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

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AIM: To study the mechanisms of hyporesponsiveness of HBV-specific CD4+ T cells by testing T\(_{\text{h}1}\) and T\(_{\text{h}2}\) 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\(_{\text{h}1}\) and T\(_{\text{h}2}\) 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 \(\beta\)2 and IL-4 was significantly lower in the patients than in healthy subjects with HBCag stimulation. Although populations of CD4+CD25+ Foxp3+ 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\(_{\text{h}1}\) antibody compared with control antibody (\(P < 0.01, 0.34\% \pm 0.12\% vs 0.15\% \pm 0.04\%\)). Deletion of CD4+CD25+ T cells increased the amount of HBCag-specific T\(_{\text{h}1}\) antibody when compared with lymphocytes reconstituted using regulatory T cells (\(P < 0.01, 0.03\% \pm 0.02\% vs 0.18\% \pm 0.05\%\)).

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\(_{\text{h}2}\)-committed cells in response to HBsAg.

Key words: Hepatitis B virus; Regulatory T cells; IL-10; FOXP3; T\(_{\text{h}1}\)

INTRODUCTION

Hepatitis B virus (HBV) is a noncytopathic DNA virus which causes chronic hepatitis and hepatocellular carcinoma as well as acute hepatitis\(^8\). 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\(^8\). 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\(^6\). However, previous reports have indicated that HBV-specific T cells restored by lamivudine treatment are insufficient to completely suppress HBV replication\(^9\).

In our previous study, we observed a defect in recovery of HBCag-specific T\(_{\text{h}1}\) cells despite restoration of CTLs, although they showed limited functions\(^10\). Since type 1 helper T (T\(_{\text{h}1}\)) cells are believed essential for immunity against intracellular pathogens\(^11\), 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 T1 and T2 commitment. The critical transcription factors for commitment of T cells to the T1 or T2 pathway are T-bet or GATA-3 respectively. 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 T1/T2 commitment in chronic hepatitis B.

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

In this study we examined the mechanisms of hyporesponsiveness of HBV-specific CD4<sup>+</sup> T cells by evaluating the T1/T2 commitment and activity of Treg 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 (Inhibition %) | DNA (LGE/mL) | 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 HBeAg 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<sup>5</sup> cells/mL in complete medium in the presence of HBsAg (29 µg/mL) or HBeAg (10 µg/mL) for 24 h. Thereafter, CD4<sup>+</sup> cells (4 × 10<sup>5</sup> cells) were separated from the stimulated PBMCs using anti-CD4-coated magnetic beads (Dynalab M-450 CD4, Dynal, Oslo, Finland) for quantification of mRNAs.

#### Quantified real time PCR

Total cellular RNA was extracted from CD4<sup>+</sup> 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 DNA 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, Foster City, CA).
IL-10 and IFN-gamma secretion assay

Purified PBMCs were stimulated at $1 \times 10^7$ cells/mL in complete medium with or without HBcAg (10 μg/mL) for 9 h at 37°C. 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°C) and further incubated in a closed tube for 45 min at 37°C under slow continuous rotation. Cells were washed and IL-10- or IFN-gamma-secreting cells were stained by adding 10 μL of IL-10- or IFN-gamma-Detection Antibody (PE-conjugated) together with anti-CD4-PerCP and anti-CD25-FITC. In some experiments, FITC fluorescence was amplified by FASCER kit-FITC (Miltenyi Biotec). Selected samples were stained with anti-CD14-FITC, anti-CD3-PerCP, anti-CD25-FITC, anti-HLA-DR-APC (BD Biosciences). Cells were analyzed by FACSCalibur.

To assess the effects of IL-10 on the HBcAg-specific IFN-gamma production by CD4+ T cells, PBMCs were stimulated at $1 \times 10^7$ 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°C. Cells were then used for IFN-gamma-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 Biosciences) after cell surface markers including CTLA-4 were stained. Subsequently, intracellular CTLA-4 was stained and the cells were analyzed by FACSCalibur.

Depletion of Treg 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-gamma production, 2 sets of lymphocyte preparations were reconstituted. The first set, designated as Treg, was the mixture of all three fractions and contained 5%-7% CD4+CD25+ cells. The second set, designated as Treg, was a 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 one-way 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 T1/T2 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).

IL-10 secreting cells in response to HBCAg were enriched in CD4+CD25+ lymphocytes

Involvement of the suppressive cytokine IL-10 in suppression of T1/T2-commitment 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-secreting CD4+ T cells was readily detectable in patients with CHB (Figure 2A) and these IL-10 secreting cells in CD4+ T cells showed CD25high 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+CD25high 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+CD25high cells (Figure 3). Our next question was whether Treg cells increased in number or were induced by HBcAg stimulation. Therefore, the population of CD4+CD25high 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 T1 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 T1 responses to HBcAg stimulation, the effect of anti-IL-10 antibody on T1 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
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 HBoAg (10 µg/mL) or HBsAg (29 µg/mL) for 24 h. A: HBoAg 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 the following formula: relative amount = 2^ΔCT, where ΔΔCT was given by subtracting ΔCT (non-stimulated cells) from ΔCT (stimulated cells). The ΔCT value was determined by subtracting the GAPDH Ct value from the target Ct 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-secreting (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+CD25high 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.
antibody (0.15% ± 0.04%, P < 0.01, Figure 5C).

**Treg depletion restores the response of IFN-γ-secreting CD4+ T cells to HBeAg**

Similar to the effect of anti-IL-10 antibody, depletion of Treg induced the recovery of HBeAg-specific T cell response. Treg were depleted by a CD4+CD25+ T cell separation kit (Figure 6A) and the cultures were reconstituted by mixing separated fractions. Treg culture contained 0.5% (mean) of CD4+CD25+ cells on average, while Treg+ culture contained 3.5% of CD4+CD25+ cells on average (Figure 6B). The number of IFN-γ-secreting CD4+ cells in response to HBeAg significantly increased in Treg culture by 6-fold (0.03% ± 0.02%, mean ± SD of 9 cases) compared with that in Treg+ 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 Treg 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 HBeAg has been reported to contribute to the resolution and seroconversion of HBV infection in chronic hepatitis B. However, in the previous study we were unable to detect the recovery
of HBcAg-specific T\(\gamma\)1 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 T\(\gamma\)1 or T\(\gamma\)2 by HBcAg stimulation, while HBsAg stimulation favored T\(\gamma\)2 deviation in chronic hepatitis C.

It may be possible that T\(\gamma\)reg cells are one of the mediators of the suppression of T\(\gamma\)1 response to HBcAg as suggested by the results of an increased population of IL-10-secreting CD4\(^+\)CD25\(^{\text{hi}}\) cells. This indicates the presence of an inducible T\(\gamma\)reg population which is specific for HBcAg and produces IL-10, as well as a natural T\(\gamma\)reg 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-\(\gamma\), in both healthy subjects and patients with chronic hepatitis B\(^{[32]}\), and cause an increase in IL-10-producing T lymphocytes and monocytes \textit{in vivo}\(^{[33]}\). Our data indicate lack of HBcAg-specific T\(\gamma\)1 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.

T\(\gamma\)reg cells may be a common feature of immune suppression in chronic viral infection. In HIV infection, appearance of T\(\gamma\)reg cells in peripheral blood has been shown to have a suppressive role in CTL development against HIV antigen\(^{[4]}\). In patients with chronic hepatitis C, the evolution of inducible T\(\gamma\)reg cells specific for HCV antigens has been reported\(^{[34]}\) and the presence of CD8\(^+\) T\(\gamma\)reg cells homing to suppress local inflammation in the liver has also been reported in HCV infection\(^{[35]}\). Thus T\(\gamma\)reg 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 T\(\gamma\)reg cells\(^{[38]}\). Although natural T\(\gamma\)reg population may also contribute to the suppression of CD4\(^+\) T cell response from the results of CD4\(^+\)CD25\(^{\text{deple}}\) depletion, the population of CD4\(^+\)CD25\(^{\text{hi}}\) T cells \textit{ex vivo} was not different between normal subjects (5.73\% \(\pm\) 1.87\%) and patients with chronic hepatitis B (4.73\% \(\pm\) 1.15\%) similar to the results of Franzese \textit{et al}\(^{[39]}\), while Stoop \textit{et al} have reported the increased T\(\gamma\)reg population in peripheral blood of patients with CHB\(^{[40]}\). The change in T\(\gamma\)reg population and its contribution to pathogenesis needs to be evaluated by comparing various HBV diseases.

Manipulation of activity of T\(\gamma\)reg 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 Treg cells[31] may be 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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