High frequency of circulating HBcAg-specific CD8 T cells in hepatitis B infection: a flow cytometric analysis

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SUMMARY
Viral antigen-specific T cells are important for virus elimination. We studied the hepatitis B virus (HBV)-specific T cell response using flow cytometry. Three phases of HBV infection were studied: Group A, HBeAg (+) chronic hepatitis; Group B, HBeAb (+) HBV carrier after seroconversion; and Group C, HBsAb (+) phase. Peripheral T cells were incubated with recombinant HB core antigen (HBcAg), and intracytoplasmic cytokines were analysed by flow cytometry. HBcAg-specific CD4 and CD8 T cells were identified in all three groups and the number of IFN-γ-positive T cells was greater than TNF-α-positive T cells. The frequency of IFN-γ-positive CD4 and CD8 T cells was highest in Group C, compared with Groups A and B. No significant difference in the HBcAg-specific T cell response was observed between Group A and Group B. The HBcAg-specific CD8 T cell response was diminished by CD4 depletion, addition of antibody against human leucocyte antigen (HLA) class I, class II or CD40L. Cytokine-positive CD8 T cells without HBcAg stimulation were present at a high frequency (7 of 13 cases) in Group B, but were rare in other groups. HBcAg-specific T cells can be detected at high frequency by a sensitive flow cytometric analysis, and these cells are important for controlling HBV replication.

Keywords flow cytometry hepatitis B core antigen hepatitis B virus immune response intracytoplasmic cytokine T cell

INTRODUCTION
Hepatitis B virus (HBV) is a DNA virus that causes acute and chronic infection in humans [1]. Although HBV itself is non-cytopathic, chronic infection results in liver cirrhosis and hepatocellular carcinoma [2]. HBV is usually cleared by the immune response to viral antigens in adults. Vigorous helper T cell and cytotoxic T cell (CTL) responses have been demonstrated in patients who cleared HBV in acute infection. Strong CTL responses have been shown against the epitopes in HB core antigen as well as surface antigens [3,4]. The immune response to the core antigen (HBcAg) was suggested to be the most important for viral clearance [5–7]. In contrast, the HBV-specific immune response is weak and hard to detect in patients with chronic infection.

Characterization of the HBV-specific helper T cell and CTL responses has been cumbersome and difficult to apply for clinical use. The helper T cell response has been studied using either a proliferation assay or cytokine release into medium, while the CTL response has been studied using a limiting dilution and conventional chromium release assay. These assays require several days to obtain results.

Flow cytometric analysis of intracytoplasmic cytokines is a method to detect cytokine production at a single cell level and requires only hours to obtain results [8–10]. This technique has been applied to study T cell functions in patients with autoimmune diseases and cancers [11]. Furthermore, this method in combination with in vitro antigen stimulation enabled analysis of the antigen-specific T cell response ex vivo [12].

In the present study, we utilized flow cytometric analysis of intracytoplasmic cytokines to study the CD4 and CD8 T cell response to HB core antigen in different groups of patients infected with HBV.

MATERIALS AND METHODS
Patients and virological status
Twenty-seven adult Japanese subjects were studied. All cases were negative for antibody to hepatitis C virus (HCV Ab). Written informed consent was obtained from each patient, and the study
was approved by the institutional Ethics Committee for Human Research. These cases were divided into three subgroups and details of these patients are summarized in Table 1. Group A consisted of nine cases with chronic hepatitis positive for HBsAg, HBeAg and HBV DNA. Diagnosis of chronic hepatitis was based on elevated serum alanine aminotransferase (ALT) activity and histological examination. Group B consisted of 13 cases of HBV carriers positive for HBsAg and HBeAg but negative for HBV DNA. Group C consisted of five cases positive for HBsAg and HBeAg but negative for HBV DNA (past HBV infection). Serum transaminase levels in Groups B and C had been in the normal range for at least the last 6 months. Controls included seven healthy volunteers (negative for HCV Ab, HBsAg and HBsAb) and a vaccinated volunteer positive for HBsAb.

Virological examinations were performed as follows: HBsAg, HBsAb, HBeAg and HBeAb were detected using a commercially available radioimmunoassay (RIA) kit (Abbott Laboratories, North Chicago, USA), HBV DNA assayed by a nucleic acid hybridization method and antibody to HCV measured using a commercially available ELISA kit (Kokusai-shiyaku, Kobe, Japan).

**Table 1. Clinical and virological profiles**

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Virological state</th>
<th>Age (years)</th>
<th>T.Bil. (mg/dl)</th>
<th>AST (IU/l)</th>
<th>ALT (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(female/male)</td>
<td>HBs Ag/Ab</td>
<td>HBeAg/Ab</td>
<td>HBV DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>9(6/3)</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>35.1±11.4</td>
</tr>
<tr>
<td>Group B</td>
<td>13(5/8)</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td>44.4±14.4</td>
</tr>
<tr>
<td>Group C</td>
<td>5(1/4)</td>
<td>-/+</td>
<td>-/+</td>
<td>-</td>
<td>45.0±13.7</td>
</tr>
</tbody>
</table>

**HBcAg and T cell stimulation**

Recombinant HBcAg (rHBcAg) was synthesized in Escherichia coli [13] and was provided generously by Dainabott Co., Ltd, Tokyo, Japan. The purity of rHBcAg was confirmed using SDS-PAGE.

Heparinized peripheral blood was collected from each patient. One ml was placed in a 15-ml Greiner tube and incubated for 5 h in a humidified CO₂ atmosphere. For T cell stimulation, 5–25 μg rHBcAg was added to medium containing 2 μg anti-CD28 MoAb (Pharmingen, San Diego, CA, USA) and 20 U IL-2 (Sigma-Aldrich Japan, KK, Co., Ltd, Tokyo, Japan). Preliminary experiments showed that the addition of both anti-CD28 MoAb and IL-2 to the culture medium allowed a higher frequency of antigen-specific response without increased background stimulation (Fig. 1). Brefeldin A (Sigma-Aldrich Japan, KK, Co., Ltd, Tokyo, Japan) was added for the last 4 h at a final concentration of 10 μg/ml. After incubation, red blood cells were lysed by FACS lysing solution (Becton Dickinson, San Jose, CA, USA). After centrifugation for 5 min, the pellet was resuspended in 2 ml of freezing medium (10% DMSO in PBS with 2% BSA). Finally, the cells were frozen at −70°C. Negative controls were incubated as above but without the antigen.

For positive controls, whole blood was incubated with SEB at a concentration of 10 μg/ml and treated as mentioned above.

**Antibodies**

Monoclonal antibodies (MoAb) against CD8 (clone SK 1, peridinin chlorophyl protein [PerCP] labelled), CD4 (clone SK 3, PerCP labelled), IFN-γ (clone 25723-11, fluorescein isothiocyanate [FITC] labelled), TNF-α (clone 6401-1111, phycoerythrin [PE] labelled), were obtained from Becton Dickinson (San Jose, CA, USA). MoAbs against CD28 (clone CD28-2), HLA-A, B, C (clone G46–2·6), HLA-DR, DP, DQ (clone Tu39) and CD40L (clone TRAP1) were obtained from Pharmingen (San Diego, CA, USA).

**Immunofluorescent staining**

The frozen cell preparation was rapidly thawed in a 37°C water bath and divided into 12 × 75-mm Falcon polystyrene tubes (Becton Dickinson, San Jose, CA, USA). After washing once with buffer (PBS containing 0.5% BSA and 0.05% sodium azide), the cells were incubated at room temperature in the dark for 15 min with PerCP-labelled anti-CD4 or -CD8 MoAb. Then 500 μl of FACS permeabilizing solution was added and incubated for 10 min. After washing again, FITC-labelled anti-IFN-γ or -TNF-α MoAb and PE-labelled anti-CD69 MoAb were added and incubated for 30 min in the dark. After a final wash, the cells were resuspended in 500 μl of PBS containing 1% paraformaldehyde.

**Flow cytometric analysis**

Three-colour analysis was performed using FACS Caliber or FACSScan. For each analysis, 10 000–20 000 events gated on CD4 or CD8 expression were acquired. As isotype controls, concentration matched FITC-labelled mouse IgG2a antibody and PE-labelled mouse IgG1 antibody were used.

Cells double-positive for CD69 and intracytoplasmic cytokine were taken as specifically activated cells. Such cells were located in the upper right region of the dot plot profile. The HBcAg-specific T cell response was determined as follows:

\[
\text{HBcAg-specific T cells} \% = \frac{\text{the number of cytokine-positive cells with HBcAg}}{\text{the number of total CD4 or CD8 T cells}} \times 100
\]

Samples that contained cytokine-positive cells at greater than 0.1% of the cell population were identified as positive.
Inhibition experiment for CD8 T cell activation

Four inhibition experiments were performed to analyse the mechanism of CD8 T cell activation. First, CD4 T cells were depleted using magnetic beads coated with anti-CD4 monoclonal antibody (Dynabeads M-450 CD4, Dynal AS, Oslo, Norway) as recommended by the manufacturer. Whole blood was diluted with an equal volume of RPMI medium and an appropriate amount of beads were added. The sample was incubated for 30 min at 4°C and the tube was placed on a magnet to remove the beads coated with CD4 T cells. Secondly, anti-HLA-A, -B, -C antibody

Fig. 1. Addition of IL-2 and anti-CD28 to the medium allows a higher frequency of antigen-specific response without increasing the background level. The upper panels show CD4 T cells and the lower panels show CD8 T cells. (a) and (d): Background with IL-2 and anti-CD28 antibody. (b) and (e): Antigen stimulation without IL-2 and anti-CD28 antibody. (c) and (f): antigen stimulation with IL-2 and anti-CD28 antibody.
(10 μg/ml) was added to the medium and HBcAg stimulation was performed as mentioned above. Thirdly, anti-HLA-DR, -DP, -DQ antibody (10 μg/ml) was added to the medium. Lastly, anti-CD40L antibody (10 μg/ml) was added to the medium.

**Statistics**

Fisher’s PSLD post-hoc test was used to compare the percentage of cytokine-positive cells in each group.

**RESULTS**

**Methodological reliability**

T cells from healthy subjects were stimulated with SEB and used as a positive control. Both CD4 and CD8 T cells were positive for intracytoplasmic IFN-γ and TNF-α. Three cytokine-positive T cells were also positive for CD69, an early activation marker (Fig. 2). The positive intracytoplasmic cytokines were also demonstrated in SEB-stimulated CD4 and CD8 T cells in all three groups.

However, T cells from healthy subjects, with or without HBcAg stimulation, showed no intracytoplasmic cytokine staining, and were used as a negative control. A vaccinated volunteer also showed no intracytoplasmic cytokine staining (data not shown).

**Cytokine production without HBcAg stimulation**

Without HBcAg stimulation, a high frequency of IFN-γ-positive CD8 T cells was demonstrated in Group B (Table 2). Seven of 13

<table>
<thead>
<tr>
<th></th>
<th>CD4</th>
<th></th>
<th>CD8</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IFN-γ</td>
<td>TNF-α</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>Frequency of cytokine-positive cells without HBcAg stimulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>0.019±0.022</td>
<td>0.018±0.023</td>
<td>0.046±0.055</td>
</tr>
<tr>
<td>Group B</td>
<td>0.037±0.048</td>
<td>0.041±0.060</td>
<td>0.32±0.62</td>
</tr>
<tr>
<td>Group C</td>
<td>0.0080±0.013</td>
<td>0.018±0.020</td>
<td>0.028±0.033</td>
</tr>
<tr>
<td>Frequency of HBcAg-specific cytokine-positive cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>0.31±0.42</td>
<td>0.061±0.10</td>
<td>0.30±0.40</td>
</tr>
<tr>
<td>Group B</td>
<td>0.17±0.15</td>
<td>0.080±0.099</td>
<td>0.18±0.20</td>
</tr>
<tr>
<td>Group C</td>
<td>1.4±1.4</td>
<td>0.008±0.082</td>
<td>4.5±5.9</td>
</tr>
</tbody>
</table>

Fig. 2. Dot plot profile of CD8 T cells for one representative case from Group C: (a) an isotype control; (b) CD8 T cells without HBcAg; (c) CD8 T cells with HBcAg; and (d) CD8 T cells with SEB stimulation. (c) Note the strong response (15.18%) following stimulation with HBcAg.
After HBcAg stimulation, IFN-γ-positive CD8 T cell response was demonstrated in Group C. The IFN-γ-positive CD8 T cell population was 0.30 ± 0.40% (mean ± s.d.) in Group A, 0.18 ± 0.20% in Group B and 4.5 ± 5.9% in Group C. The CD8 T cell population positive for HBcAg-specific IFN-γ was significantly higher in Group C compared with that in Groups A or B (Fig. 4).

The TNF-α-positive CD8 T cell population was 0.097 ± 0.18% in Group A, 0.061 ± 0.13% in Group B and 0.54 ± 0.77% in Group C. The percentage was also significantly higher in Group C than that in Group A or B.

The CD4 T cell population positive for IFN-γ was 1.4 ± 1.4% in Group C, 0.31 ± 0.42% in Group A and 0.17 ± 0.15% in Group B. The percentage was significantly higher in Group C than that in Groups A or B. However, the TNF-α-positive CD4 T cell population did not differ among the three groups.

**Mechanism of CD8 T cell activation**

To analyse the mechanism of CD8 T cell activation, four experiments were performed. Depletion of CD4 T cells from the incubation diminished HBcAg-specific IFN-γ-positive CD8 T cells (Fig. 5), suggesting that antigen-specific helper CD4 T cells are necessary for CD8 T cell activation. Addition of the antibody to HLA class I to the culture medium also decreased the number of IFN-γ-positive CD8 T cells. CD8 T cell activation was restricted by HLA-class I (Fig. 6). Addition of anti-HLA class II antibody or anti-CD40L antibody to the medium also blocked both CD4 and CD8 T cell activation (Fig. 7).

**DISCUSSION**

Intracellular cytokine staining and multi-parameter flow cytometry is a feasible way of determining the levels of antigen-specific T cells among human circulating lymphocytes [11]. Antigen-specific cytokine-secreting cells have recently been reported both for circulating CD4 [12] and cloned CD8 + CTLs [14]. Although the precise mechanism for CD8 T cell activation is not known, dendritic cells or macrophages may play an important role in antigen presentation and activation [15,16].

Utilizing this technique, we analysed the HBcAg-specific T cell response in three different phases of HBV infection. A high frequency of circulating HBcAg-specific CD4 and CD8 T cells were demonstrated in chronic HBV carriers as well as in subjects who had recovered from HBV infection. The most vigorous response was demonstrated in HBsAb-positive subjects who are supposed to have cleared HBV. The number of IFN-γ-positive CD4 and CD8 T cells was significantly higher in the HBsAb-positive group than in the HBcAg-positive or the HBsAb-positive groups. On the other hand, no significant difference was observed between HBcAg-positive and HBsAb-positive carriers.

Recently, Chisari et al. reported that a small amount of HBV persists years after acute HBV infection [17,18]. Cytotoxic T lymphocytes (CTL) specific for HBV have also been
demonstrated in such subjects, suggesting an important role for CTL in suppressing reactivation of HBV. The present finding may be consistent with their study. The presence of circulating HBcAg-specific T cells at a high frequency might result from continuous stimulation by residual HBV. It is well known that the reactivation of HBV may occur in subjects positive for HBsAb or HBeAb after immunosuppressive therapy for autoimmune and renal diseases [19] or chemotherapy for cancer [20]. Furthermore, HBV reactivation has been demonstrated in transplanted livers from living related donors who are positive for HBcAb [21]. The HBV-specific T cell response may play a role in the suppression of HBV reactivation.

The HBcAg-specific T cell response was also demonstrated in both HBeAg-positive and HBeAb-positive subjects with chronic infection. This is inconsistent with previous reports, where HBcAg-specific T cells were rarely detected in patients with chronic hepatitis. Little or no immune response to HBV antigens in HBV carriers has been suggested as the reason why infection persists in carriers [22].

One reason for the discrepancy is that the present method is more sensitive at detecting the antigen-specific T cell response than previous methods. Previous methods used cytokine production or a limiting dilution and chromium release assay, which is cumbersome and time-consuming. Recently, Tsai et al. [5] reported a T cell response in chronic hepatitis patients, especially during acute exacerbation. If the previous method is sensitive enough, the T cell response might be detected in chronic HBV carriers, although the response may be weaker than that observed in acute infection.

Previous studies have shown that the CD8 CTL response is important for elimination and suppression of HBV replication [23]. The CTLs specific for viral antigens are known to possess IFN-γ and TNF-α. Although we have not clarified the function of the HBcAg-specific IFN-γ-positive CD8 T cells, it is conceivable that the HBV-specific CTLs are present in this cell population. Recently, HBV-specific CD8 T cells were analysed using a new method of HLA A2-tetramers [24]. This might be a better method to analyse antigen specific CD8 T cells and the function of IFN-γ-positive CD8 T cells and the mechanism of activation should be analysed using this sophisticated method in future studies.

There remain many unanswered questions. What is the mechanism of viral clearance (seroconversion) in chronically infected patients and what triggers the process? The present study demonstrated that HBcAg-specific CD8 T cell activation was mediated through both helper CD4 T cells and antigen presentation by HLA class I. This suggests that the function of antigen-presenting cells (APC) is key for activation of CD8 T cells. In transgenic mouse models and even in human HBV infection, the function of APC is shown to be depressed [25]. Administration of antigens through immunogenic routes may activate APC, which had become anergic to antigens. Vaccination of the HBV antigens might be a solution for reactivation of both anergized APC and the T cell response in chronic HBV carriers [26,27].

In summary, the present study demonstrated the presence of a high frequency of HBcAg-specific CD4 and CD8 T cells not only in recovered but also in chronically infected patients. These T cells may play an important role in controlling viral clearance and reactivation.

ACKNOWLEDGEMENTS

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Fig. 5. Effect of CD4 T cell depletion on cytokine production of HBcAg-specific CD8 T cells for one representative case from Group C. (a) Isotype control, (b) and (d) CD4 and CD8 T cells, respectively, without HBcAg, (c) and (e) CD4 and CD8 T cells, respectively, with HBcAg and (f) HBcAg-stimulated CD8 T cells with CD4 T cell depletion. (f) Note diminution of IFN-γ-positive CD8 T cells following depletion of CD4 T cells.

Fig. 6. Effect of addition of an anti-HLA class I antibody on HBcAg-specific cytokine production in CD4 (upper panels) and CD8 T cells (lower panels) in one representative case from Group C. (a) Isotype control, (b) and (d) CD4 and CD8 T cells, respectively, without HBcAg, (c) and (e) CD4 and CD8 T cells, respectively, with HBcAg, and (f) HBcAg-stimulated CD8 T cells plus anti-HLA class I antibody. Note the reduction in HBcAg-specific IFN-γ-positive CD8 T cells in the presence of the anti-HLA class I antibody.
Fig. 7. Addition of anti-HLA class II antibody or anti-CD40L antibody blocks the HBcAg-specific response in both CD4 (upper panels) and CD8 (lower panels) T cells. (a) and (d) CD4 and CD8 T cells, respectively, with HBcAg (b) and (e) CD4 and CD8 T cells, respectively, with HBcAg in the presence of an anti-HLA class II antibody and (c) and (f) CD4 and CD8 T cells, respectively, with HBcAg in the presence of an anti-CD40L antibody.
REFERENCES


