Selective functional deficit in dendritic cell – T cell interaction is a crucial mechanism in chronic hepatitis B virus infection

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SUMMARY. A defect in specific T cell immunity has long been assumed to be the central mechanism of persistent Hepatitis B virus (HBV) infection. Recent studies on HBV transgenic mice have suggested, however, that functional deficit of dendritic cells (DC) was an underlying cause for the T cell dysfunction. The functions of monocyte-derived DC were determined by studying 75 subjects that included chronic hepatitis B patients with low or high HBV load; antibody to hepatitis B surface antigen (anti-HBs) positive individuals who had recovered completely from previous acute HBV infection; healthy donors who had received hepatitis B vac- cination and were anti-HBs positive; and immunologically naïve to HBV or the vaccine individual. Impaired interactions between monocyte-derived DC and T cells were shown in chronic HBV infection patients, especially in those with active virus replication. The dysfunctions included: (i) failure of DC to increase human leukocyte antigen (HLA-II), B7 expression and interleukin-12 secretion in responses to hepatitis B surface antigen (HBsAg), (ii) defective induction of T cell proliferative response to HBsAg, (iii) failure to activate T cells to produce cytokines and (iv) deficit in the induction of antigen specific cytotoxic T lymphocytes (CTLs).

In vitro treatment of DC with tumour necrosis factor-α improved HLA-II and B7 expression, as well as Th cell and CTL responses. It is concluded that defective DC-T cell interactions may account for the specific T cell immune defects in chronic HBV infection. Immunotherapy that aims at restoring DC functions could offer a new opportunity for effectively managing persistent HBV infections.

Keywords: chronic hepatitis B infection, dendritic cells, functional deficits, HBV, T cells.

INTRODUCTION

The World Health Organization has estimated that some 350 million people worldwide are chronically infected with hepatitis B virus (HBV) [1]. These individuals are prone to the development of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Though the exact mechanisms of persistent HBV infection have not been fully elucidated, defects in host cellular immune responses are nonetheless crucial in causing viral persistence. In those who progress to chronic HBV infection, oligoclonal T helper (Th) responses with weak or undetectable virus-specific cytotoxic T lymphocyte (CTL) activity [2] have been demonstrated. This contrasts with the vigorous polyclonal Th cell and CTL responses against viral capsid and envelope antigen evoked in self-limiting acute HBV infection. It has been reported that permanent clearance of HBV from chronic hepatitis B patients can be achieved in patients upon adoptive transfer of bone-marrow cells from donors who have acquired the immunity from natural HBV infection but not from vaccination [3]. These observations suggest that T cell immunity determines the outcome of HBV infection, which may either be recovery with eradication of the virus from the host, or a state of immune tolerance with persistence of the virus [4].

The Th cell immune response to HBV infection is orchestrated through the activities of antigen presenting cells (APC). In HBV transgenic (Tg) mice, infusion of cytokine-activated dendritic cells (DC) from either Tg or syngeneic non-Tg mice could elicit hepatitis B surface antigen (HBsAg)-specific CTL response [5]. These results suggest strongly that immune tolerance to the transgene could be attributed to the impaired APC function. As most human chronic HBV infections are acquired either intra utero, perinatally, or horizontally, and are at different clinical stages, APC...
function from chronic HBV infections may be different from those in Tg mice. The present study was designed to study functions of DC-T cell interactions in human chronic HBV infections, and to decipher the functional deficits in such interactions, especially with regards to active virus replication.

METHODS

Study subjects

Patients with chronic HBV infection and healthy individuals were enrolled in the study (Table 1). Thirty patients, all ethnic Chinese, who had not received any specific treatment for at least 6 months prior to this study, were recruited and divided into two groups: patients whose serum HBsAg was negative with undetectable or low HBV DNA level (group 1), and those who were HBsAg positive with high viral DNA level (group 2). Control subjects were composed of healthy donors who had completely recovered from previous acute HBV infection (group 3, recovered donors), those previously vaccinated with the HB vaccine (group 4, vaccinated donors) or those immunologically naïve to HBV or HB vaccination (group 5, naïve donors).

Serological and biochemical analysis

Serum HBsAg, HBeAg, anti-HBs, anti-HBe and anti-HBc were assayed by enzyme-linked immunosorbent assay (ELISA) kits (BIOKIT, Barcelona, Spain), according to the manufacturer’s instructions. HBV DNA was extracted from plasma using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA), and quantification was performed by LightCycler PCR (LC-PCR) using primers, 5’-AACATGGAGAACATCATTCC (forward) and 5’-AGCGATAACCAGGACAAGTTT reverse, which yielded a 203 bp product. A donor fluorescein probe (5’-ATTGAGAGAAGTCCACCAGAGACTAGAC-fluorescein) and acceptor LightCycler-Red 640 probe (5’-LC-Red 640-CTGTGGTATTGTGAG GATTCTTGTCAACAAG) directed to the 203 bp product were designed for the assay. LC-PCR was carried out using the DNA Master Hybridization Probes Kit and LightCycler (Roche Diagnostics, Carlsbad, CA, USA) according to the manufacturer’s instructions. A 10-fold serial dilution ranging from 0.015 to 150 pg/mL of calibrator 5 from the Hybrid Capture II assay (Digene Corp, Gaithersburg, MD, USA) was employed as quantification controls. The levels of serum alanine aminotransferase (ALT) were measured by a multiple-point rate colorimetric method with the Vitros 950 dry-chemistry analyzer (Ortho Clinical Diagnostics Inc., Ravitan, NJ, USA).

Preparation of dendritic cells

Monocyte-derived DC were generated from peripheral blood mononuclear cells (PBMC) using the method described by
Bender et al. [6] with modification. Monocytes were obtained by adherence after plating out 5 × 10^6 cells/mL of PBMC in culture flasks at 37 °C for 1 h, followed by the removal of nonadherent cells. DC precursor enriched adherent monocytes were harvested after overnight culture and further cultured in RPMI supplemented with 10% foetal bovine serum (FBS; Gibco-BRL, Grand Island, NJ, USA), 0.2 mM L-glutamine, 100 µg/mL of streptomycin and 20 µg/mL of gentamicin (10%-RPMI), containing 1000 U/mL of GM-CSF (granulocyte/macrophage colony stimulating factor), a generous gift of Immunex, Thousand Oaks, CA, USA) and 500 U/mL of interleukin (IL)-4 (R&D System Inc., Minneapolis, MN, USA). On day 6, DC were purified by two rounds of immunomagnetic depletion (Dynabeads, Oslo, Norway) using monoclonal antibodies against CD3, CD8, CD14, CD16, CD19 and CD56 (BD-PharMingen, San Diego, CA, USA). The resulting cell population was over 95% HLA-DR^high by flow cytometric analysis (FACS; FACS Calibur, BD, USA), suggesting that it consisted of mature DC (M-DC). Tumour necrosis factor (TNF)-α activated DC (A-DC) were cultured in the presence of 1000 U/mL of TNF-α (R&D System Inc.) after their purification.

**Flow cytometry analysis**

The percentages of DC were measured by FACS using Cy5/PE-, FITC- or PE-labelled monoclonal antibodies (mAbs) specific for human CD3, CD1a, CD11c, CD14, lineage 1-neg (BD-PharMingen), CD83, HLA-DR and mouse IgG1 (Coulter, Miami, FL, USA) in a flow cytometer (FACSCalibur). The expression of HLA-class II and B7 on the surface of DC was detected using FITC- or PE-labelled mAbs against HLA-DR, CD80 and CD86 (BD-PharMingen). The mean molecules/cell of HLA-class II and B7 were quantified using SPHEROTM rainbow calibration particles (SpheroTechnical, Libertyville, IL, USA) according to the manufacturer’s instructions.

**Functional lymphocyte studies**

Autologous T and B cells in nonadherent cells were separated by adsorption with sheep red blood cells. The effects of DC on CTL function and T lymphocyte proliferation were studied. For CTL assays, autologous lymphoblastoid cell lines (LCLs) were derived from Epstein–Barr virus (EBV) transformed B cells as described previously [7]. T lymphocytes were first stimulated by culturing with mature or TNF-α activated DC plus HBsAg for 10–12 days. Specific CTLs were further expanded by adding 25 IU/mL of human rIL-2 (recombinant) (R&D systems) and cultured for 2 weeks. Autologous LCLs were infected with 10 PFU/cell of a recombinant vaccinia virus expressing HBsAg (Vac-HBsAg) or with the blank virus control (Vac-blank) for 12 h, and were separately used as HBsAg specific or control targets. The CTL activity in the cultures was assayed in triplicate in a standard 4-h calcein release assay as reported previously [8, 9]. To test for the induction of T lymphocyte proliferation by DC, approximately 2 × 10^5/mL of M-DC and A-DC were incubated with 5 µg/mL of purified recombinant HBsAg (Research Diagnostics Inc., Knoxville, TN, USA) or the same concentration of tetanus toxoid (TT) (Connaught Int. Laboratories, Toronto, Canada), for 12–16 h at 37 °C, and separately mixed with autologous T cells (5 × 10^5/well) obtained from study subjects at an effector:stimulator ratio of 20:1. Phytohaemagglutinin (PHA) at 25 µg/mL (Sigma, Saint Louis, MD, USA) was added to cell cultures as positive control, while cell cultures with medium alone formed baseline controls. The culture medium was RPMI supplemented with 10% heat-inactivated human AB serum (Gemini Bio-Products Inc., Woodland, CA, USA) and all cultures were performed in triplicate in 96-well microplates for 3 days and tested as described previously [10].

**Detection of HBsAg-induced cytokines**

HBsAg-induced IL-12 secretion in DC was determined by adding HBsAg or TT (5 µg/mL respectively) to the purified DC cultures, followed by the testing of the supernatants at 2-day intervals. For evaluating the stimulatory effects of the DC on T cells in inducing cytokine-production, autologous T cells were mixed with M-DC or A-DC plus HBsAg at a ratio of 20:1, and the culture supernatants were collected at 24-h intervals thereafter. The levels of interferon (IFN)-γ, TNF-α, IL-5, IL-10 and IL-12 in culture supernatants were determined by ELISA using OptEIA kits (PharMingen, USA) according to the manufacturer’s instructions.

**Statistical analysis**

The significance of differences between groups was analysed by the paired Student’s t-test.

**RESULTS**

**Differential stimulatory responses in DC-T cells from patients with chronic HBV infection and healthy controls**

Autologous T cells from the study subjects were separately stimulated with mature M-DC-HBsAg or M-DC-TT. As shown in Table 2, the mean lymphocyte Proliferation Index of the cultures achieved by M-DC-HBsAg was significantly lower in chronic HBV patients (groups 1 and 2), compared with donors who had acquired HBV immunity from natural infection (group 3) (P < 0.02). The nonspecific proliferation responses to PHA stimulation and to an irrelevant antigen (TT) showed however no significant differences across all groups (P > 0.03). When A-DC-HBsAg was used for stimulation, both groups 1 and 2 showed increased T cell proliferation, compared with cultures stimulated by M-DC-HBsAg (Table 2).
Stimulatory effects of the M-DC on Th cells were evaluated by the secretion of cytokines – IFN-γ and TNF-α from Th-1 cells and IL-5 and IL-10 from Th-2 cells – in the supernatants of the mixed cultures at 24, 48 and 72 h post-stimulation (Fig. 1). T cells of donors who had completely recovered from HBV infection secreted the highest levels of IFN-γ, TNF-α, IL-5 and IL-10 following M-DC-HBsAg stimulation, with the production of IL-10 decreasing precipitously. M-DC-HBsAg did not induce significant secretion of IFN-γ, TNF-α and IL-5 in cultures of all patients. When the T cell cultures of group 1 and 2 were stimulated with A-DC-HBsAg, significantly higher levels of IFN-γ, TNF-α and IL-5 were produced (P < 0.03).

Results of HBsAg specific cytotoxicity are shown in Fig. 2. In group 3, the cytotoxic activity against HBsAg target cells was the highest, while no significant antigen-specific cytotoxicity was elicited in the cultures of the other groups (Fig. 2a). TNF-α activated A-DC were able to activate HBsAg specific CTLs in cultures of the patients, though at low level (Fig. 2b). At an effector:target (E:T) ratio of 30, the mean specific lysis in cultures of group 1 and group 2 reached 39 and 26%, respectively, which was about 2.5-fold higher than in cultures stimulated by nonactivated M-DC-HBsAg (Fig. 2a). The specificity of the cytotoxicity was confirmed, as there was no significant cytolysis against the control targets in the same cultures.

**Deficit in the expression of HLA-class II and B7 on M-DC in chronic HBV infection**

Expression of HLA-class II and B7 molecules on the surface of DC was measured by FACS using appropriate mAbs and rainbow calibration particles (Table 3). There was no significant difference between expression of these cell surface markers on M-DC in the absence of HBsAg in cell culture. However, under HBsAg stimulation, these markers were significantly increased in all three groups of donors (P < 0.05) but not in the two groups of patients (P > 0.30). In group 2 patients, i.e. those with high virus load, the expression of HLA-class II and CD80 was significantly lower than that in other groups (P < 0.05). The up-regulation of HLA-class II and B7 molecule expression by HBsAg in naïve donors, indicates that there are HBsAg responsive M-DCs in these donors, although they have had no previous exposure to HBV vaccination or infection. Expression of these molecules could be up-regulated markedly by TNF-α treatment (Table 4). A twofold or higher increase of the expression of these cell markers on the surface of A-DCs was found not only in the donor groups, but also in the viraemic groups (P < 0.05), indicating partial restoration of these molecules in DCs from chronic hepatitis B patients.

**Defective production of IL-12 by M-DC in chronic HBV patients**

Dendritic cells produce IL-12 which orchestrates cell-mediated immune responses. To determine if an impaired stimulatory function of DC was associated with altered production of IL-12, its secretion in the supernatants of M-DC cultures from study subjects was monitored continuously for 5 days at 2-day intervals (Fig. 3). In the absence of HBsAg from cultures, IL-12 p40 was first detected on day 1 in group 3, earlier than in the other groups. The level of IL-12 p40 was the highest on day 5, reaching 327 ± 99 pg/mL (mean ± SD) in group 3, which was approximately two times higher than the other groups. These results suggested that the DC from recovered donors could achieve sustained production of IL-12 even in the absence of antigen stimulation. In the presence of HBsAg, IL-12 p40 production by M-DC in group 3 was significantly increased (P < 0.05).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lymphocyte Proliferation Index (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M-DC-HBsAg†</td>
</tr>
<tr>
<td>1</td>
<td>2.59 ± 1.33</td>
</tr>
<tr>
<td>2</td>
<td>1.78 ± 0.66</td>
</tr>
<tr>
<td>3</td>
<td>6.48 ± 2.92*</td>
</tr>
<tr>
<td>4</td>
<td>3.31 ± 1.77</td>
</tr>
<tr>
<td>5</td>
<td>1.23 ± 0.61</td>
</tr>
</tbody>
</table>

*The level was significantly higher than that of the other groups (P < 0.02).
**The level was significantly higher than that of cultures stimulated by M-DC-HBsAg (P < 0.01).

†T cells taken from all study subjects were co-cultured with M-DC plus HBsAg (M-DC-HBsAg) and A-DCs plus the antigen (A-DC-HBsAg) and tested for T lymphocyte proliferation.
‡T cells obtained respectively from 12 study subjects of each group were co-cultured with M-DC plus tetanus toxoid (M-DC-TT) and tested for T lymphocyte proliferation.
The production of IL-12 was also increased in the donors of group 4 and 5. However, there was no significant change of IL-12 production by M-DC in the two groups of patients. Again, IL-12 production was the lowest in those with high virus load. Levels of IL-12 induced by tetanus toxoid were similar in all study groups, suggesting that the deficit in the production of IL-12 by M-DC in patients was specific to HBV.

DISCUSSION

While functional deficit of DC in HBV transgenic mice has been described [5], to date, its occurrence in chronic hepatitis B patients has not been elucidated nor confirmed. It is noted that chronic hepatitis B patients differ from HBV transgenic mice in that: (i) chronicity in patients is the result of natural infection, either intra utero, perinatally or horizontally; (ii) there are different stages in human infection, characterized by the presence or absence of virus replication and viral gene integration, fulminant hepatitis, asymptomatic carrier state, or are in the recovery phase; and (iii) the virus-host interactions may be more complicated in humans as the virus may reside only in the liver or several organs. Furthermore, studying DC function in humans is far more complicated and difficult than that in experimental animals. The major difficulties relate firstly to obtaining sufficient numbers of purified DC from patients and secondly to dissecting the functions of DC excluding the involvement of T cells. In this study, we used DC derived from peripheral blood monocytes, which is a practical approach that has been accepted by researchers. Given a higher than 95% recovery of the purified cell population for FACS analysis, comparable results from all groups have been obtained to enable meaningful analysis to be made. Methodologically only autologous T lymphocytes could be used for the determination of DC function through specific stimulation of T cell proliferation and cytokine secretion. As T cells had been isolated from the nonadherent PBMC from each study subject, and targets for cytolysis were acquired by EBV transformed B cells.
cells from the same individual, functional defects could then be specifically ascribed to defects in DC-T cell interactions.

Through an in vitro DC-specific T cell stimulation study, we have shown that the stimulatory effect of M-DC to HBsAg-specific T cells was the highest in those who had completely recovered from HBV infection. This was evidenced by marked T lymphocyte proliferation (Table 2), higher levels of IFN-γ, TNF-α, IL-5 secretion (Fig. 1) and effective induction of specific CTLs (Fig. 2). These responses indicate that the effects of DC on HBsAg specific T cells remain intact after virus clearance. In contrast, such stimulation was not observed in the two groups of chronic hepatitis B patients. Interestingly, the defect in DC-T cell stimulation was more intense in the high virus load and HBeAg+ patients (group 2) than the low virus load and HBeAg− patients (group 1). This discrepancy could be related to the direct inhibitory effects of active replicating virus on DC-T cell interaction, or due to the secondary inhibitory effects of HBeAg protein on DC or T cells.

The defects of DC-T cell interaction in chronic HBV infection could be reverted by TNF-α activation of DC (Tables 3 and 4), suggesting that the Th and CTL were partially defective. With effective stimulation of A-DC, partial restoration of the T cell and cytokine responses could be achieved. Apart from the functional defects elicited in T cells [2], the functional impairment in DC-T cell interactions could also have resulted from the failure of DC to induce IL-12 production under HBsAg stimulation (Fig. 3). Defective production of IL-12 by DC could therefore play a vital role in the development of immune tolerance to HBsAg in chronic hepatitis B patients. Yet another possible factor is the failure of HBsAg to

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**Table 3** Expression of HLA-class II and B7 on the cell surface of monocyte-derived DCs in expanded cultures in the presence or absence of HBsAg

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cell surface markers (mean molecules no./cell ± SD × 10^5)</th>
<th>CD80</th>
<th>CD86</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLA-class II</td>
<td>CD80</td>
<td>CD86</td>
</tr>
<tr>
<td>1</td>
<td>20 ± 3 25 ± 4</td>
<td>81 ± 30**</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>2</td>
<td>16 ± 3 18 ± 4</td>
<td>37 ± 12**</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>23 ± 4 36 ± 6*</td>
<td>118 ± 31**</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>21 ± 6 29 ± 9*</td>
<td>83 ± 32**</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>19 ± 5 28 ± 11*</td>
<td>77 ± 26**</td>
<td>1.7 ± 0.6</td>
</tr>
</tbody>
</table>

* The values were significantly higher than that in mature M-DC cultures in the absence of HBsAg (P < 0.05).
** Expression of cell surface markers was significantly higher in TNF-α-activated DC (A-DC) cultures than non-TNF-α-activated M-DC cultures (P < 0.02) in all five groups.
enhance expression of HLA-II and B7 on DC in chronic hepatitis B patients (Table 3). As up-regulation of HLA-II and the co-stimulatory receptor is critical for an effective immune response, the impaired expression of these molecules on DC in response to antigen stimulation may also contribute to immune tolerance. Besides, defect in production of IFN-γ, TNF-α and IL-5, as well as lack of effective CTLs in chronic hepatitis B patients have also been demonstrated in our study (Fig. 1).

By comparing the DC-T cell functions of HBV chronically infected patients with those who had recovered from acute HBV infection and vaccines, we have been able to confirm the occurrence of marked DC-T cell function impairment in chronic hepatitis B patients, notably in the setting of a high virus load. The results provide scientific rationale for the combined use of antiviral drugs to decrease virus load and immunotherapy for reverting the impaired function of DC-T cell interactions, a strategy which may prove to be

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**Table 4** Expression of HLA-class II and B7 on cell surface of DC in the presence or absence of TNF-α

<table>
<thead>
<tr>
<th>Groups</th>
<th>M-DC</th>
<th>A-DC</th>
<th>M-DC</th>
<th>A-DC</th>
<th>M-DC</th>
<th>A-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20 ± 3</td>
<td>68 ± 23*</td>
<td>1.6 ± 0.3</td>
<td>3.7 ± 0.8*</td>
<td>1.0 ± 0.2</td>
<td>2.3 ± 0.3*</td>
</tr>
<tr>
<td>2</td>
<td>16 ± 3</td>
<td>32 ± 8*</td>
<td>1.5 ± 0.5</td>
<td>3.1 ± 0.9*</td>
<td>1.0 ± 0.2</td>
<td>1.9 ± 0.5*</td>
</tr>
<tr>
<td>3</td>
<td>23 ± 4</td>
<td>75 ± 25*</td>
<td>1.8 ± 0.4</td>
<td>6.1 ± 1.4*</td>
<td>1.2 ± 0.1</td>
<td>3.3 ± 1.2*</td>
</tr>
<tr>
<td>4</td>
<td>21 ± 6</td>
<td>70 ± 27*</td>
<td>1.7 ± 0.5</td>
<td>5.0 ± 2.2*</td>
<td>1.1 ± 0.2</td>
<td>3.1 ± 0.8*</td>
</tr>
<tr>
<td>5</td>
<td>19 ± 5</td>
<td>69 ± 23*</td>
<td>1.7 ± 0.5</td>
<td>4.5 ± 1.7*</td>
<td>1.1 ± 0.3</td>
<td>2.3 ± 0.9*</td>
</tr>
</tbody>
</table>

*The values were significantly higher than those of non-TNF-α treated cells (M-DC).

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Fig. 3 Detection of IL-12 production in M-DC cultures of study subjects in the presence or absence of HBsAg. Sequential secretion of IL-12 in the supernatants of purified M-DC cultures in the presence or absence 5 µg/mL of HBsAg was detected by ELISA on day 1 and at 2-day intervals thereafter (a). Secretion of IL-12 in supernatants of M-DC cultures from groups 1–4 in the presence or absence of an 5 µg/mL tetanus toxoid (TT) was tested as control (b).
more effective for the treatment of chronic hepatitis B infection.

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