^5 cells) were separated from the stimulated PBMCs using anti-CD4-coated magnetic beads (Dynabeads M-450 CD4, Dynal, Oslo, Finland) for quantification of mRNAs.

Quantified real time PCR

Total cellular RNA was extracted from $CD4^+$ cells using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacture's instruction. Contaminating small DNA was removed by DNase I digestion using an RNase-free DNase system (Qiagen). Subsequently, total RNA was reverse-transcribed to single strand cDNA using random hexamers. In brief, the amount of extracted RNA was measured by NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE). After mixing with random primers and DEPC water, 1 μg RNA was further mixed with 5 × first strand buffer, dNTP mixture and 0.1 mol/L DTT. After preincubation (25℃, 10 min), M-MLV RT (Takara, Tokyo, Japan) and ribonuclease inhibitor were added and samples were incubated further for 60-min at 37℃. Realtime PCR was performed on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA) using predeveloped TaqMan Assay Reagents (Perkin-Elmer

Applied Biosystems) according to the manufacturer's protocol[28]. The commercially available primers and probe for the amplification of T-bet (ID Hs00203436), IFN-γ (ID Hs00174143), GATA-3 (ID Hs00231122), IL-4 (ID Hs00174122), FOXP3 (ID Hs00203958) and GAPDH were purchased from Perkin-Elmer Applied Biosystems. Amplification of IL-12R β2 was performed as previously described[29].

IL-10 and IFN-gamma secretion assay

Purified PBMCs were stimulated at 1×10^7 cells/mL in complete medium with or without HBcAg (10 μg/mL) for 9 h at 37℃. Cells were washed by adding 2 mL of cold buffer and resuspended in 90 μL of cold medium. After the addition of 10 μL of IL-10- or IFN-gamma-capture Reagent, cells were incubated for 5 min on ice. Thereafter, cells were diluted with 1 mL of warm medium (37℃) and further incubated in a closed tube for 45 min at 37℃ under slow continuous rotation. Cells were washed and IL-10- or IFN-γ-secreting cells were stained by adding 10 μL of IL-10- or IFN-γ-Detection Antibody (PE-conjugated) together with anti-CD4-PerCP and anti-CD25-FITC. In some experiments, FITC fluorescence was amplified by FASER kit-FITC (Miltenyi Biotec). Selected samples were stained with anti-CD14-FITC, anti-CD3-PerCP, anti-HLA-DR-APC (BD Biosciences). Cells were analyzed by FACSCalibur.

To assess the effects of IL-10 on the HBcAg-specific IFN-γ production by $CD4^+$ T cells, PBMCs were stimulated at 1×10^7 cells/mL in complete medium with or without HBcAg (10 μg/mL) and with or without anti-human IL-10 monoclonal antibody at the indicated concentration for 9 h at 37℃. Cells were then used for IFN-γ-secretion assay and analyzed by FACSCalibur.

Intracellular and surface CTLA-4 staining

In order to analyze the expression of total CTLA-4 in $CD4^+CD25^+$ cells, cells were fixed and permeabilized using BD cytofix/cytoperm solution (BD Bioscience) after cell surface markers including CTLA-4 were stained. Subsequently, intracellular CTLA-4 was stained and the cells were analyzed by FACSCalibur[30].

Depletion of T_{reg} cells

By using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec), three fractions of lymphocytes were obtained; lymphocytes depleted of $CD4^+$ cells (fraction 1), purified $CD4^+CD25^-$ lymphocytes (fraction 2) and purified $CD4^+CD25^+$ cells (fraction 3). To test the effect of $CD4^+CD25^+$ cells on HBcAg-specific IFN-γ production, 2 sets of lymphocyte preparations were reconstituted. The first set, designated as T_{reg}^+, was the mixture of all three fractions and contained 5%-7% $CD4^+CD25^+$ cells. The second set, designated as T_{reg}^-, was the mixture of fractions 1 and 2, and contained 0.5% (mean) of $CD4^+CD25^+$ cells.

Statistical analysis

Differences in the amounts of cytokines produced were analyzed by oneway ANOVA between patients with CHB and healthy controls. The frequencies of cytokine-secreting cells were analyzed by Mann-Whitney *U* test. Both tests were run using SPSS ver. 10. A level of $P < 0.05$ was considered as being statistically significant.

RESULTS

Expression of mRNA relating to T_H1/T_H2 commitment in $CD4^+$ cells

In CHB patients, HBcAg significantly suppressed the expression of mRNAs for T-bet ($P < 0.01$), IL-12R β2 ($P < 0.05$) and IL-4 ($P < 0.05$) compared with those of healthy volunteers (Figure 1A). In addition, the expression levels of mRNAs for IFN-γ and GATA-3 were below 1.0 in response to HBcAg stimulation (Figure 1A). On the other hand, HBsAg induced the upregulation of GATA-3 mRNA compared with healthy volunteers ($P < 0.01$) while the expression level of T_H1 related mRNA (T-bet, IFN-γ, and IL-12R β2) remained unchanged (Figure 1B).

IL-10 secreting cells in response to HBcAg were enriched in $CD4^+CD25^+$ lymphocytes

Involvement of the suppressive cytokine IL-10 in suppression of T_H1-comittement of HBcAg-stimulated cells was evaluated by enumeration of IL-10-secreting cells. Since the cells secreting IL-10 were mostly found in the $CD3^+$ population, cells were further studied by staining with antibodies to CD4 and CD25. A population of IL-10-sereting $CD4^+$ T cells was readily detectable in patients with CHB (Figure 2A) and these IL-10 secreting cells in $CD4^+$ T cells showed $CD25^{high}$ expression (Figure 2B), while there were no such responding cells in healthy subjects (Figure 2C). In addition, when the cells were stimulated with HBsAg, no IL-10 producing $CD4^+CD25^{high}$ cells were detected (Figure 2D). The percentage of HBcAg-specific IL-10 secreting $CD4^+$ cells in all patients with CHB was 0.10% ± 0.04 % (mean ± standard deviation), and the population was more prominent in $CD4^+CD25^{high}$ cells (Figure 3). Our next question was whether T_{reg} cells increased in number or were induced by HBcAg stimulation. Therefore, the population of $CD4^+CD25^{high}CTLA\text{-}4^+$ T cells was compared between CHB patients and healthy subjects (Figure 4A). However, no statistical difference in the population with this phenotype was found between normal subjects and CHB patients (Figure 4B).

Recovery of IFN-γ-secreting cells by the addition of anti-IL-10 antibody

Low response of HBcAg-specific T_H1 cells defined by IFN-gamma-secreting $CD4^+$ T cells in response to HBcAg stimulation was indicated by the lack of statistical difference in that population between patients with CHB and normal subjects (Figure 5A). To further assess the role of IL-10 in the suppression of T_H1 responses to HBcAg stimulation, the effect of anti-IL-10 antibody on T_H1 response was evaluated by addition of anti-IL-10 cultures. The population of $CD4^+$ T cells was comparable when cultured with and without anti-IL-10 antibody (Figure 5B). In the presence of anti-IL-10 antibody, the population of IFN-γ-secreting $CD4^+$ T lymphocytes in response to HBcAg significantly increased (2.3-fold, 0.34% ± 0.12%; mean ± SD of 9 cases) compared to culture with a control

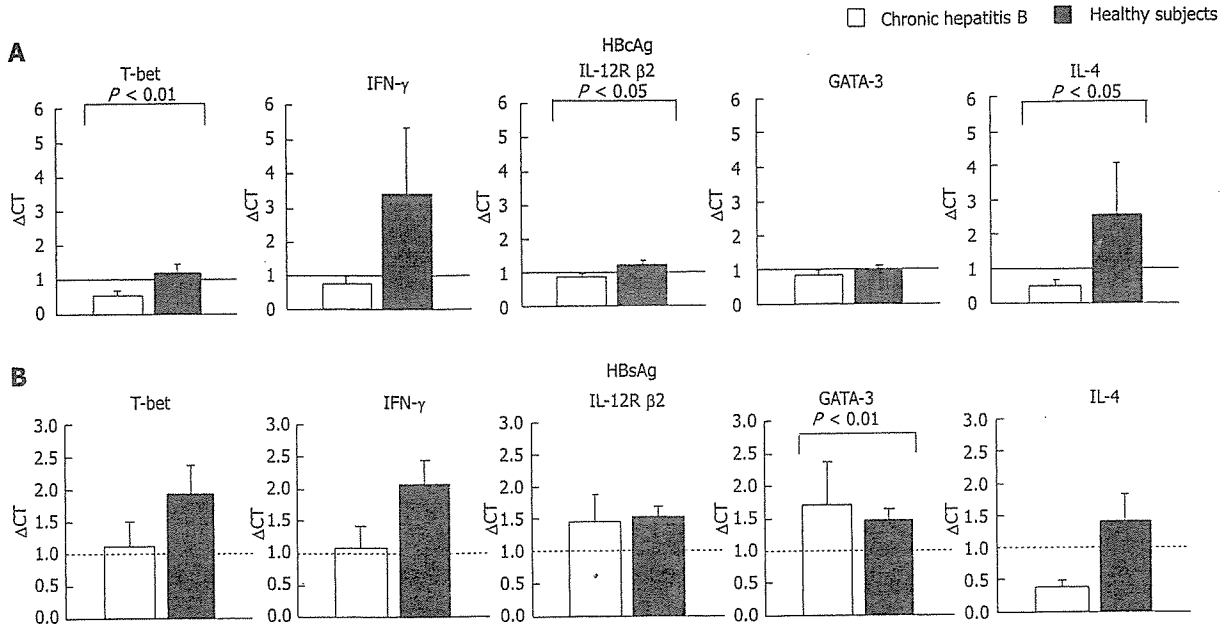

Figure 1 Comparison of levels of mRNAs for T-bet and GATA-3 after stimulation with HBsAg and HBcAg with mRNAs for IFN-gamma, IL-10 and IL-4. Total cellular RNA was extracted from $CD4^+$ T cells after the stimulation of PBMCs with HBcAg (10 μg/mL) or HBsAg (29 μg/mL) for 24 h. **A**: HBcAg stimulation; **B**: HBsAg stimulation. Levels of mRNA for T-bet, GATA-3, IFN-γ, IL-12R β2 and IL-4 were quantified by TaqMan PCR. GAPDH was used as an internal control. Relative amount of target mRNA was calculated using comparative CT method. The expression level of mRNAs of the non-stimulated sample in each subject is represented as 1.0 and relative amount of target mRNA in a stimulated sample was calculated using the as following formula: relative amount = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ was given by subtracting ΔC_T (non-stimulated cells) from ΔC_T (stimulated cells). The ΔC_T value was determined by subtracting the GAPDH C_T value from the target C_T value. The validation experiments were performed in advance for all the target mRNAs to demonstrate that efficiency of each target and GAPDH are approximately equal.

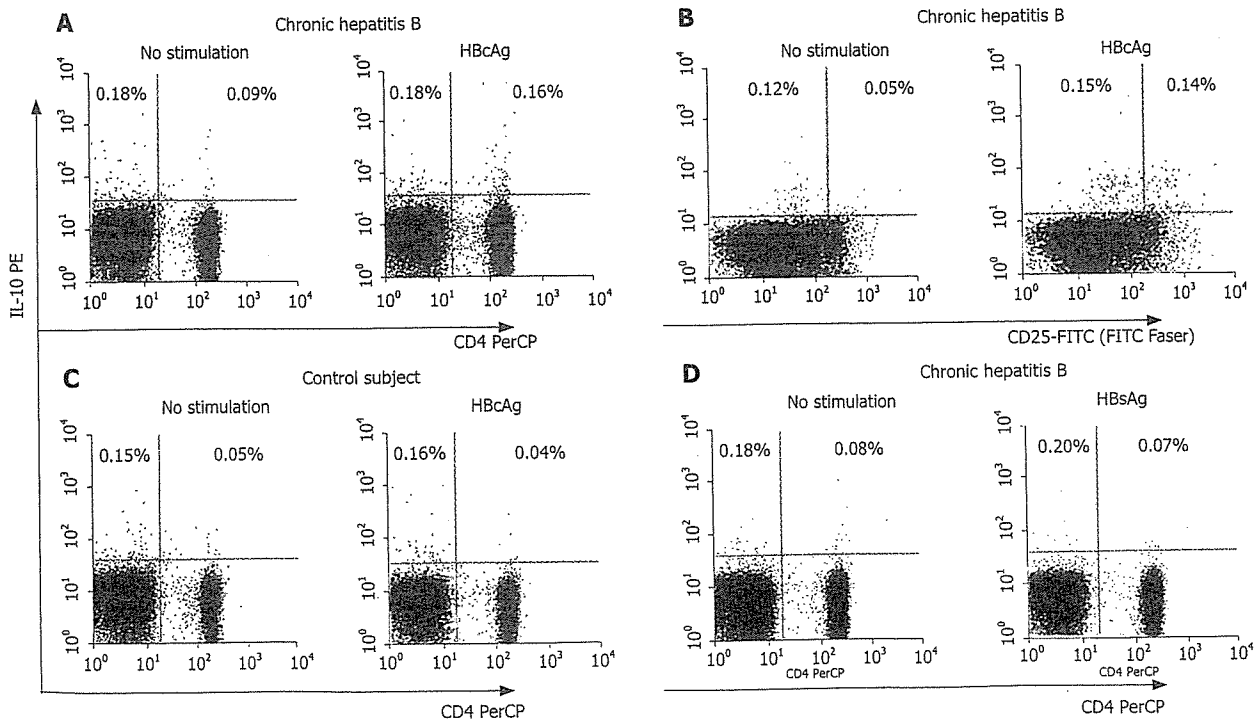

Figure 2 FACS analysis of HBcAg-specific production of IL-10 in patients with hepatitis B. Cellular source of HBcAg-specific production of IL-10 was identified by staining for IL-10-secretion (PE-labeled), anti-CD3-PerCP, anti-CD4-PerCP and anti-CD25-FITC. Representative dot plots of IL-10-secreting $CD4^+$ T cells in a patient with CHB (**A**) and IL-10-secreting $CD4^+CD25^{high}$ T cells in a patient with CHB (**B**). For the control, IL-10-secreting cells in a healthy subject with HBcAg stimulation (**C**) and in a patient with CHB with HBsAg stimulation (**D**) were also shown. Numbers shown in the dot plots indicate percentage of the cells in the quadrant region.

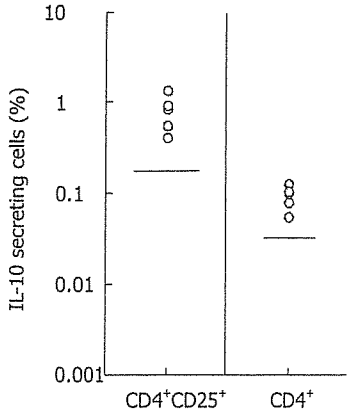

Figure 3 Increased populations of HBcAg-specific IL-10-producing $CD4^+$ or $CD4^+CD25^{high}$ T cells in patients with chronic hepatitis B. Population of IL-10 secreting cells in $CD4^+$ T cells and in $CD4^+CD25^+$ T cells was evaluated in patients with CHB. Frequencies of HBcAg-specific IL-10 secreting cells were calculated by subtracting percentage in non-stimulated samples from percentage in HBcAg-stimulated samples. Upper limits of normal subjects (mean ± 2SD of 5 subjects) were shown by straight lines in the plots (0.14% for $CD4^+CD25^+$ cells and 0.027% for $CD4^+$ cells). A FITC faser kit (BD Bioscience Pharmingen) was used in some experiments of ease separation of positive events by enhancing fluorescence intensity.

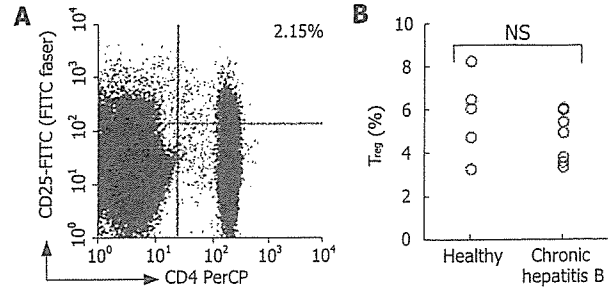

Figure 4 Comparison of $CD4^+CD25^{high}$ T cell population between patients with hepatitis B and healthy subjects. The cells that express CD4, $CD25^{high}$ and CTLA-4 were identified by flow cytometry. Representative dot plots of an *ex vivo* sample of a patient with CHB is shown (**A**), numbers shown in the dot plot indicates percentage of cells in the quadrant lesion. Percentage of $CD4^+CD25^+$ T cells was shown for patients with CHB and healthy subjects (**B**).

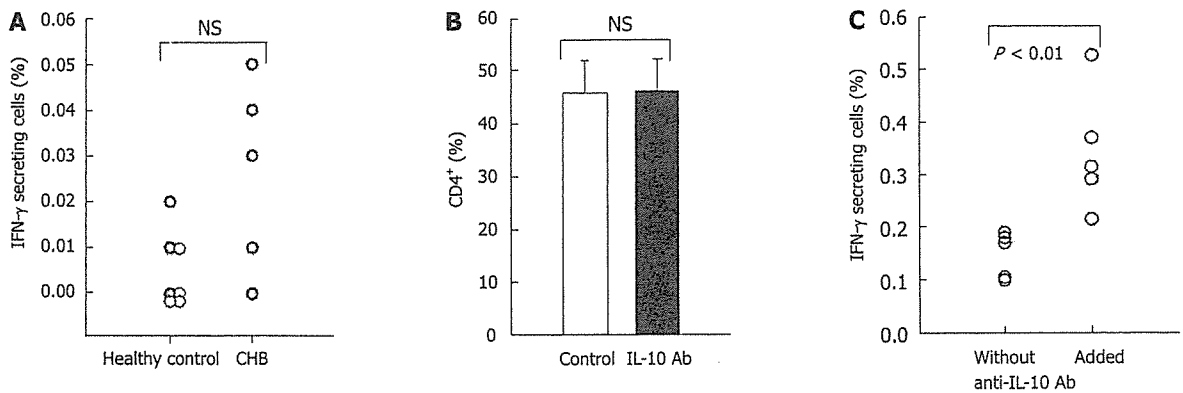

Figure 5 Addition of neutralizing anti-IL-10 antibody restores HBcAg-specific production of IFN-γ by $CD4^+$ in patients with hepatitis B. PBMCs obtained from 5 patients with CHB and 7 healthy subjects were stimulated with HBcAg (10 μg/mL) for 9 h and thereafter cells were stained for IFN-γ-secretion (PE) and anti-CD4-PerCP to determine the population of HBcAg-specific T_H1 being identified as IFN-γ^+ cells in $CD4^+$ T cells (**A**). Anti-IL-10 neutralizing antibody or isotype-matched control antibody were added to the culture during stimulation with HBcAg. The addition of anti-IL-10 antibody did not affect the percentage of $CD4^+$ T cells (**B**). In culture with anti-IL-10 antibody, numbers of HBcAg-specific T_H1 were significantly higher than those in culture with a control antibody (**C**).

antibody (0.15% ± 0.04 %, $P < 0.01$, Figure 5C).

T_{reg} depletion restores the response of IFN-γ-secreting $CD4^+$ T cells to HBcAg

Similar to the effect of anti-IL-10 antibody, depletion of T_{reg} induced the recovery of HBcAg-specific T_H1 response. T_{reg} were depleted by a $CD4^+CD25^+$ T cell separation kit (Figure 6A) and the cultures were reconstituted by mixing separated fractions. T_{reg}^- culture contained 0.5% (mean) of $CD4^+CD25^+$ cells on average, while T_{reg}^+ culture contained 3.5% of $CD4^+CD25^+$ cells on average (Figure 6B). The number of IFN-γ-secreting $CD4^+$ cells in response to HBcAg significantly increased in T_{reg}^- culture by 6-fold (0.03% ± 0.02%, mean ± SD of 9 cases) compared with that in T_{reg}^+ culture (0.18% ± 0.05%, $P < 0.01$, Figure 6C). Expression level of FOXP-3 and CTLA-4 was analyzed in 3 separate fractions to verify that $CD4^+CD25^+$ cells exhibited typical characteristics of T_{reg} cells. Fraction 3 ($CD4^+CD25^+$) expressed higher FOXP-3 than fraction 2 ($CD4^+CD25^-$) by 3.7 fold and fraction 1 ($CD4^-$) by 7.8 fold. The percentage of total CTLA-4 expression in fraction 1, fraction 2 and fraction 3 was 0.45%, 2.71% and 32.71% respectively.

DISCUSSION

The response of T cells to HBcAg has been reported to contribute to the resolution and seroconversion of HBV infection in chronic hepatitis B[31]. However, in the previous study we were unable to detect the recovery

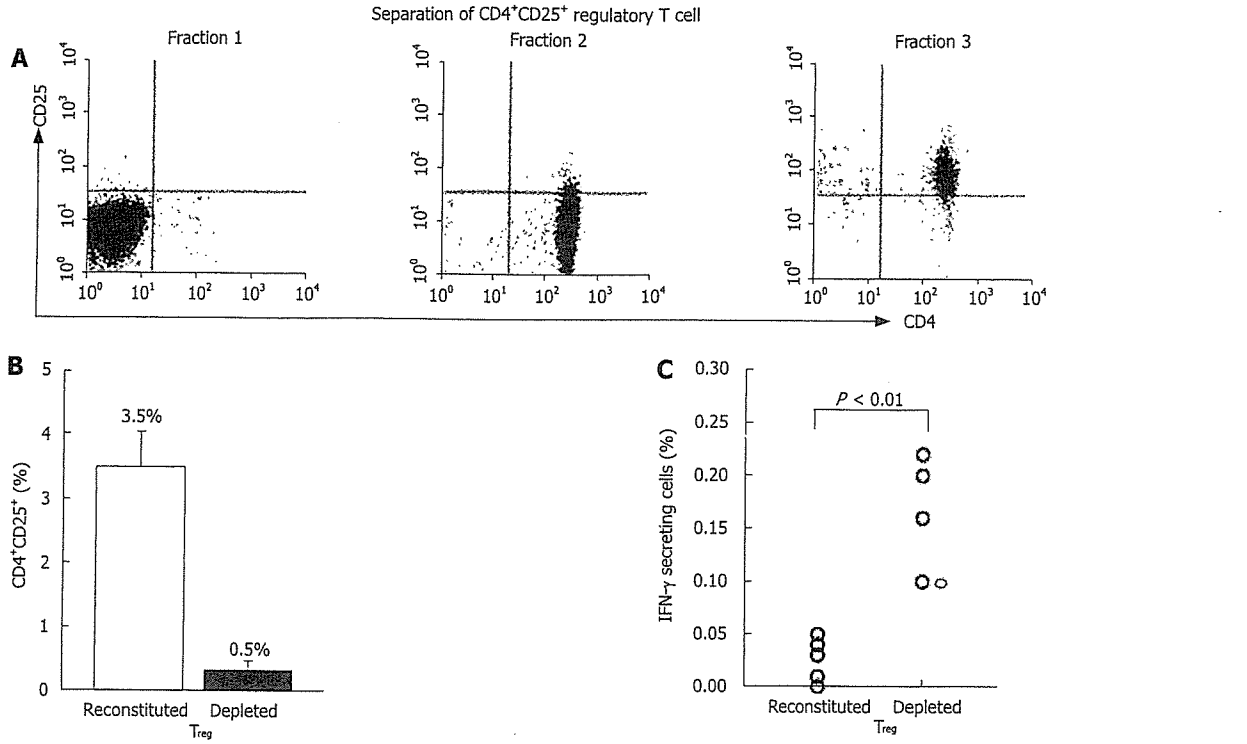

Figure 6 Depletion of $CD4^+CD25^+$ T cells from PBMCs increases HBcAg-specific production of IFN-γ in patients with hepatitis B. Using the differential expression of CD4 and CD25, cells were separated into 3 fractions; fraction 1 consisted of $CD4^-$ cells, fraction 2 consisted of $CD4^+CD25^-$ cells and fraction 3 consisted of $CD4^+CD25^+$ cells (**A**). Thereafter, 2 sets of lymphocyte preparations were reconstituted by remixing fractions 1, 2 and 3 or by remixing fractions 1 and 2 (**B**). They were stimulated with HBcAg to finally stain a $CD4^+IFN\text{-}\gamma^+$ population (**C**).

of HBcAg-specific TH1 despite the substantial increase in HBV-specific CTLs in patients receiving lamivudine therapy[11]. The results raised a question about the profound suppression of $CD4^+$ T cell response to HBV in patients with chronic hepatitis B. The current results showed that polarization of $CD4^+$ T cells was suppressed when the cells were stimulated with HBcAg in patients with chronic hepatitis B. The mechanisms underlying this suppression of $CD4^+$ T cells were through suppression of either direction to TH1 or TH2 by HBcAg stimulation, while HBsAg stimulation favored TH2 deviation in chronic hepatitis C.

It may be possible that Treg cells are one of the mediators of the suppression of TH1 response to HBcAg as suggested by the results of an increased population of IL-10-secreting $CD4^+CD25^{high}$ cells. This indicates the presence of an inducible Treg population which is specific for HBcAg and produces IL-10, as well as a natural Treg population in patients with CHB. However, the role of HBcAg is controversial, since it can induce IL-18, a monokine that stimulates T lymphocytes and macrophages to produce IFN-γ, in both healthy subjects and patients with chronic hepatitis B[32], and cause an increase in IL-10-producing T lymphocytes and monocytes *in vitro*[33]. Our data indicate lack of HBcAg-specific TH1 response in CHB patients, although the results of IL-18 are not available. Our study was conducted on a small scale with 9 patients and the hyporesponsiveness of HBV-specific T cells should be investigated in studies with larger populations.

Treg cells may be a common feature of immune suppression in chronic viral infection. In HIV infection, appearance of Treg in peripheral blood has been shown to have a suppressive role in CTL development against HIV antigen[34]. In patients with chronic hepatitis C, the evolution of inducible Treg cells specific for HCV antigens has been reported[35] and the presence of $CD8^+$ Treg cells homing to suppress local inflammation in the liver has also been reported in HCV infection[36]. Thus Treg cells may have diverse effects during chronic viral infection; suppression of cellular immune response to eliminate the virus and the suppression of unfavorable tissue damage by the cellular immune response to the virus[37]. In addition, there has been a report of different clinical features in patients with chronic hepatitis C, namely a higher prevalence of cryoglobulinemia in patients with lower Treg cells[38]. Although natural Treg population may also contribute to the suppression of $CD4^+$ T cell response from the results of $CD4^+CD25^+$-depletion, the population of $CD4^+CD25^{high}$ T cells *ex vivo* was not different between normal subjects (5.73% ± 1.87%) and patients with chronic hepatitis B (4.73% ± 1.15%) similar to the results of Franzese *et al*[39], while Stoop *et al* have reported the increased Treg population in peripheral blood of patients with CHB[40]. The change in Treg population and its contribution to pathogenesis needs to be evaluated by comparing various HBV diseases.

Manipulation of activity of Treg cells specific for HBcAg may become one of the potent options in future therapy. An immunomodulating approach, which is indicated by successful use of GITR (glucocorticoid-

induced TNF-alpha receptors) to suppress activity of T_{reg} cells[41], may become beneficial in patients with CHB.

In summary, this report demonstrates underlying mechanisms of suppression of immune responses to HBcAg in chronic HBV infection. A therapeutic approach to the molecules or cell types involved in these mechanisms may contribute to the improvement of prognosis in patients with chronic hepatitis caused by persistent replication of HBV.

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S- Editor Wang J **L- Editor** Alpini GD **E- Editor** Bai SH

Journal of General Virology (2006), 87, 1175–1180 DOI 10.1099/vir.0.81525-0

Short Communication

Analysis of the entire nucleotide sequence of hepatitis B virus genotype B in the Philippines reveals a new subgenotype of genotype B

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The entire nucleotide sequences were determined for hepatitis B virus (HBV) genotype B (HBV/B) genomes extracted from five patients in the Philippines and designated GenBank AB219426, AB219427, AB219428, AB219429 and AB219430. The serotype of the first four isolates was *ayw* and that of GenBank AB219430 was *adw*. Divergences of entire sequences were 1·0–2·0 % between the first four isolates and 3·8–4·2 % between these four and GenBank AB219430. Phylogenetic-tree analysis revealed that, worldwide, HBV/B comprises five subgenotypes: B1, B2, B3, B4 and the new Philippines group, designated B5. Divergences of the entire genome sequences between four isolates in subgenotype B5 and isolates from other countries (subgenotypes) were 4·4–4·8 % with Vietnam (B4), 2·9–3·5 % with Indonesia (B3), 4·7–5·1 % with China (B2) and 5·4–6·0 % with Japan (B1). Similarly, GenBank AB219430 showed the lowest divergences: 3·4 % with the isolate from Indonesia (B3), 5·0 % with Vietnam (B4), 5·4 % with China (B2) and 6·1 % with Japan (B1). This is the first report of entire nucleotide sequences of HBV/B from the Philippines and the results show that these sequences belong to a new subgenotype, B5. The present study identified that HBV/B isolates throughout the world are divided genetically into five subgenotypes, the relationships between geographical distances and the genetic distances of HBV/B being well-correlated.

Received 13 September 2005
Accepted 6 January 2006

Hepatitis B virus (HBV) is an aetiological agent of liver cirrhosis and hepatocellular carcinoma and it remains a major health problem worldwide, especially in Asian countries (Lee, 1997). The subtypes of HBV have been determined serologically by the antigenic determinants of the HBV surface antigen (HBsAg), common determinant *a* and two mutually exclusive determinant pairs, *d/y* and *w/r*. As subtype, i.e. serotype, is mainly defined by a limited number of amino acids, one point mutation in the HBsAg gene could change the serotype in a patient (Okamoto *et al.*, 1987). Therefore, serotyping has its limitations for the classification of HBV.

In 1988, Okamoto and colleagues proposed classifying HBV according to genotype, the genetic classification of HBV based on the comparison of entire genomes, and found an intergroup divergence of 8 % or more (Okamoto *et al.*, 1988). As the classification of HBV by genotype is based on full genome sequences, it is more reliable.

Eight different genotypes of HBV, named A–H, have been determined to date (Okamoto *et al.*, 1988; Norder *et al.*, 1992; Stuyver *et al.*, 2000; Arauz-Ruiz *et al.*, 2002). The distribution of genotypes throughout the world has been confirmed, with genotype A being predominant in North

The GenBank/EMBL/DDBJ accession numbers for the complete-genome sequences described in this paper are AB219426–AB219430.

A table showing a comparison of nucleotide similarities in the precore and core gene of the present five isolates from the Philippines and representative HBV/B isolates from four other subgenotypes, B1–B4, with isolates from HBV/C and B1 is available in JGV Online.

Africa and north-western Europe, genotype B in South-East Asia, genotype C in North-East Asia, genotype D in northern Europe and the Middle East, genotype E in Africa, genotype F in South America and genotype G in France, Germany, the USA and Mexico (Magnius & Norder, 1995; Lindh *et al.*, 1997; Stuyver *et al.*, 2000). Genotype H was recently reported to be detected in South and Central America (Arauz-Ruiz *et al.*, 2002).

There have been some reports that clinical outcomes vary with HBV genotypes. For example, for genotypes B and C, which are characteristic of Asia, genotype B has been found to cause HBe seroconversion more frequently than genotype C, and those infected with HBV/B appear to have better prognoses (Sunitha *et al.*, 2000).

The division of HBV/B into four subgenotypes, B1, B2, B3 and B4, based on the sequence divergence, has recently been reported (Norder *et al.*, 2004). Ba (B2, B3 and B4) has recombined with genotype C and has a higher prevalence of HBe antigen, so it manifests a more severe clinical course than Bj (B1) (Sugauchi *et al.*, 2002). Thus, HBV needs to be examined with regards not only to its genotype, but also to its subgenotypes.

The Philippines are located in South-East Asia, where HBV is hyperendemic. It is reported that about 10 % of the population of the Philippines are HBV carriers, and that chronic HBV infection is a major health problem (Lingao *et al.*, 1989), although to our knowledge, few data on HBV genotype distribution in the country are available. We obtained sera containing HBV isolates from carriers in the Philippines and investigated their features.

The serum samples were provided by St Luke's Medical Center, Quezon City, the Philippines. Quezon City has a population of about 2 100 000 and is the biggest of the cities in the Metro Manila, the capital of the Republic of the Philippines. The patients were HBV carriers being followed at St Luke's. All patients were negative for antibody to *Hepatitis C virus* and human immunodeficiency virus and were not intravenous drug abusers. The five patients were a 30-year-old male, a 48-year-old female, a 21-year-old male, a 60-year-old male and a 54-year-old male. They were all positive for HBe antigen and negative for anti-HBe. We explained the purpose of this study and collected serum samples with informed consent.

For amplification of HBV DNA by PCR, nucleic acids were extracted from 200 µl serum as described previously (Niitsuma *et al.*, 1995). For analysis of the entire nucleotide sequence, we divided the entire HBV genome into six overlapping segments and amplified each segment. Extracted DNA was subjected to the first round of PCR with each set of primers. PCR was performed with TaKaRa Ex *Taq* for 35 cycles (consisting of denaturation for 1 min at 93 °C, annealing for 1 min at 55 °C and extension for 1 min at 74 °C), followed by an extension cycle at 74 °C for 8 min. The second round of PCR was carried out for 30 cycles consisting of the same protocol as those in the first round. The primers for the first and the second PCR rounds were as reported previously (Shan *et al.*, 2002).

We used the standard numbering system in this report, the numbering of the bases commencing at the cleavage site for the restriction enzyme *Eco*RI in the preS2 region and the full length of 3215 bp being counted.

We used each set of sequencing primers for nucleotide sequences of HBV isolates shown previously (Shan *et al.*, 2002). Direct sequencing of the PCR products was carried out by a fluorescence autosequencer (model 377; Perkin Elmer Applied Biosystems), using a BigDye Terminator Sequencing kit (Perkin Elmer Applied Biosystems) according to the manufacturer's instructions. Six overlapping segments were joined and phylogenetic analysis of the sequences of HBV clones was performed by the neighbour-joining method with the aid of CLUSTAL W (http://www.ddbj.nig.ac.jp/search/clustalw-j.html).

We compared the clones determined in our present study with eight reported HBV clones and confirmed their genotype to be B by phylogenetic analysis. The names of clones and the genotypes of their HBV sequences used in the

Table 1. Percentage divergences of entire nucleotide sequences among HBV/B isolates from five subgenotypes

HBV/B subgenotype	GenBank accession no.	Country of origin	1	2	3	4	5	6	7	8	9
1. B5	AB219426	Philippines	–	1·5	1·5	1·0	3·8	4·5	3·0	4·9	5·6
2. B5	AB219427	Philippines		–	2·0	1·7	4·2	4·7	3·5	5·1	6·0
3. B5	AB219428	Philippines			–	1·7	3·8	4·4	2·9	4·7	5·4
4. B5	AB219429	Philippines				–	4·0	4·8	3·3	5·0	5·8
5. B3	AB219430	Philippines					–	5·0	3·4	5·4	6·1
6. B4	AB031267	Vietnam						–	4·2	4·0	5·0
7. B3	AB033554	Indonesia							–	4·8	5·4
8. B2	AF282917	China								–	4·4
9. B1	D00329	Japan									–

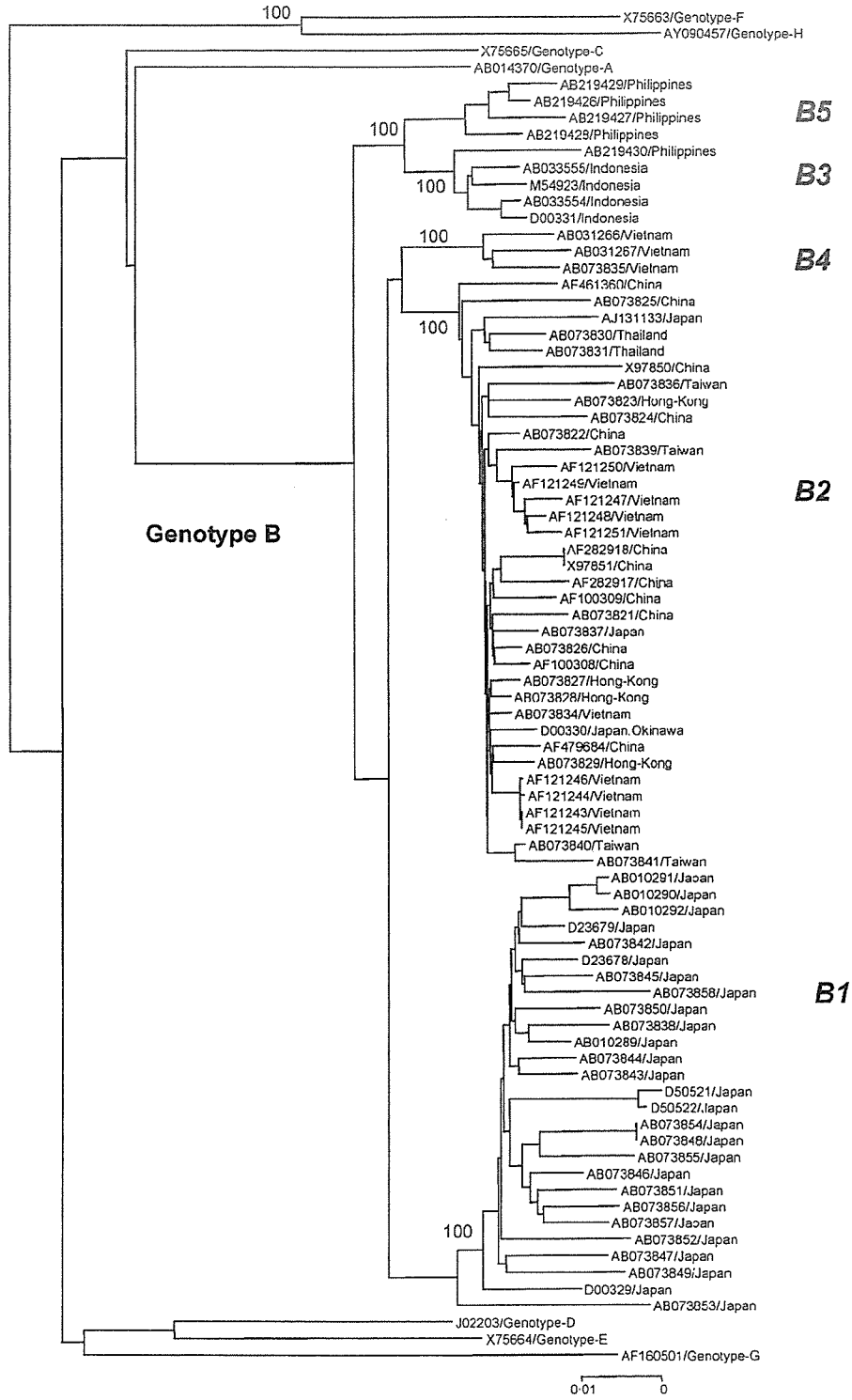

Fig. 1. Phylogenetic tree of entire nucleotide sequences constructed by the neighbour-joining method using the present five isolates, HBV/B isolates and other HBV genotype (A, C, D, E, F, G and H) isolates retrieved from DDBJ/GenBank. Bootstrap values are indicated for each group.

analysis were as follows: GenBank AB014370 (genotype A), X97850 (genotype B), X75665 (genotype C), J02203 (genotype D), X75664 (genotype E), X75663 (genotype F), AF160501 (genotype G) and AY090457 (genotype H).

We used four representative clones from four subgenotypes of HBV/B, i.e. B1–B4, in addition to the present HBV/B clones from the Philippines for the study of the divergences in the entire sequences among the subgenotypes of HBV/B in Asian countries. The GenBank accession numbers of HBV sequences and countries from which they were derived were as follows: D00329 (Japan, B1); AF282917 (China, B2); AB033554 (Indonesia, B3); and AB031267 (Vietnam, B4).

We compared the precore and core sequences of our five clones from the Philippines and four previous isolates from subgenotypes B1–B4 with HBV/C and HBV/B1 by following the method of Sugauchi *et al.* (2002, 2003). The GenBank accession numbers and the countries from which they were derived are as follows: HBV/C, AB014378 (Japan) and D23684 (Japan); HBV/B1, AB073845 (Japan) and AB073855 (Japan).

Entire nucleotide sequences of five HBV/B isolates from the Philippines were determined. They were registered with DDBJ/GenBank under the accession numbers AB219426, AB219427, AB219428, AB219429 and AB219430.

Serotypes were determined from aa 122 and 160 in the HBs gene. GenBank AB219430 was found to be of serotype *adw* and AB219426, AB219427, AB219428 and AB2194294 were of serotype *ayw*.

We constructed a phylogenetic tree by using the entire nucleotide sequences determined from the five isolates, other HBV/B isolates retrieved from DDBJ/GenBank and additional representative genotypes (A, C, D, E, F, G and H). The HBV isolates are illustrated in Fig. 1, the HBV/B isolates detected worldwide being divided into five groups: subgenotypes B1, B2, B3, B4 and the Philippines group. We suggest the latter to be a new HBV/B subgenotype, B5. Among the present five isolates, four isolates belonged to subgenotype B5 and the other was subgenotype B3.

Divergences in the entire genome sequences of the present five isolates with HBV/B subgenotype isolates registered with GenBank are shown in Table 1. The four isolates in subgenotype B5 showed 1·0–2·0 % divergence from each other. Among the sequences registered with GenBank, AB033554 (subgenotype B3, serotype *adw*) from Indonesia showed the lowest divergences with the four isolates, 2·9–3·5 %. These four isolates showed divergences of 3·8–4·2 % with GenBank AB219430 and, similarly, 4·4–4·8 % with Vietnam (B4), 4·7–5·1 % with China (B2) and 5·4–6·0 % with Japan (B1).

On the other hand, similar examination showed that GenBank AB219430 has the lowest divergence, i.e. 3·4 %, with AB033554 (B3). Furthermore, GenBank AB219430 showed 5·0 % divergence from isolates from Vietnam (B4), 5·4 % with China (B2) and 6·1 % with Japan (B1).

In addition, we constructed a phylogenetic tree by using the partial nucleotide sequences (369 bp in the HBs region) determined from 30 HBV/B isolates from the Philippines (Fig. 2). As for the four HBV/B subgenotypes, they were not as obvious in this tree as in that constructed from entire nucleotide sequences. To the contrary, the 17 isolates constructed an obvious subgroup with the serotype *ayw*, with one exception, which is characteristic for B5.

Similar to the study reported by Sugauchi *et al.* (2002), a comparison of nucleotide similarities from positions 1814 to 2452 in the precore and core gene of the present five isolates from the Philippines and representative HBV/B isolates from the other four subgenotypes, B1–B4, with isolates from HBV/C and B1 is shown in Supplementary Table S1 in JGV Online. The divergences confirmed that HBV/B in the Philippines had recombined with HBV/C in the precore and core gene, like in B2, B3 and B4. In this study, we determined the entire nucleotide sequences of five HBV/B isolates from the Philippines, where data on HBV have been scant.

We showed that HBV/B isolates from all over the world, including the Philippines, can be divided into five subgroups in the phylogenetic tree based on entire nucleotide sequences, whose geographical distances and genetic distances are well-correlated. There were long genetic distances among the subgroups, i.e. subgenotypes. In general, subgenotypes vary by >4·0 % at the nucleotide level over the entire genome, as described by Norder *et al.* (2004) and Kramvis *et al.* (2005). In our study, the divergence of the entire-genome nucleotide sequences between GenBank AB219427 (B5) and D00329 (B1) was 6·0 %, that with AF282917 (B2) was 5·1 %, that with AB033554 (B3) was 3·5 % and that with AB031267 (B4) was 4·7 %.

We suggest that the subgroup composed of four HBV/B isolates from the Philippines is a new subgenotype, B5, for the following three reasons. First, a significant bootstrap value was confirmed on the phylogenetic-tree analysis. Second, the divergences between B5 and the other four subgenotypes, B1, B2, B3 and B4, were nearly 4 %. Third, as for the serotypes, B3 was *adw* and B5 was *ayw*. We confirmed an additional 25 HBV clones in the Philippines to be genotype B from the sequence analysis of 369 bp (nt 278–646) in the HBs region. Phylogenetic-tree analysis of partial nucleotide sequences also showed the subgenotype B5. Seventeen clones among the 30 isolates, including the current four isolates, belonged to B5. Their serotypes were *ayw* with one exception (*adw*). This may be derived from just one point mutation in the HBsAg region. HBV infection is known to show clinical differences among genotypes. Therefore, these five HBV/B subgenotypes might show clinical differences, as previously reported for B1 (Bj) and B2 (Ba) (Orito *et al.*, 2001).

This is the first report of the entire nucleotide sequences of HBV/B from the Philippines, where there have been scant data on HBV. It shows that they belong to a novel HBV/B

Fig. 2. Phylogenetic tree of partial nucleotide sequences (nt 278–646 in the HBs region) constructed by the neighbour-joining method using the present 30 isolates, HBV/B isolates and other HBV genotype (A, C, D, E, F, G and H) isolates retrieved from DDBJ/GenBank. The 25 isolates are described as PHL-number-serotype. Isolates classified in different subgroups on the basis of phylogenetic analysis by entire and partial nucleotide sequences are marked with an asterisk.

subgenotype, B5. Further studies, including other genotypes and their clinical relevance, should be conducted.

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Hepatology Research 34 (2006) 104–110
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Th1 response during ribavirin and interferon-α combination therapy in chronic hepatitis C

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Received 29 October 2004; received in revised form 4 March 2005; accepted 9 November 2005
Available online 27 December 2005

Abstract

Ribavirin and interferon-α induce Th1 polarization of human CD4+ T cells. The study was conducted to investigate the whether cellular immune response during ribavirin/interferon-α therapy is associated with viral eradication by examining mRNA expression of molecules relevant to Th1 and Th2 polarization in CD4+ cells of 13 patients with chronic hepatitis C (seven patients with sustained viral response and six with transient response). Peripheral CD4+ T lymphocytes at 0, 4 and 24 weeks of treatment were tested. There were no significant differences in the mRNA levels at each point of time of the treatment between patients with sustained viral response and those with transient response. The percent increase in mRNA level of the IL-12R β2 chain from the baseline to the end of the treatment was significantly higher in patients with sustained viral response (15.3 ± 6.1%) than in those with transient response (−1.6 ± 4.7%, $p < 0.05$). There was no significant difference in percent changes in level of IL-12R β1 chain mRNA between the two groups. In conclusion, the results of this study indicate that the increase of Th1 response is related to the inflammatory activity in the liver and possibly to ribavirin and interferon-α therapy. It is also suggested that the measurement of Th1 response has the potential to distinguish patients with relapse from those with sustained virus response.

Keywords: CD4+ T-lymphocyte; Chronic hepatitis C; Interferon-γ; Interle3ukin-12 receptor; Ribavirin

1. Introduction

Ribavirin, a synthetic guanosine nucleoside analogue, is widely used for the treatment of chronic hepatitis C in combination with interferon (IFN)-α [1]. Combination therapy with ribavirin and IFN-α has been shown to have a higher response rate both at the end of treatment and at the end of follow-up when compared with IFN-α monotherapy [2,3]. However, there are two types of viral response after the loss of serum HCV RNA during treatment, that is, sustained virus loss and relapse of viral replication after withdrawal of treatment, the latter of which may be due to incomplete immune-mediated removal of infected cells [4–6].

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doi:10.1016/j.hepres.2005.11.002

Table 1
Baseline characteristics of the 13 patients with chronic hepatitis C

Characteristics	Total	SVR	REL	*p*
N	13	7	6	
Gender (male/female)	11/2	6/1	5/1	NS
Age in years, median (range)	41 (20–62)	31 (20–54)	51 (41–62)	<0.05
Source or type of infection, *n* (%)				
Blood transfusion	3	1	2	
Tattoo	0	0	0	NS
Sporadic, or unknown	10	6	4	
ALT IU/l, median (range)	85 (54–608)	85 (54–608)	114 (77–275)	NS
HCV genotype				
Genotype 1	6	1	5	<0.05
Genotype 2 + 3	7	6	1	
HCV RNA kcopies/ml, median (range)	350 (260–>850)	350 (260–>850)	250 (250–650)	NS
Fibrosis score				
0	4	3	1	
1	1	1	0	NS
2	2	0	2	
3	1	0	1	
4	1	1	0	
Undetermined	4	2	2	
Activity score				
1	3	3	0	
2	6	2	4	NS
Undetermined	4	2	2	

Development of an HCV-specific Th1 response is observed with the resolution of acute infection with virus clearance [7,8], while loss of the Th1 response is associated with the development of chronic infection [9]. Therefore, it is expected that failure to develop an effective cellular immune response during ribavirin/IFN-α treatment in chronic HCV infection may be associated with viral persistence.

In previous studies in vitro, we have shown that both ribavirin and IFN-α may have a modulating effect on the Th1 polarization of human peripheral CD4+ T cells through the up-regulation of mRNA for the interleukin (IL)-12 receptor β2 chain (IL-12R β2 chain) in patients with hepatitis C and the normal volunteers [10,11]. To investigate whether such cellular immune response during combination therapy of ribavirin and IFN-α may be associated with sustained viral response, we compared the expression of mRNA for molecules, which are relevant to the development of Th1 and Th2 in CD4+ T cells ex vivo between patients with sustained viral response and those with transient viral response.

2. Patients and methods

2.1. Patients and treatment

The study included 13 patients with serologically (anti-HCV antibodies and HCV RNA positive) and histologically demonstrated chronic hepatitis C. Informed consent was obtained from each subject included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institution's human research committee. The patients entered in the study received recombinant IFN-α at a dose of 6 or 10 mU daily for the first 2 weeks and thrice weekly for the subsequent 22 weeks. They were also received ribavirin at a dose of 600 mg/day (300 mg twice a day) for patients weighing <60 kg and 800 mg/day (400 mg twice a day) for patients weighing >60 kg. Further characteristics of the patients are listed in Table 1. As control subjects, 15 healthy individuals were also included in the study (six males and nine females, age 48.9 ± 9.7 years old). Samples for analysis of mRNA level were obtained just before the beginning of treatment (W0), at 4 weeks of the treatment (W4) and at the end of the treatment (W24). After sampling, the blood was immediately processed for separation of peripheral blood mononuclear cells (PBMC) separation (as described below).

2.2. Preparation of PBMC and extraction of cellular RNA

PBMC were isolated from patients by gradient centrifugation over Ficoll Hypaque. PBMC (1×10^6 cells in a volume of 500 μl) were resuspended in phosphate buffered saline (PBS). Using anti-CD4 antibody coated magnetic beads (Dynabeads CD4, Dynal Biotec, Oslo, Norway), CD4+ lymphocytes were separated according to the manufacturer's instructions. For the measurement of cytokine mRNA level in CD4+ cells, total RNA was isolated using Isogen (Nippon Gene, Toyama, Japan). cDNA was synthesized from