Antiviral Research 96 (2012) 59–64

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Strong and multi-antigen specific immunity by hepatitis B core antigen (HBcAg)-based vaccines in a murine model of chronic hepatitis B: HBcAg is a candidate for a therapeutic vaccine against hepatitis B virus

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ARTICLE INFO

Article history:
Received 11 April 2012
Revised 24 July 2012
Accepted 24 July 2012
Available online 1 August 2012

Keywords:
Chronic hepatitis B
Immune therapy
Hepatitis B core antigen
Dendritic cells

ABSTRACT

Experimental evidence suggests that hepatitis B core antigen (HBcAg)-specific cytotoxic T lymphocytes (CTL) are essential for the control of hepatitis B virus (HBV) replication and prevention of liver damage in patients with chronic hepatitis B (CHB). However, most immune therapeutic approaches in CHB patients have been accomplished with hepatitis B surface antigen (HBsAg)-based prophylactic vaccines with unsatisfactory clinical outcomes. In this study, we prepared HBsAg-pulsed dendritic cells (DC) and HBcAg-pulsed DC by culturing spleen DC from HBV transgenic mice (HBV TM) and evaluated the immunomodulatory capabilities of these antigens, which may serve as a better therapy for CHB. The kinetics of HBsAg, antibody levels against HBsAg (anti-HBs), proliferation of HBsAg- and HBcAg-specific lymphocytes, production of antigen-specific CTL, and activation of endogenous DC were compared between HBV TM vaccinated with either HBsAg- or HBcAg-pulsed DC. Vaccination with HBsAg-pulsed DC induced HBsAg-specific immunity, but failed to induce HBcAg-specific immunity in HBV TM. However, immunization of HBV TM with HBcAg-pulsed DC resulted in: (1) HBsAg negativity, (2) production of anti-HBs, and (3) development of HBsAg- and HBcAg-specific T cells and CTL in the spleen and the liver. Additionally, significantly higher levels of activated endogenous DC were detected in HBV TM immunized with HBcAg-pulsed DC compared to HBsAg-pulsed DC ($p < 0.05$). The capacity of HBcAg to modulate both HBsAg- and HBcAg-specific immunity in HBV TM, and activation of endogenous DC in HBV TM without inducing liver damage suggests that HBcAg should be an integral component of the therapeutic vaccine against CHB.

1. Introduction

Despite the considerable information regarding the viral life cycle, epidemiology, immunology, pathogenesis and prevention of hepatitis B virus (HBV), there has been a lack of significant developments in treating patients with chronic hepatitis B (CHB). Several antiviral drugs have been developed for treating CHB patients during the last three decades. However, controversy remains about their therapeutic efficacy. A systemic review of the National Institutes of Health (NIH) Consensus Development Conference, which assessed all randomized clinical trials on antiviral drugs in CHB patients from 1989 to 2008, revealed that antiviral drug treatment did not improve the clinical outcomes and all intermediate outcomes in CHB patients in any credible randomized-controlled trial (Shamliyan et al., 2009; Wilt et al., 2008). However, others have shown that these drugs could block or delay the progression of liver disease in CHB patients (Liaw, 2009; Lin et al., 1999). Although it is difficult to determine the underlying causes of these discrepancies, as different investigators used different criteria in their therapeutic evaluations, it is generally accepted that an ongoing treatment regimen for CHB with antiviral drugs is not satisfactory, and has low efficacy and considerable adverse effects. In addition, it is now clear that antiviral drugs possess poor immunomodulatory capabilities, which may be responsible for their ineffective control of HBV replication and inadequate prevention of liver damage in CHB (Lok and McMahon, 2007; Liaw and Chu, 2009).

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http://dx.doi.org/10.1016/j.antiviral.2012.07.011

Clinical and experimental evidence suggests that the replication of HBV DNA and progression of liver damage is under control in many CHB patients, even those not receiving any antiviral drug therapy. The magnitude and nature of host immunity to HBV is important in regulating these pathological events in CHB. In support of this concept, Maini et al. (2000) demonstrated that CHB patients that are capable of controlling HBV replication and liver damage harbor higher frequencies of hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg)-specific cytotoxic T lymphocytes (CTL) compared to those that express high levels of HBV and have progressive liver damage. Taken together, it appears that the restoration of host immune responses to HBV-related antigens may have therapeutic implications in CHB patients.

Based on these observations, polyclonal immunomodulators, such as cytokines, growth factors, and other immune mediators, were used in CHB patients. However, they had limited therapeutic efficacy and considerable side effects in CHB patients (Sprengers and Janssen, 2005). Subsequently, an antigen-specific immunotherapeutic approach, or vaccine therapy, was developed for CHB patients, which used commercially available prophylactic hepatitis B (HB) vaccines for treating CHB patients. Different investigators used different types of vaccines with different immunization protocols, and thus, it is difficult to assess the real therapeutic implications of vaccine therapy in CHB patients (Wang et al., 2010; Hoa et al., 2009; Pol et al., 2001). Indeed, it appears that the HBsAg-based vaccine may not be an effective immunotherapeutic approach in CHB. A well-planned clinical trial in 80 patients with CHB used a HBsAg-based vaccine in combination with another antiviral drug also failed to exhibit substantial therapeutic effect (Vandepapelière et al., 2007). Conversely, Heathcote et al. (1999) used a HBcAg epitope-based vaccine in CHB patients and achieved moderate therapeutic effects. Recently, Luo et al. (2010) reported that antigen-pulsed dendritic cells (DC) containing epitope of HBsAg and HBcAg had therapeutic effects in hepatitis B e antigen (HBeAg)-negative patients, but not in HBeAg-positive patients.

These clinical trials with HBsAg- and HBcAg-based vaccines have raised more questions than solutions regarding immune therapy for CHB patients, as the mechanisms of action of HBsAg- or HBcAg-based vaccine in CHB have not yet been explored. However, most cellular and molecular events following vaccination with either HBsAg or HBcAg could not be evaluated in CHB patients due to ethics, safety, technical, and procedural limitations.

To develop proper insights about immunogenecity of HBsAg- or HBcAg-based therapeutic vaccines in CHB, the role of DC in adaptive immunity has been examined. DC, the most potent antigen-presenting cells, are responsible for processing and presenting antigens for induction of antigen-specific immune responses in normal conditions as well as in the immune tolerance state (Steinman and Banchereau, 2007). Studies have shown that the phenotypes and functions of DC are distorted in chronic HBV infections (van der Molen et al., 2004). One way to circumvent immune tolerance state is to produce antigen-pulsed DC and use them as a vaccine. In fact, cancer antigen-pulsed DC and HBsAg-pulsed DC have been used to induce cancer-specific immunity and HBsAg-specific immunity in cancer patients and CHB patients, respectively, when antigen-specific immune responses could not be properly induced by only cancer antigen or HBsAg (Banchereau and Palucka, 2005; Steinman and Banchereau, 2007; Akbar et al., 2010a).

The present preclinical study assessed the immunomodulatory mechanisms of HBsAg and HBcAg in a murine model of HBV, specifically HBV transgenic mice (TM). After immunizing HBV TM with antigen-pulsed DC, the immune responses of HBsAg-pulsed DC or HBcAg-pulsed DC were compared in the spleen and liver. This study may provide further insight into developing an immune therapy for CHB patients.

2. Methods

2.1. Mice

HBV TM (official designation, 1.2HB-BS10) were prepared by microinjecting the complete genome of HBV plus 619 bp of HBV DNA into the fertilized eggs of C57BL/6 mice. HBV TM are known to express HBV DNA and mRNAs of 3.5, 2.1, and 0.8 kbp of HBV in the liver (Araki et al., 1989). HBV DNA were also detected in the liver, and HBsAg was found in the sera of all HBV TM. Eight-week-old male C57BL/6 mice were purchased from Nihon Clea (Tokyo, Japan). Mice were housed in polycarbonate cages in our laboratory facilities, and maintained in a temperature- and humidity-controlled room (23 ± 1 °C) with a 12-h light/dark cycle. All mice received humane care, and the study protocol was approved by the Ethics Committee of the Graduate School of Medicine, Ehime University, Japan. Eight-week-old C3H/He mice (Nihon Clea) were used in an allogenic mixed leukocyte reaction (MLR).

2.2. Detection of HBV-related markers

HBsAg levels and antibodies against HBsAg (anti-HBs) in sera were estimated with a chemiluminescence enzyme immunoassay (Special Reference Laboratory, Tokyo, Japan) and expressed as IU/ml and mIU/ml, respectively, as previously described (Akbar et al., 2010b).

2.3. Isolation of T lymphocytes, B lymphocytes, and DC

We have previously described in detail the methodology for isolating spleen cells and liver nonparenchymal cells (NPCs) (Akbar et al., 2010b; Chen et al., 2011; Yoshida et al., 2010). To produce a single cell suspension from the spleen, spleens were cut into pieces and passed through a 40-μm-pore nylon filter (BD Falcon, Durham, NC, USA). The resulting cells were collected and suspended in culture medium containing RPMI 1640 (Iwaki, Osaka, Japan) with 10% fetal calf serum (Filtron PTY Ltd., Brooklyn, Australia).

To retrieve liver NPCs, liver tissues were cut into pieces, homogenized, passed through 70-μm-pore steel meshes (Morimoto Yakuhin Co., Matsuyama, Japan), and suspended in 35% percoll (Sigma Chemical, St. Louis, MO, USA). After centrifugation for 15 min at 450 × *g* at room temperature, a high-density cell pellet was collected and suspended in culture medium.

T lymphocytes were isolated from the spleen single cell suspension or liver NPC by a negative selection column method using a mouse pan T isolation kit (Miltenyi Biotec, Bergish Gladbach, Germany), according to the manufacturer's directions (Chen et al., 2011; Yoshida et al., 2010).

DC were isolated from single cell suspensions of spleen and liver NPC using a density column (specific gravity 1.082), plastic adherence, re-culturing on plastic surface, and depletion of macrophages and lymphocytes or via positive selection of $CD11c^+$ cells with flow cytometry, as described (Akbar et al., 2010b, Chen et al., 2011).

2.4. Preparation of antigen-pulsed DC for immunizing HBV TM

HBsAg and HBcAg were purchased from Tokyo Institute of Immunology (Tokyo, Japan). Murine antigen-pulsed DC were prepared based on data from preliminary studies and according to our previous report (Akbar et al., 2010b; Miyake et al., 2010). Briefly, spleen DC were cultured with phosphate buffered solution (PBS) (unpulsed DC) or pyruvate dehydrogenase complex (PDC,

Sigma Biochemical, St. Louis, MO, USA) or HBsAg or HBcAg in culture medium for 48 h. DC were recovered from the cultures and washed five times with PBS. The viability of DC was assessed with a trypan blue exclusion test. The production of cytokines and T cell stimulatory capacities of antigen-pulsed DC were assessed *in vitro*.

2.5. Immunization schedule

HBV TM with comparable sera levels of HBsAg were used for this study. HBV TM were injected with either 5×10^6 unpulsed DC or 5×10^6 PDC-pulsed DC or 5×10^6 HBsAg-pulsed DC, or 5×10^6 HBcAg-pulsed DC. All vaccinations were done via the intraperitoneal route, six times, at an interval of 2 weeks. HBV TM were bled from the tail vein at different time intervals for assessments of various immunological parameters. HBV TM were sacrificed at different times after the initiation of immunization to estimate vaccine-induced cellular immune responses in the spleen and liver.

2.6. Lymphoproliferative assays

As described previously, murine lymphocytes were cultured in the absence or presence of different antigens to evaluate antigen-specific cellular immune responses (Akbar et al., 2010b; Chen et al., 2011; Yoshida et al., 2010). All cultures were performed in 96-well U-bottom plates (Corning Incorporated, New York, NY, USA). ^{3}H-thymidine (1.0 μCi/ml, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was diluted in sterile PBS, added to the cultures for the last 16 h, and harvested automatically via a multiple cell harvester (LABO MASH, Futaba Medical, Osaka, Japan) onto filter paper (LM 101–10, Futaba Medical). [^{3}H]-thymidine levels of incorporation were determined with a liquid scintillation counter (Beckman LS 6500, Beckman Instruments, Inc., Fullerton, CA, USA) at blastogenesis. Triplicate cultures were assayed routinely and the results were expressed as counts per minute (cpm). The stimulation index was calculated as the ratio of cpm obtained in the presence of antigen or antigen-pulsed DC to that obtained without antigen or in presence of only DC or irrelevant antigen-pulsed DC (i.e., control culture). A stimulation index >3.0 was considered significant.

2.7. ELISPOT assay

$CD8^+$ T lymphocytes (1×10^5) were stimulated with the antigen in presence of mitomycin C-treated spleen adherent cells in an IFN-γ coated ELISPOT plate (Mabtech, Nacka Strand, Sweden) for 24 h (Akbar et al., 2010a; Yoshida et al., 2010). Subsequently, biotinylated antibodies (2A5-biotin, Mabtech) were added into the wells. After 2 h of incubation, the plates were incubated with streptavidin–alkaline phosphatase for 1 h. After washing the plates, the substrate solution, BCIP/NBT, was added. The reaction was stopped by washing the plates extensively with tap water. The numbers of spot-forming units (SFU) were counted using an ELISPOT reader (KS ELISPOT, Carl Zeiss, Thornwood, NY, USA), and subtracted from the numbers of background SFU of control wells.

2.8. Estimation of cytokine levels

Various cytokine levels were estimated in culture supernatants using a commercial kit for the cytometric bead array method, as previously described (Akbar et al., 2010b; Yoshida et al., 2010). Cytokines levels were calibrated to the mean fluorescence intensities of the standard negative control, standard positive control, and samples with Cytometric Bead Array software (BD Biosciences Pharmingen, San Jose, CA, USA) on a Macintosh computer (SAS Institute, Cary, NC, USA).

2.9. Statistical analysis

Data are shown as mean ± standard deviation (SD). Differences were compared using the Student's *t* test. For differences determined by the *F* test, the *t* test was adjusted for unequal variances (Mann–Whitney's *U*-test). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of specificity of the experimental system

To assess the specificity of the experimental system, we immunized normal C57BL/6 mice twice with 10 μg of HBsAg or 10 μg of HBcAg, or 10 μg of PDC to induce antigen-specific lymphocytes in normal mice. Antigen-pulsed DC were prepared by culturing spleen DC from normal C57BL/6 mice with different antigens. HBsAg-, HBcAg-, and PDC-pulsed DC induced significant lymphoproliferation in mice immunized with HBsAg-, HBcAg-, and PDC, respectively. However, HBsAg-specific lymphoproliferation was not detected in mice immunized with HBcAg-pulsed DC or PDC-pulsed DC. Additionally, HBcAg and PDC-specific lymphocytes could not be retrieved from mice immunized with non-relevant antigens (Table 1). HBcAg-pulsed DC induced significantly higher levels of antigen-specific lymphoproliferation compared to HBsAg-pulsed DC (stimulation index, 31 ± 5 versus 15 ± 3.2, $N = 5$, $p < 0.05$) (Table 1).

After assessing the immunogenecity of antigen-pulsed DC of normal C57BL/6 mice *in vitro*, we prepared antigen-pulsed DC from HBV TM. Antigen-pulsed DC from HBV TM produced significantly higher levels of IFN-γ and IL-12 compared to unpulsed DC ($p < 0.05$). They also induced antigen-specific lymphoproliferation in normal mice immunized with the respective antigens (data not shown).

3.2. HBsAg negativity and anti-HBs production by antigen-pulsed DC

To assess if antigen-pulsed DC were capable of inducing HBsAg negativity and anti-HBs production in HBV TM, we checked HBsAg and anti-HBs in these mice at different times after vaccinations. HBsAg and anti-HBs levels were assessed in the sera of HBV TM before (0), and after 2, 4, and 6 vaccinations with various combinations of antigen-pulsed DC. All HBV TM expressed HBsAg in the sera, and anti-HBs were not detected in any of the mice prior to vaccination. In each group, 15 HBV TM were included for analyses.

Immunization of HBV TM with unpulsed DC or PDC-pulsed DC did not result in significant alteration in serum HBsAg levels.

Table 1
Antigen-specific proliferation of T cells by antigen-pulsed dendritic cells.

Lymphocytes	Dendritic cells (DC)	Stimulation index
HBsAg-immunized mice	PDC-pulsed DC	1.0
	HBsAg-pulsed DC	15 ± 3.2*
	HBcAg-pulsed DC	1.7 ± 0.5
HBcAg-immunized mice	PDC-pulsed DC	1.0
	HBsAg-pulsed DC	1.9 ± 0.5
	HBcAg-pulsed DC	31 ± 5.2*
PDC-immunized mice	HBsAg-pulsed DC	1.0
	HBcAg-pulsed DC	1.8 ± 0.6
	PDC-pulsed DC	9.2 ± 2*

Normal C57BL/6J mice were immunized with HBsAg, HBcAg, and PDC. Antigen-pulsed DC were prepared by culturing DC with different antigens, as described in the Section 2. Mice were sacrificed 4 weeks after the second immunization, and spleen cells were stimulated with different types of DC. The levels of blastogenesis in cultures containing T cells and irrelevant antigen-pulsed DC were regarded to as a stimulation index of 1.0. Data are presented as mean and standard deviation of five separate experiments. Stimulation index > 3.0 was regarded to as significant antigen-specific proliferation. *<0.05 vs.T cells stimulated with irrelevant antigen-pulsed DC.

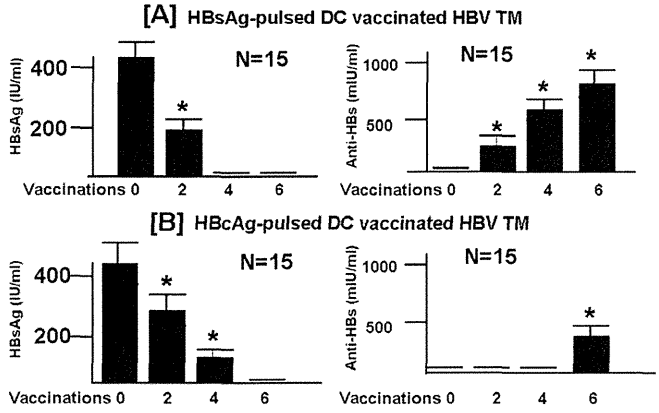

Fig. 1. HBsAg negativity and development of anti-HBs in HBV TM immunized with (a) HBsAg-pulsed DC ($n = 15$) and (B) HBcAg-pulsed DC ($n = 15$). HBsAg and anti-HBs were estimated in the sera by chemiluminescence enzyme immunoassay and expressed as IU/ml and mIU/ml, respectively. Data are presented as mean and standard deviation. 0, 2, 4, and 6 represent before vaccination, after two vaccinations, after four vaccinations and after six vaccinations, respectively. $^*p < 0.05$ compared to before vaccination (0).

Additionally, anti-HBs were not detected in HBV TM immunized with unpulsed DC and PDC-pulsed DC (data not shown).

HBsAg and anti-HBs levels at different time points after administration of antigen-pulsed DC are presented in Fig. 1. Eight of the 15 HBV TM immunized with HBsAg-pulsed DC became negative for HBsAg after two vaccinations, and all HBV TM became negative for HBsAg after four vaccinations. High levels of anti-HBs were induced in HBV TM after six vaccinations with HBAg-pulsed DC (Fig. 1A).

Interestingly, immunization with HBcAg-pulsed DC also resulted in a downregulation of HBsAg in HBV TM. All HBV TM immunized with HBcAg-pulsed DC became negative for HBsAg after six vaccinations. Anti-HBs were also detected in all HBV TM immunized with six vaccinations with HBcAg-pulsed DC (Fig. 1B).

3.3. Proliferation of antigen-specific spleen T lymphocytes in HBV TM due to immunization with antigen-pulsed DC

In order to develop insights about role of antigen-pulsed DC on proliferative responses of T lymphocytes, *in vitro* studies were accomplished with spleen T lymphocytes of HBV TM. Six vaccinations with unpulsed DC or PDC-pulsed DC did not induce HBsAg- and HBcAg-specific lymphocytes in HBV TM, as the spleen T lymphocytes of these mice did not show significant proliferation following stimulation with HBsAg or HBcAg *in vitro* (data not shown).

Antigen-specific proliferation of spleen T lymphocytes in HBV TM immunized with HBsAg-pulsed DC is presented in Table 2, panel A. The proliferation levels of T lymphocytes before vaccination were considered a stimulation index of 1.0. T lymphocytes from the spleen of HBV TM immunized with HBsAg-pulsed DC exhibited significant T cell proliferative responses to HBsAg (stimulation index 17.4 ± 4.3, $n = 5$). However, T lymphocytes of HBV TM immunized with HBsAg-pulsed DC did not demonstrate any HBcAg-specific immune responses (Table 1, panel A). Conversely, spleen T lymphocytes from HBV TM immunized with HBcAg-pulsed DC showed significant levels of proliferation in response to both HBsAg (stimulation index, 28.4 ± 3.8, $N = 5$) and HBcAg (stimulation index, 41.2 ± 4.1, $N = 5$).

3.4. Immunization of HBV TM with HBcAg-pulsed DC induced HBcAg- and HBsAg-specific IFN-γ producing CD8$^+$ cytotoxic T lymphocytes (CTL) in the liver

To compare the capacities of HBsAg-pulsed DC or HBcAg-pulsed DC to induce antigen-specific immune responses in the liver of

Table 2
Antigen-specific T cells in the spleen and the liver of HBV TM immunized with antigen-pulsed DC.

HBV TM immunized with	HBsAg-specific T cell proliferation	HBcAg-specific T cells proliferation
(A) *Antigen-specific T cells in the spleen of HBV TM immunized with antigen-pulsed DC*		
HBsAg-pulsed DC	17.4 ± 4.3	1.2 ± 0.4
HBcAg-pulsed DC	28.4 ± 3.8	41.2 ± 4.1
HBV TM immunized with	HBsAg-specific ELISPOT	HBcAg-specific ELISPOT
(B) *IFN-γ-secreting CD8 + T-cells in the liver of HBV TM immunized with antigen-pulsed DC*		
Unpulsed DC	13 ± 4	11 ± 3
PDC pulsed DC	13 ± 3	12 ± 3
HBsAg-pulsed DC	213 ± 23*	17 ± 6
HBcAg-pulsed DC	453 ± 32*	623 ± 38*

Hepatitis B virus (HBV) transgenic mice (TM) were injected with unpulsed DC, pyruvate dehydrogenase complex (PDC)-pulsed DC, hepatitis B surface antigen (HBsAg)-pulsed DC, or hepatitis B core antigen (HBcAg)-pulsed DC, six times every 2 weeks. HBV TM were sacrificed 2 weeks after the last vaccination.
Panel A: Spleen T cells were evaluated for antigen-specific proliferation in lymphoproliferative assay. The proliferation levels of T lymphocytes before vaccination were regarded as stimulation index of 1.0. Data are presented as mean and standard deviation of five separate experiments.
Panel B: Liver CD8$^+$ T cells were stimulated with HBsAg or HBcAg on an ELISPOT plate to assay IFN-γ production. Data are presented as mean and standard deviation of five separate experiments. *<0.05 vs. HBV TM immunized with unpulsed DC or PDC-pulsed DC.

HBV TM, HBsAg-specific and HBcAg-specific CTL were enumerated among liver NPC. Immunization of HBV TM with unpulsed DC or PDC-pulsed DC did not induce significant numbers of CTL (i.e., IFN-γ producing CD8$^+$ T) in the liver following stimulation with HBsAg or HBcAg. Considerable numbers of CTL were detected among liver CD8$^+$ T lymphocytes of HBV TM immunized with HBsAg-pulsed DC following stimulation with HBsAg *in vitro* (213 ± 23, $N = 5$) but not with HBcAg (17 ± 6, $N = 5$) (Table 2, panel B). Conversely, very high proportions of both HBcAg-specific CD8$^+$ CTL (623 ± 38, $N = 5$) and HBsAg-specific CD8$^+$ CTL (453 ± 32, $N = 5$) were detected in the liver of HBV TM immunized with HBcAg-pulsed DC (Table 2, panel B).

3.5. Increased production of proinflammatory cytokines by liver NPC from HBV TM immunized with HBcAg-pulsed DC compared to those immunized with HBsAg-pulsed DC

To evaluate cytokine production by liver NPC from HBV TM immunized with HBsAg-pulsed DC or HBcAg-pulsed DC, Liver NPC from HBV TM immunized with HBsAg- or HBcAg-pulsed DC were cultured with HBsAg or HBcAg. IFN-γ and TNF-α levels were significantly higher in HBV TM immunized with HBsAg- or HBcAg-pulsed DC compared to unpulsed HBV TM ($p < 0.05$) (Fig. 2). However, the levels of both cytokines were significantly higher in HBV TM immunized with HBcAg-pulsed DC compared to those immunized with HBsAg-pulsed DC ($p < 0.05$) (Fig. 2).

3.6. Increased activation of endogenous DC via administration of HBcAg-pulsed DC in HBV TM

Although antigen-pulsed DC induced antigen-specific T lymphocytes in HBV TM, it was necessary to assess if antigen-pulsed DC can activate endogenous DC of these mice. Four weeks after six vaccinations with antigen-pulsed DC in HBV TM, DC were isolated from HBV TM to assess the functional capacities of endogenous DC. DC were cultured with allogenic T lymphocytes from

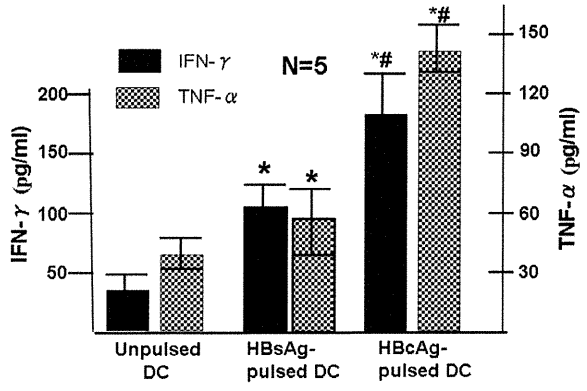

Fig. 2. Significantly higher levels of IFN-γ and TNF-α production via liver NPC in HBV TM immunized with HBcAg-pulsed DC compared to those immunized with HBsAg-pulsed DC. Liver NPC from HBV TM immunized with unpulsed DC or HBsAg-pulsed DC or HBcAg-pulsed DC were cultured with HBsAg or HBcAg and the levels of IFN-γ and TNF-α were estimated by CBA method. Mean and standard deviation of IFN-γ (black bar) and TNF-α (checkered bar) levels produced via liver NPC following immunization with unpulsed DC, HBsAg-pulsed DC, and HBcAg-pulsed DC in five separate experiments. $^{*}p < 0.05$ vs. HBV TM immunized with unpulsed DC. $^{\#}p < 0.05$ vs. HBV TM immunized with HBsAg-pulsed DC.

C3H/He mice, and T cell proliferation levels in allogenic MLR and cytokines in culture supernatants were estimated. The allostimulatory capacities of DC were significantly higher in HBV TM immunized with HBsAg- or HBcAg-pulsed DC compared to DC from HBV TM immunized with unpulsed DC (Fig. 3). HBV TM immunized with HBcAg-pulsed DC showed significantly higher T cell proliferation levels than DC from HBV TM immunized with HBsAg-pulsed DC (Fig. 3A).

As shown in Fig. 3B, IFN-γ and TNF-α levels were also significantly higher in culture supernatants of allogenic MLR containing DC from HBV TM immunized with HBsAg- or HBcAg-pulsed DC compared to those containing DC from HBV TM immunized with unpulsed DC ($p < 0.05$). The levels of IFN-γ were significantly higher in cultures containing DC from HBcAg-pulsed immunized HBV TM compared to HBsAg-pulsed immunized HBV TM ($p < 0.05$).

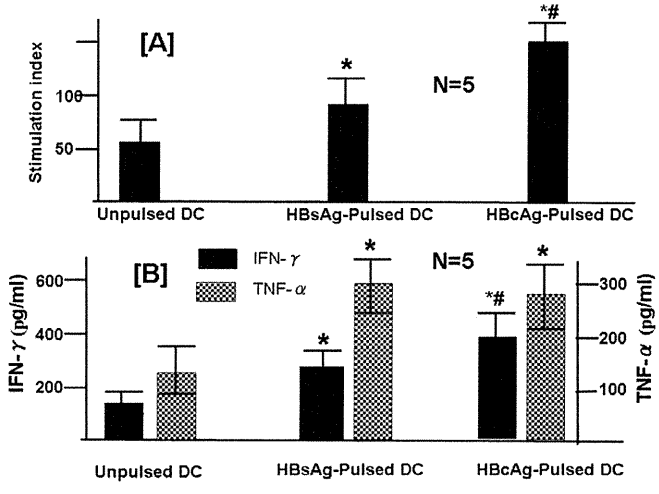

Fig. 3. Increased immunogenicity of DC in HBV TM immunized with HBcAg-pulsed DC compared to HBV TM immunized with HBsAg-pulsed DC. DC were cultured with allogenic T lymphocytes from C3H/He mice, and T cell proliferation levels in allogenic MLR and cytokines in culture supernatants were estimated. (A) Allostimulatory capacity of DC from HBV TM immunized with unpulsed DC or HBsAg-pulsed DC or HBcAg-pulsed DC. (B) Production of IFN-γ (Black bar) and TNF-α (checkered bar) in culture containing DC from HBV TM immunized with unpulsed DC or HBsAg-pulsed DC, or HBcAg-pulsed DC. Data are presented as mean and standard deviation of the stimulation index and cytokines in five separate experiments. $^{*}p < 0.05$ vs. HBV TM immunized with unpulsed DC. $^{\#}p < 0.05$ vs. HBV TM immunized with HBsAg-pulsed DC.

4. Discussion

Antigen-based immune therapy (vaccine therapy) has emerged as a potential therapeutic approach for CHB patients, as it is based on the concept of controlling HBV replication and preventing liver damage in CHB by inducing and maintaining HBV-specific immune responses. Investigators have shown that non HBV-specific immune responses are mainly responsible for impaired control of HBV replication and progressive liver damages in CHB patients, whereas, HBV-specific immune responses, especially HBcAg-specific CTL, are related to control of HBV replication and containment of liver damages in CHB patients (Bertoletti and Maini, 2000). CHB patients that are capable of controlling HBV replication and liver damage harbor higher frequencies of HBV-specific immunocytes, especially HBcAg-specific CTL, compared to those that express high levels of HBV and have progressive liver damage (Maini et al., 2000). These facts show that vaccine therapy can be regarded as an evidence-based therapeutic approach for CHB patients, however, there is still controversy regarding the therapeutic strategies of using vaccines. Although several variables may be important in this context, such as the nature of the antigen, dose of antigen, duration of therapy, and nature of adjuvant, it is of utmost importance to develop further insight regarding the nature of antigens that are proposed to be used as therapeutic vaccines in CHB. After the first clinical trial on HBsAg-based vaccine therapy conducted by Pol et al. (1994), several studies during the last 15 years have pointed to the inherent limitations of HBsAg-based vaccines, even if these therapies are given in combination with antiviral drugs (Pol et al., 2001; Vandepapelière et al., 2007) or loaded on DC or other immunocytes (Akbar et al., 2010a). Both Hoa et al. (2009) and Akbar et al. (2010a) found that HBsAg-based vaccine therapy induced HBsAg-specific immunity and anti-HBs in some CHB patients, but these were not translated into therapeutic efficacy, as adequate levels of HBcAg-specific immunity was not induced.

The data from these studies suggests that HBcAg-pulsed DC are capable of: (1) inducing HBsAg negativity in the sera, (2) developing anti-HBs in the sera, and (3) inducing both HBsAg and HBcAg-specific T cells and CTL in the spleen and the liver. The induction of HBcAg-specific immunity was expected in HBV TM immunized with HBcAg-pulsed DC. However, the effects of HBcAg-pulsed DC on HBsAg-specific immunity in HBV TM is worthy of consideration in the context of immune therapy. Our data corroborates previous reports on the wide-spread immunomodulatory capacities of HBcAg as an adjuvant to HBsAg-specific immunity (Lobaina et al., 2005; Aguilar et al., 2004).

In the present study, we also explored the mechanisms underlying the immunomodulatory effects of HBcAg. It was found that the strong immunomodulatory capabilities of HBcAg may be due to an establishment of an inflammatory hepatic microenvironment, induction of HBcAg-specific CTL in the liver, and activation of host DC. Lee et al. (2009) has reported that HBcAg activates innate immunity. Although we have not checked activation levels of cells of innate immunity in this study, increased production of pro-inflammatory cytokines by liver NPC of HBV TM immunized with HBcAg-pulsed DC compared to those immunized with HBsAg-pulsed DC provide an indirect support for the notion that innate immunity may be stimulated by HBcAg. However, this remains to be confirmed in future in more details.

Taken together, HBcAg should be an integral part of a therapeutic vaccine against chronic HBV infection. However, factors, such as the dose of antigen and duration of therapy, should be properly determined prior to the development of therapeutic vaccines

against CHB. In a previous report (Akbar et al., 2010b), we could not induce HBsAg-specific immune responses by HBcAg-based immunization by loading spleen DC with 10 μg of HBcAg and administrating 2 million DC twice. In this study, we loaded DC with 50 μg of HBcAg and administered 5 million HBcAg-pulsed DC six times. Thus, additional studies would be required to determine the influence of factors, such as dose and duration of therapy, in restoring immunity in CHB.

The clinical utility of the data presented herein may not be translatable to the human condition, as there are fundamental differences between HBV TM and CHB patients. In addition, HBV TM do not demonstrate all of the different features of HBV-related pathogenesis, as they have no evidence of liver injury and exhibit very low levels or almost no circulating HBV DNA. Thus, the implications of these findings need to be confirmed in CHB patients, and the role of HBcAg should be further assessed in humans. The major limitation of the present study lies in the fact that human consumable and commercially developed HBcAg are seldom available for clinical trials in human. To address this issue, we have been conducting a clinical trial with a human consumable HBcAg/HBsAg conjugate vaccine in CHB patients. Preliminary outcomes suggest that the HBsAg/HBcAg-based vaccine induces HBV negativity in 50% of subjects, diminishes liver damage in almost all of the patients, and induces HBsAg- and HBcAg-specific immune responses (Akbar et al., 2010c). However, the relative contribution of HBsAg and HBcAg in this protocol could not be assessed properly. A future study has been designed in which only human consumable HBcAg will be used as a therapeutic vaccine in CHB.

It is still unclear whether HBcAg-based or a conjugate vaccine containing both HBcAg and HBsAg may be required in the design of an evidence-based immunotherapeutic approach against CHB. The findings of the present study, as well as the clinical observations with the HBcAg/HBsAg-based vaccine (Akbar et al., 2010c) in CHB, indicate that HBcAg should be an integral part of a therapeutic vaccine against CHB.

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Dendritic Cell-Based Immune Therapy in Liver Diseases

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Abstract: The field of immune therapy is currently undergoing a shift in focus, from non antigen-specific immune modulator-based immune therapy to antigen-based vaccine therapy to more sophisticated cell-based vaccine applications. Dendritic cells (DCs) are rare leukocytes that are uniquely potent in their ability to capture, process and present antigens to T cells. By culturing DCs with viral antigens or tumor-associated antigens or different cellular products, immunogenic or tolerogenic DCs can be produced. When antigen-pulsed DCs are administered, an increase in the functional capacities of cells of innate immune system is observed. Also, patients administered with antigen-loaded DCs exhibit an augmentation of helper T cells, cytotoxic T cells, and plasma cells activities. Patients with liver diseases exhibit distorted immune responses to invading pathogens or cancer cells or autoantigens. On the other hand, recovery from liver diseases is usually associated with restoration of host immunity. In this review, we would discuss about rationale and strategies of immune therapy including DC-based therapy in liver diseases.

Keywords: Antigen-pulsed dendritic cells, Antigen-specific immunity, Dendritic cells, Immune therapy, Liver diseases, Non antigen-specific immunity.

THE LIVER: A REGULATOR OF HOST IMMUNITY

The liver is a vital organ that performs a variety of functions. In an adult, it weighs about 1500 g, i.e., approximately one-fortieth of total adult body weight. However, the liver is a highly vascular organ that receives one-quarter of total circulating blood flow, with oxygenated blood being delivered through the systemic circulation, a phenomenon similar to other organs. The liver also receives venous blood *via* the portal system. Thus, various noxious materials enter the liver together with gut-derived portal venous blood. In addition to synthetic, metabolic and excretory functions, it is also an immunologically active organ. The liver harbors immunocytes of both the innate and adaptive immune systems. It contains considerably higher proportions of natural killer (NK) cells, the representative cells of the innate immune system, as compared to other parenchymal tissues and lymphoid organs. Moreover, large numbers of lymphocytes reside in the liver with their frequencies elevated several fold in many pathological conditions. In addition, specialized cells with certain immune regulatory functions are also present in the liver. The microenvironment of the liver allows different immunocytes to establish close contact with vascular endothelium and hepatocytes for designing the nature of hepatic immunity.

The liver is regarded as a tolerogenic organ and has been designated the graveyard of lymphocytes [1, 2]. Several food ingredients and toxic materials that enter the liver through the portal circulation are detoxified in the liver without inducing immune responses. In addition, the liver handles a variety of metabolic products without inducing aberrant immune responses. In the context of transplantation, liver transplants are usually accepted in the recipients in spite of HLA mismatch. This is because different immune regulatory cells of the liver also bear tolerogenic properties.

Despite the dominant tolerogenic properties of the liver, its microenvironment also supports immune responses and inflammatory processes. Hepatocytes are damaged and destroyed in different pathological conditions, mainly, if not solely, by immune-mediated mechanisms [3]. Immune-mediated damage and destruction of hepatocytes may continue for decades; with the ultimate outcome being the progression of hepatic fibrosis, distortion of lobular hepatic architecture of the liver, and carcinogenesis of hepatocytes.

ANTIGEN-PRESENTING DENDRITIC CELLS (DCS)

DCs are regarded as being the most potent professional antigen-presenting cells (APCs). Although many other APCs are able to present antigens, DCs are especially efficient at presenting antigenic peptides to naïve immunocytes for the induction of primary immune responses [4, 5]. After the discovery of DCs by Steinman and Cohn in 1973, thousands of reports have been published about the ontogeny, phylogeny, subtypes, localization, and function of DCs. Some features of DCs are cited in relevant chapters of this issue, and so we will not provide a detailed description of these cells. However, relevant points relating to DCs that are important in the development of DC-based therapy will be discussed here. DCs are bone-marrow-derived immunocytes widely distributed in almost all tissues of the body. DCs are also a member of the innate immune system. They migrate quickly to tissues that are infiltrated with harmful agents. Large numbers of DCs are also detected in inflamed mucosal tissues where they also produce varieties of cytokines including type-1 interferon (IFN). DCs act as bridges

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1573-3955/12 $58.00+.00

between the innate and the adaptive immune system. These cells are capable of recognizing microbial agents, harmful entities, altered cells, and malignant cells. Recognition of harmful, dangerous and non-dangerous entities by DCs is especially important for the development of DC-based therapy. After recognition, different agents are internalized and processed by the DCs. These events may cause phenotypic and functional alterations of the DCs. The levels of maturity of the DCs are dependent on the nature of the DCs, the nature of the antigens, the local microenvironment and several other factors; some of which are yet to be explored. DCs with antigenic peptides usually migrate to lymphoid tissues and interact with clonally selected immunocytes to induce antigen-specific immune responses or immune tolerance. The efficacy of DC-based therapy is dependent on (1) the efficacy of antigen capture by the DCs, (2) cleaving of the antigen into epitopes within the DCs, (3) intracellular migration of antigenic epitopes, (4) expression of antigenic epitopes on the DCs, (5) migration of the DCs to lymphoid tissues, and (6) functional abilities of the DCs to induce immune responses and immune tolerance. In addition, proper functioning of effecter immunocytes in a purposeful manner also determines the therapeutic capacities of DC-based immune therapy. Induction of immune tolerance seems to be the main function of DCs in normal conditions. DCs are capable of maintaining homeostasis in normal conditions and they also respond to danger signals in the case of pathological conditions. In fact, DCs regulate host immunity in both health and disease.

DC-BASED THERAPY IN LIVER DISEASES

The pathogenesis, clinical presentations, therapy, and prognosis of liver diseases are variable. Autoimmune mechanisms seem to be related with the pathogenesis of autoimmune hepatitis (AIH), although the exact nature of these is yet to be clarified. Immune-mediated mechanisms are responsible for liver damage and persistence of viral replication in patients with chronic hepatitis B (CHB) and chronic hepatitis C (CHC) [3]. Like most cancers, improper host immunity may underlie the pathogenesis of hepatocellular carcinoma (HCC). Taken together, various pathological processes underlie the pathogenesis of different liver diseases. However, it is expected that critical questions would arise if immune therapy were considered a practical and rational scientific approach to treat these diseases. To address this basic query, we will discuss the genesis of immune therapy in liver diseases. Finally, the concept of DC-based therapy will be described with recent progress in this field.

Limited Therapeutic Efficacies of Traditional Therapeutic Regimens Against Chronic Liver Diseases and the Emergence of the Concept of Immune Therapy as an Alternate Therapeutic Approach

Present concepts of immune therapy against liver disease involve either upregulating or down regulating host immunity with immune therapy because studies have shown that immune responses of these patients are decreased, impaired or distorted. The exact nature of the immune response in patients with AIH is not completely understood at this point; however, circumstantial evidence indicates that the magnitudes of several parameters of immune responses are exacerbated in these patients. Although immune suppressor agents down regulate the progression of liver disease in AIH, these drugs are usually given over a prolonged period of time or even throughout entire life [6]. However, prolonged use of immune suppressor drugs in AIH is endowed with severe side effects. In other words, non antigen-specific immune therapy by immune suppressive agents seems to have a limited effect in AIH. Because little is known about target antigens in patients with AIH, antigen-specific therapeutic interventions have not been applied in these patients. In this context, the exact nature of DC-based immune therapies in patients with AIH is still elusive. Tolerogenic DCs may be used in patients with AIH, but this type of immune therapy is not expected to yield better outcomes because generalized immune suppression by tolerogenic DCs may not be a better therapeutic strategy compared to immune suppressive drugs. However, if a new set of tolerogenic DCs can be developed that target only the liver or liver-related antigens, the arena of DC-based therapy may be expanded for all patients with AIH.

The concept of DC-based therapy in patients with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections has emerged from issues different to that of DC-based therapy for AIH. Approximately 2 billion people in the world bear evidence of past or ongoing infections with HBV. However, considerable levels of liver damage are detected in only some HBV-infected subjects. The replication of HBV and HBV-induced liver damage are strictly regulated by the host immune system. In some individuals, HBV replication remains at low levels for decades without any features of progressive liver damage. In others, liver damage is not significant even in the presence of high HBV replication. However, some patients with chronic HBV infection exhibit considerable liver damage with high, moderate or low levels of HBV replication. It is usually assumed that host immune responses regulate the extent of HBV replication and liver damage. However, little has been clarified about the nature of beneficial and detrimental immune responses in patients with CHB. Despite this, several antiviral agents have been used in these patients for containment of HBV replication and for minimizing liver damage. A final assessment has shown that these drugs produce limited therapeutic efficacies and cause significant side effects [7]. Some drugs that block HBV replication by inhibiting DNA polymerase may be used over a prolonged period or for the entire life of the patient. In addition, the use of these drugs is related to the emergence of mutant HBV that has the potential to cause severe liver damage.

The scenario of therapeutic options for patients with chronic hepatitis C (CHC) is more or less similar to that of patients with CHB, yet antiviral drugs do exhibit considerable potential for treating these patients [8]. However, patients infected with certain HCV genotypes and with very high levels of HCV RNA respond poorly to antiviral drugs. In addition, maintenance of sustained therapeutic effects in HBV and HCV-infected subjects represent a major challenge for clinicians.

Patients with HCC are traditionally treated by surgical resection, medical ablation of the cancer mass of various

natures, anti-cancer drugs, and radiotherapy. However, recurrence of HCC nodules and unsatisfactory prognosis of conventional therapeutic approaches also suggests that alternative therapy should be developed for treating HCC.

Taken together, the present regimens of different therapeutic interventions seem to have limited efficacy along with considerable side effects. Considering the burden of liver diseases on national and international health care delivery systems, the need for novel and alternative therapeutic approaches is paramount for patients with liver disease. As host immunity regulates the pathological processes of different liver diseases, immune-based therapy has been applied in these patients for the last 2-3 decades. However, poor clinical outcomes of ongoing regimens of immune therapy are also not inspiring in patients with liver disease. These realities have unmasked a new type of cell-based immune therapy for patients with liver disease using DCs as an adjuvant (DC-based therapy). Before we introduce DC-based therapy for liver disease, the scope and limitation of other immune-based protocols in liver disease will first be discussed.

Non Antigen-Specific Immune Therapy in Liver Disease

Limited efficacy and considerable side effects of antiviral drugs in patients with chronic HBV and HCV infection has led to investigations into alternative therapeutic approaches. As the acquisition and progression of chronic HBV and HCV infection as well as the ultimate prognosis of HCC are mainly regulated by nature and the magnitude of the host immune response, the concept of immune-based therapy has surfaced. Initially, it was shown that host immunity is diminished in patients with chronic HBV and HCV infections. Accordingly, immune therapy was accomplished in these patients through the administration of agents that upregulate host immune responses. A group of immune modulatory agents, such as cytokines, growth factors, and immune modifying agents were applied (Table **1**). Unfortunately, it was difficult to assess the true therapeutic potential of these immune modulators in HBV and HCV-infected subjects [9-19] because most of these studies were conducted as pilot studies and the long-term effects, especially off-treatment efficacies of these agents were not properly reported. Few clinical trials have been accomplished with non antigen-specific immune modulators in patients with HCC.

Antigen-Specific Immune Therapy in Chronic Liver Diseases

It has recently become evident that antigen-specific immune modulators may be therapeutically advantageous in patients with CHB and CHC because varying magnitudes of HBV and HCV-specific immune responses have the potential to provide antiviral and liver protecting properties. The outcomes of antigen-specific immune therapy in CHB and CHC are shown in Table **2**. The utilization of antigen-specific immune responses as a therapeutic modality has been seen mostly in patients with CHB [20-25]. Clinical trials were done with a hepatitis B surface antigen (HBsAg)-based vaccine in patients with CHB. Although vaccine therapy was safe for these patients, the therapeutic outcome of this therapy was controversial. Initial studies indicate that vaccine therapy has antiviral and immune modulator potential in patients with CHB, yet other studies have not found any notable therapeutic effect in CHB. To have better therapeutic effects, antigen-specific immune therapy has also been done as combinational therapy with antiviral drugs. The outcome of combined antiviral and immune therapy seems to be better, but, off- treatment follow up data has not been published to draw a conclusion about this [26-28].

The nature of different antigens in patients with HCV infection has not been properly elucidated. Thus, there is a paucity of information about the nature of antigens in HCV

Table 1. Antigen Non-Specific Immune Therapy with Polyclonal Immune Modulators in Patients with Chronic Hepatitis Patients B and C

Immune Therapeutic Agents	Type of Study	Therapeutic Effect	References
Chronic Hepatitis B			
Interferon-gamma	Open-level trial	Less hepatic fibrosis	[9]
Interleukin-2	RCT	No effect	[10]
Interleukin-12	Phase I/II studies	No notable effects	[11]
Granulocyte-macrophage colony stimulating factor	Pilot study	Inconclusive	[12]
Thymosin-alpha 1	RCT	Moderately effective	[13]
Alpha-galactosylceramide	Phase I/II	No effect	[14]
Propagermanium	RCT	Moderate effect	[15]
Chronic Hepatitis C			
Recombinant interleukin-2	Pilot study	No added benefit	[16]
Interleukin-10	Pilot study	Dichotomy about liver damage and HCV RNA	[17]
Interleukin-11	Pilot study	Side effects: edema of lower extremities in all patients	[18]
Interleukin-12	Open level study	Poorly tolerated and lack of notable response	[19]

Immune therapy with non antigen-specific immune modulators in patients with chronic hepatitis B and chronic hepatitis C. *RCT; Randomized-controlled trial.

Table 2. Immune Therapy with Hepatitis B Vaccine and Combination of Antiviral and Immune Therapy in Patients with Chronic Hepatitis B

Type of Study	Nature of Vaccine	Therapeutic Effect	References
Vaccine Monotherapy			
Pilot study	HBsAg-based,	Moderate effect	[20]
Pilot study	HBsAg-based,	Limited effects	[21]
Pilot study	HBsAg-based	Inconclusive	[22]
Pilot study	HBsAg-based	Non-effective	[23]
Pilot study	HBcAg epitope,	Ineffective	[24]
Pilot study	DNA vaccine	Immunogenic	[25]
Combination Therapy: Vaccine Plus Antiviral or Immune Modulator			
Pilot studies	Lamivudine plus HBsAg-based vaccine	Effective during therapy	[26]
Pilot study	Clevudine plus HBsAg-based vaccine	Effective	[27]
Randomized-controlled trial	HBsAg-based vaccine plus lamivudine	Ineffective	[28]

infection that have the potential to induce therapeutic immunity in patients with CHC. However, studies have assessed the therapeutic efficacies of antigen-based or HCV DNA vaccines in patients with CHC [29-34] (Table **3**).

Patients with HCC have also been treated with HCC-related antigens. Even though these antigens are expressed in the liver of the patients, the therapeutic effects of immune responses against these antigens are not clearly known.

CONCEPT OF DC-BASED IMMUNE THERAPY IN LIVER DISEASES

Different clinical trials in patients with CHB, CHC and HCC during the last 30 years has revealed that antigen-specific immune therapies may have better therapeutic potential, yet the ongoing regimen of these therapies is still not satisfactory (Tables **2** and **3**). The positive point in antigen-specific immune therapy lies in the fact that these therapeutic modalities are safe for human use. The major limitation of these immune-based therapeutic approaches is related to their divergent effects in different clinical trials.

Finally, studies on the mechanisms of action of immune therapy and contemporary interventions targeting cellular and molecular mechanisms of immune responses have revealed that the mere administration of antigens may not be the best approach for inducing responses against tolerogenic antigens in patients with liver disease. The ongoing regimens of antigen-specific immune therapy have mainly been accomplished in patients with CHB by administrating a commercially available HBsAg-based vaccine emulsified in different adjuvants. On the other hand, the antigens that have been used in CHC and HCC are HCV-related and HCC-related. These antigens have been administered to patients with liver disease with the expectation that APC-like DCs would recognize these antigens. These antigens would then be processed and presented by DCs for the induction of antigen-specific immunity. Finally, it has been presumed that immune responses to administered vaccines and antigens would control viral replication, liver damage or liver cancer. However, several other variables relating to the induction and functioning of therapeutic immunity in these diseases have not been properly considered during the design of such antigen-specific immune therapies against liver diseases. These variables will be discussed later in the context of (1) the nature of antigens, (2) the nature of adjuvants, (3) the route of administration of antigens, (4) protective effects of immunity, and (5) the microenvironment of patients with liver disease.

In addition to these variables, several studies have shown that the antigen processing and presentation capacities of APCs, especially those of DCs, are impaired in patients with

Table 3. Antigen-Specific Immune Therapy in Patients with Chronic Hepatitis C

Type of Study	Nature of Vaccine	Therapeutic Effect	References
Vaccine Monotherapy			
Core protein-derived vaccine	Phase I study	Safe, immunogenic, insignificant virological responders	[29]
HCV 1b-derived peptide	Phase I study	Limited effect	[30]
Synthetic peptide vaccine IC41	Phase II study	Limited effect	[31]
V-5 Immunitor	Open-level trail	Limited effect	[32]
CIGB-230 DNA vaccine	Phase I clinical trial	Inspiring outcome	[33]
Envelope protein E1	Pilot study	Inspiring outcome	[34]

almost all liver diseases [4]. In some cases, the expression of costimulatory antigens are reduced, in others, the functions of DCs are impaired. Furthermore, DCs do not necessarily have potent migratory capacities, or they might undergo apoptosis. These limitations should be addressed when designing immune therapeutic protocols, the efficacy of which will not be dependent on endogenous DCs of the patients with liver disease. This can be addressed, at least to a reasonable level, by preparing antigen-pulsed DCs. In this scenario, DCs are cultured with antigens *in vitro*. DCs engulf antigens and process them during culture, an environment different to the *in vivo* environment of these patients [4, 5]. In fact, studies have shown that when immune responses are not induced after administrating antigen and adjuvant alone, they can be induced if antigens are loaded on DCs. These observations provided the rationale for implementing DC-based therapy in liver disease.

DC-BASED IMMUNE THERAPY IN LIVER DISEASE

Chronic HBV Infection

In Vitro Studies

Several investigators have prepared HBV-related antigen-pulsed DCs *in vitro* and checked their immune modulator capacities in culture. In most cases, HBsAg was used to load the DCs. Use of HBsAg was done mainly for the following reasons:

1. Human consumable forms of HBsAg are commercially available.
2. Patients with CHB exhibit impaired humoral and cellular immune responses to HBsAg.
3. Antibody to HBsAg (anti-HBs) represents a protective antibody against the HBV.
4. Development of anti-HBs in the sera of patients with chronic HBV infection, either due to therapy or spontaneously, is related to the recovery from disease.

The protective role of HBsAg-specific immune responses in HBV uninfected normal subjects is well known. However, it is not clear whether HBsAg-specific immune responses have therapeutic roles in subjects with chronic HBV infection. *In vitro* studies have documented that HBsAg-pulsed DCs induce HBsAg-specific immune responses *in vitro* when immunocytes from murine HBV carriers, HBV transgenic mice (HBV TM) or patients with CHB were cultured with HBsAg-pulsed DCs. HBsAg-pulsed DCs also cause T helper polarization and increase production of proinflammatory cytokines from lymphocytes of HBV TM and patients with CHB [35, 36].

Preclinical Trial with HBsAg-Pulsed DCs in HBV TM

Some investigators including us have conducted preclinical studies using HBsAg-pulsed DCs in HBV TM to assess the therapeutic potential of these approaches [35, 36]. A study that we reported in 2004 showed that only 2 administrations of HBsAg-pulsed DCs induced antibody to HBsAg (anti-HBs) in almost all HBV TM that expressed considerable levels of HBsAg before therapeutic manipulation [35]. The antiviral potential of HBsAg-pulsed DCs could not be properly assessed because the levels of HBV DNA in the sera were too low in most HBV TM of this series. Moreover, the role of HBsAg-pulsed DCs on liver damage could not be explored because HBV TM does not display evidence of liver damage. Administration of HBsAg-pulsed DCs was safe for all HBV TM because elevation of alanine aminotransferase (ALT) or histological evidence of liver damage was not detected in any of the HBV TM. Shimizu *et al.* also showed antiviral potential of HBsAg-pulsed DCs in HBV TM [36].

HBsAg-pulsed DCs are also potent inducers of anti-HBs in immune suppressed HBV TM, where immune suppression has been induced by daily administration of FK-506 [37]. Thus, immune modulations by HBsAg-pulsed DCs have been confirmed in HBV TM, but inherent limitations of this model do not allow extensive analyses of the therapeutic effects of HBsAg-pulsed DCs (antiviral effect and minimizing liver damage potentiality).

HBsAg-Pulsed DCs in Patients with CHB

Only few attempts have been made to use antigen-pulsed DCs in patients with CHB. Prior to using HBsAg-pulsed DCs in patients with CHB, HBsAg-pulsed DCs were prepared for human use and administered in normal volunteers to assess the safety of this approach. HBsAg-pulsed DCs were found to be safe for all normal volunteers and no generalized or liver-related adverse effects were detected in any of the subjects [38]. Following this, HBsAg-pulsed DCs were administered to patients with CHB and their safety was confirmed in these studies [39, 40]. Regarding antiviral capacity, conflicting data prevails: Chen *et al.* showed that HBV DNA was reduced in patients with CHB after immunization with HBsAg-pulsed DCs [39]. However, we did not find any significant reduction of HBV DNA from the administration of HBsAg-pulsed DCs in patients with CHB [40]. HBsAg-specific humoral and cellular immune responses have been detected in some patients with CHB due to administration of HBsAg-pulsed DCs, yet without any visible antiviral effects [40].

HBcAg-Pulsed DCs and their Immunogenicity *In Vitro* and *In Vivo*

HBcAg-pulsed DCs have also shown potent immune modulatory capacities *in vitro*. There is paucity of information about the therapeutic effects of HBcAg-pulsed DCs *in vivo*. One study recently reported that DCs pulsed with HBsAg and HBcAg are capable of inducing HBcAg-specific cellular immunity in HBV TM [41]. Human trials with DCs pulsed with peptides of HBsAg and HBcAg have been reported with some clinical efficacy in HBeAg-negative subjects [42].

HCV Related Antigen-Pulsed DCs and their Immunogenicity *In Vitro* and *In Vivo*

Murine DCs have been loaded with IFN alpha or ribavirin or both. In addition, antigen-pulsed DCs have been prepared by culturing DCs with NS3, NS5a antigen, NS5a mRNA, or core antigens of HCV. HCV antigen-pulsed DCs induced antigen-specific immunity in normal mice and in

mice with HCV infections. Furthermore, phase I clinical trials with HCV antigen-pulsed DCs in patients with CHC has been reported [43-45]. However, it is still elusive as to the nature of HCV-related antigens that should be used for loading DCs for therapeutic purposes because some HCV antigens such as core antigen and NS3 antigen have been shown to block DC maturation [46].

DC-Based Therapy in Patients with HCC

Without treatment, the 5-year survival rate of HCC is less than 5%. Treatment of HCC depends on the presence of comorbidity; tumor size, location and morphology; and the presence of metastatic diseases. Complete surgical resection followed by hepatic transplantation offers the best long-term survival, but this can be applied in few patients. All other therapies are palliative. At present, radiofrequency ablation, ethanol injection, chemoembolization, selective internal radiation therapy, and systemic administration of biological and chemotherapeutic agents are used to treat HCC patients (reviewed in reference [47]).

As a part of DC-based therapy, DCs have been loaded with HCC-related antigens by various methods to prepare HCC-related antigen-pulsed DCs [48-51]. As a source of HCC-related antigens, either HCC cell lines or HCC-related antigens have been used. Pulsing has been done by cell culture methods or by transfection techniques. In some cases, DCs have been fused with HCC cell lines or HCC lysates. *In vitro* studies have shown that HCC-pulsed DCs express various costimulatory molecules and produce proinflammatory cytokines. Moreover, these DCs induce antigen-specific immunity when administered to animal models of HCC. HCC-pulsed DCs have also been administered in patients with HCC (Table **4**). The data shows that HCC-pulsed DCs induce antigen-specific immunity, cause better survival, and induce decreased size of HCC nodules [52-58]. Antigen-pulsed DCs have been administered in patients with HCC using various routes. These include parental as well as intratumoral administration of antigen-pulsed DCs in patients with HCC.

DC-Based Therapy in Autoimmune Liver Diseases

DC-based therapy has been accomplished in animal models of autoimmune liver diseases. In a preliminary study, we prepared an animal model of primary biliary cirrhosis (PBC). Pyruvate dehydrogenase complex (PDC)-pulsed regulatory DCs were prepared by culturing bone marrow-derived DCs with PDC and interleukin-10. These DCs were administered to a mouse model of PBC. Administration of PDC-pulsed DCs caused decreased concentration of antimitochondrial antibody (personal communication), and the extent of the PBC lesion in the liver of the mouse showed variable outcomes due to the administration of the PDC-pulsed DCs.

DC-BASED THERAPY IN LIVER DISEASE: PAST, PRESENT AND FUTURE

The concept of DC-based immune therapy in patients with liver disease is logical because distorted immune responses are seen in most of these patients. However, proper design of DC-based immune therapy is time-consuming and requires further understanding of the host immunity in liver disease. For the last three decades, the general assumption has been that immune responses are decreased in patients with CHB, CHC and HCC, and exacerbated in patients with AIH. However, this is an oversimplification of a very complex issue. In fact, not all parameters of immune responses are decreased in patients with CHB, CHC and HCC. Furthermore, not all parameters of immune responses are upregulated in patients with AIH. Patients with CHB and CHC tend to harbor an inflamed mucosal microenvironment, and proinflammatory cytokines are elevated in liver tissue in many of these patients. Patients with HCC are known to have high levels of tumor-necrosis factor (TNF), a potent proinflammatory cytokine. Regarding antigen-specific immunity, all patients with CHB express humoral immunity to hepatitis B core antigen (anti-HBc) and many patients also express antibody to hepatitis B e antigen (anti-HBe). HBsAg-specific immune responses are also detected in patients with CHB. Antibody to HBsAg (anti-

Table 4. Dendritic Cells-Based Immune Therapy in Patients with Hepatocellular Carcinoma

DC-Based Therapy	Type of Study	Therapeutic Effect	References
Chronic Hepatitis B			
HBsAg-pulsed DC	Pilot study	Antiviral potential	[39]
HBsAg-pulsed DC	Pilot study	Safe, partially immunogenic, no antiviral efficacy	[40]
DC pulsed with epitopes of surface and core antigen of HBV	Clinical trial	Moderately effective	[42]
Hepatocellular Carcinoma			
Autologous tumor-pulsed DC	Pilot study	Moderately effective	[52]
Alpha-fetoprotein pulsed DC	Phase I/II trial	Immune modulation	[53]
Pulsing of DC in cancer nodule in situ	Pilot study	Safe	[54]
Tumor lysate-pulsed DC	Pilot study	Safe and better 1-year survival	[55]
Autologous DC after conformal radiotherapy	Open level study	Safe and immunogenic	[56]
Tumor lysate-pulsed DC	Phase II study	Antitumor effects	[57]
Transcatheter embolization plus DC	Pilot study	Insufficient efficacy	[58]

HBs), although present in most patients with CHB, may not be detected by conventional techniques. Anti-HCV is so common in patients with CHC that it has been used for a long time to diagnose HCV infection. Thus, decreased non antigen-specific or antigen-specific immune responses are neither a valid concept nor a scientific logic in patients with CHB, CHC and HCC. The present scientific know how indicates that patients with CHB, CHC and HCC harbor a distorted immune response. It is really difficult to make a distinction between decreased and distorted immune responses. Extensive studies are required to assess the type of immune responses that would be necessary for containment of HBV, HCV and cancer cells in HCC, and also to minimize liver damage in these diseases. Insights into different HCV and HCC-related antigens are yet to be established. Thus, to develop an effective immune therapeutic strategy would require extensive basic information about these diseases. However, the DCs as regulators of immunity are capable of inducing both immune responses and immune tolerance. Thus, DCs can restore the necessary immunity in therapeutic applications. Concern does remain about strategy development.

DEVELOPMENT OF EVIDENCE-BASED THERAPY FOR LIVER DISEASES USING DCS

DC-based therapy is in its infancy. This is relevant for different pathological conditions that include liver disease. DC-based therapy may be an effective therapeutic strategy if the following variables are addressed properly and scientifically.

Antigens

Investigators have shown that HBV is contained in the liver of patients with CHB due to the immune modulatory capacities of HBcAg-specific cytotoxic T lymphocytes (CTL). On the other hand, anti-HBs are required to neutralize circulating HBV. Thus, both HBcAg- and HBsAg-specific immune responses including antigen-specific CTL are necessary for controlling HBV replication in such patients. Thus, DC-based immune therapy should incorporate both HBcAg and HBsAg. In addition, other HBV-related antigens, such as HBxAg and polymerase antigens may be needed to prepare antigen-pulsed DCs for developing better therapeutic immune strategies against HBV. Current data on the immune-mediated control of HCV is unclear. Further basic studies are essential for developing antigen-pulsed DCs against HCV infection. It remains elusive as to the nature of protective antigens in HCV infection. Although various antigens are detected after HCC progression, the precise nature of these antigens must be exclusively analyzed before using them for DC-based therapy against HCC. On the other hand, clinical trials should be conducted with different antigens and their immune modulatory capacities be elucidated to establish information on the most effective antigens applicable for treating HCC.

Method of Production of Antigen-Pulsed DCs

It is generally assumed that the culture of DCs and antigens would lead to the production of a suitable DC population. This is not only ambitious, but also counter productive. Both immunogenic and tolerogenic antigen-pulsed DCs are prepared by culturing DCs with antigens. However, as immunogenic antigen-pulsed DCs would induce antigen-specific immunity, tolerogenic antigen-pulsed DCs would cause immune tolerance. Culture conditions should be optimized for preparing antigen-pulsed DCs for specific purposes. Proper attention should be given regarding the (1) nature of the DCs, (2) source of the DCs, (3) dose of the antigen, (4) nature of the antigen, and (5) incubation time for preparing antigen-pulsed DCs. When antigen-pulsed DCs are prepared, they should not contain soluble antigens, and the antigen-pulsed DCs should be checked for viability, expression of costimulatory antigens, production of cytokines, and capacity to induce proliferation of antigen-specific lymphocytes *in vitro*.

In addition, the source of DC is one of important variables for preparation of antigen-pulsed-DC. DC has been isolated from almost all lymphoid organs and tissues. However, mostly monocyte-derived DC has been used for DC-based therapy in human diseases. Also, different subtypes of DC have been detected. These include myeloid DC, lymphoid DC, and plasmacytoid DC. Antigen-pulsed DC has mainly been prepared from myeloid DC or a bulk population of DC. However, both myeloid and palsmacytoid DC may be used in future for development of better regimens of DC-based therapy.

METHOD OF ADMINISTRATION OF ANTIGEN-PULSED DCS

Studies have shown that immunogenicity of antigen-pulsed DCs depends on routes of administration. This needs to be confirmed for different types of liver disease. Although the parental route is normally used to administer antigen-pulsed DCs, a pilot study should be performed to assess comparative therapeutic benefits from intradermal and subcutaneous, and intravenous or tissue administration. Mucosal routes should also be explored because the mucosal immune system is extensively distributed throughout the body.

Dose and Duration of Therapy

Dose and duration of DC-based therapy is an important aspect in liver disease. Antigen-pulsed DCs induce antigen-specific CTLs, helper T cells and antibodies. These CTLs may later kill the activated DCs that are delivered with subsequent injections and so this type of administration should be carefully planned.

DC-BASED THERAPY AS A PART OF COMBINATION THERAPY

Combination therapy might be needed for treating patients with CHB, CHC and HCC with antigen-pulsed DCs. Patients with CHB harbor abundant amounts of HBV and HBV-related antigens, especially HBsAg in the sera and in the liver. Similar conditions are also seen in patients with CHC. Patients with HCC also develop millions of cancer cells. The presence of these cells may hinder the induction and maintenance of antigen-specific immunity from antigen-pulsed DCs. Thus, antiviral drugs may be necessary for

lowering HBV or HCV load prior to administering DC-based therapy. It may also be imperative to reduce the cancer through operation or ablation methods before administrating antigen-pulsed DCs. In fact, combination of antiviral- and immune-based therapies has been used for different liver diseases, with polyclonal immune modulators or antigens given as immune modulatory agents. Thus, antigen-pulsed DCs may be incorporated as a part of such combination therapies in liver disease.

CONCLUDING REMARKS

The concept of immune therapy for treating different liver diseases is a comparatively new one whereby various practical points have yet to be optimized. In addition, immune therapy in liver disease is yet to receive a general consensus among physicians and hepatologists. Most immune-based therapies targeting liver disease have been conducted in pilot studies or clinical trials. In fact, DC-based therapy is in its infancy and few clinical trials have been conducted in patients with liver disease. This is also the case for other pathological conditions, except cancer. DC-based therapies are not intended to upregulate or down regulate host immunity in liver disease. Instead, they should be aimed at fixing the distorted immunity of patients with various liver diseases. The challenge lies in its application in the clinical setting. As discussed earlier in this chapter, we must first gain extensive insight into the pathogenesis of different liver diseases and then evaluate whether immune therapy would be advantageous in that specific condition. Careful attention should also be given to the nature of the antigens, the method of DC preparation, the technique of production of antigen-pulsed DCs *in vitro*, and the route of administration. Moreover, this should all be accomplished with due consideration of the safety of the patients. It is a challenging job. However, if DC-based therapy can be optimized for different liver diseases, it would then be easy to develop DC-based therapies for other pathological conditions. Thus, it is imperative that immunologists, hepatologists and cellular biologists initiate collaborative approaches in order to address these challenges to better improve future immune-based therapies.

ACKNOWLEDGEMENTS

The study was supported in part by a grant-in-aid from the 'United States-Japan Cooperative Medical Science Program' and the 'International Health Cooperation Research', from Ministry of Health and Welfare, Japan.

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Original Paper

Digestion

Digestion 2012;86:1–5
DOI: 10.1159/000337529

Received: November 17, 2011
Accepted: February 28, 2012
Published online: June 8, 2012

Biochemical, Virological, Immunological and Histopathological Features of 702 Incidentally Detected Chronic Hepatitis B Virus Carriers in Bangladesh

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Key Words

Incidentally detected hepatitis B virus carrier · Developing country · Liver damages · Public health problem · Hepatitis B virus control

Abstract

Background/Aims: Simultaneous assessment of biochemical, virological, and histological parameters of incidentally detected chronic hepatitis B virus (HBV)-infected subjects in Bangladesh were done to develop strategies for containment of HBV and management of liver diseases of these patients. ***Methods:*** A total of 702 chronic HBV carriers detected incidentally were enrolled in the study. Levels of HBV DNA and alanine aminotransferase (ALT) in sera were measured. The extent of hepatic inflammation and liver fibrosis was evaluated in all patients by examining liver biopsy specimens. ***Results:*** Of the 702 patients, 358 (50.7%) exhibited HBV DNA levels $>10^5$ copies/ml. ALT levels were above the upper limit of normal (ULN; >42 U/l) in more than 50% of the patients. High levels of HBV DNA ($>10^5$ copies/ml), increased ALT (>1.0 × ULN), moderate hepatic inflammation (HAI-NI ≥7) and severe hepatic fibrosis (HAI-F ≥3) were detected in 60 patients. ***Conclusion:*** As considerable numbers of apparently healthy subjects are unaware of the fact that they are chronically infected by HBV, many of whom have already developed progressive liver damage, emergency strategies would be needed for the containment and management of HBV infection in developing countries.

Introduction

In the last three decades, important advances in our understanding of the natural course of hepatitis B virus (HBV) infection have been made. It has been shown that chronic HBV infection can be divided into different phases, which may not be sequential. Patients may present (1) in a state of immune tolerance, (2) with hepatitis B e antigen (HBeAg)-positive chronic HBV, (3) with HBeAg-negative chronic HBV, or (4) as an inactive hepatitis B surface antigen (HBsAg) carrier [1, 2]. Patients with chronic HBV (both HBeAg-positive and HBeAg-negative) are assumed to be at greater risk of developing complications such as liver cirrhosis, hepatic decompensation and hepatocellular carcinoma [3]. Accordingly, major liver organizations, such as the American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of the Liver (EASL), and

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the Asian-Pacific Association for the Study of the Liver (APASL), consistently recommend antiviral therapy for patients with liver damages and complications [4–6].

However, Kumar et al. [7] reported that a fair proportion of incidentally detected HBV carriers, who were unaware of their HBV infection, had features of progressive liver damages. We also have detected severe liver damages in some HBeAg-negative patients with low HBV DNA and normal alanine aminotransferase (ALT) levels [8]. Studies in Egypt and Pakistan have revealed similar findings [9, 10]. Chu and Liaw [11] stated that asymptomatic HBV should not be viewed as an innocent long-lasting condition with a good prognosis because a considerable number of such patients develop liver cirrhosis.

We have encountered several hundred HBsAg-positive individuals in Bangladesh during routine clinical practice. All of them were apparently healthy, had never complained of liver diseases, and had not been vaccinated. All patients assumed that they did not need any antiviral treatment or active management because they were asymptomatic and apparently healthy. Also, they were unaware that they were potential source of HBV infection. This study was conducted to gain an insight into the virological, immunological, biochemical and histological features of these patients. The outcome of the study provided important insights about the management of such patients in Bangladesh and other countries.

Materials and Methods

Patients

All subjects were apparently healthy without any features of liver-related diseases when they first visited the hospital. They came to the hospital for testing of serum HBsAg (1) before traveling to a foreign country for work (compulsory requirement to obtain a working visa for most developed countries), (2) on the advice of their physician because of pregnancy, (3) before receiving HBV vaccine, or (4) before donating blood for transfusion.

When HBsAg was detected in the serum, the patient was requested to follow different public health measures. Patients were also advised to seek HBsAg testing after 6 months and to contact our clinical monitor if they experienced any physical anomaly. When HBsAg was detected 6 months after the first test, the patients were regarded as being chronically infected with HBV.

A total of 1,072 HBsAg-positive subjects were diagnosed as being chronic HBV carriers. Inclusion criteria for the study included: (1) positive for HBsAg, (2) negative for serological markers of hepatitis C virus, IgM antibodies to hepatitis A virus and hepatitis E virus, (3) having an alcohol consumption of <20 g/day, and (4) had no evidence of pregnancy. None of the patients had received any antiviral drugs (treatment-naïve) or immune modulators for treatment of HBV infection. A review board of the 'Viral Hepatitis Foundation, Bangladesh' provided permission to accomplish the study. A total of 743 patients gave informed written consent for a liver biopsy. The nature and purpose of the study were explained to all patients. Patients were excluded from further analyses if adequate amounts of liver tissue were not available during liver biopsy, as recommended [12]. Finally, a total of 702 chronic HBV carriers were available for final analyses.

Biochemical and Serological Tests

Levels of ALT in serum were measured commercially. The cutoff for the upper limit of normal (ULN) was ALT >42 U/l. HBsAg was assessed using a commercial ELISA kit (Diasorin, Fallugia, Italy). HBeAg was checked using an ELISA kit (Abbott Labs, Chicago, Ill., USA).

Quantification of Serum HBV DNA Levels

Serum HBV DNA was quantified using an RT-PCR kit (Amplicon HBV Monitor Assay, Roche Molecular Systems, Calif., USA). The lower limit of detection was 250 copies of HBV DNA/ml.

Liver Biopsy

A percutaneous liver biopsy was performed in all patients with prior, voluntary, informed written consent. In the case of minors, consent was obtained from a legal guardian. Biopsies were performed under local anesthesia using a 16G Tru-cut biopsy needle (Cardinal Health, McGaw Park, Ill., USA). A biopsy specimen of more than 1.0 cm in length with five to six portal tracts was evaluated. Histology was graded according to the histologic activity index (HAI) using the criteria of Knodell et al. [13]. The total HAI score comprises necroinflammation (HAI-NI) and fibrosis (HAI-F) scores. The HAI-NI scale includes three components (0–10, piecemeal necrosis; 0–4, lobular necrosis and inflammation; 0–4, portal inflammation). HAI-F was graded according to severity: 0, absence of fibrosis; 1, fibrous portal expansion; 3, bridging fibrosis; 4, cirrhosis.

Statistical Analyses

Data are expressed as mean ± SD. Data were analyzed by the unpaired t test if they were normally distributed and by the Mann-Whitney rank-sum test if they were skewed. Differences were considered significant at $p < 0.05$.

Results

Baseline Information of All Patients

The baseline data of 702 patients with chronic HBV infection are given in table 1. The mean age of the patients was 27.3 years (SD 8.8 years); 534 were male and the other 168 were female. Of a total of 702 patients, 248 were expressing HBeAg in the sera (HBeAg-positive) (35.3%) and the remaining 454 (64.7%) were HBeAg-negative. The mean level of serum ALT was 52 U/l (range 4–948 U/l). The levels of HBV DNA varied considerably among patients ranging from 300 to 1×10^{12} copies/ml (median value 1.2×10^5 copies/ml).

Virological, Biochemical and Histopathological Data of the Patients with Chronic HBV Infection

As shown in table 2, comparatively higher levels of HBV DNA (1×10^5 copies/ml) were seen in increased numbers of HBeAg-positive patients compared to HBeAg-negative patients (92.3 vs. 28.4%, $p < 0.05$). The levels of ALT above ULN (>42 U/l) were detected in 141 of 248 (56.8%) HBeAg-positive patients and 206 of 454 (45.4%) HBeAg-negative patients. Thirty-seven HBeAg-positive patients and 34 HBeAg-negative patients had ALT levels more than twice the ULN (>84 U/l) (table 2). ALT levels of >126 U/l (3 ULN) were detected in 12 HBeAg-positive and 11 HBeAg-negative asymptomatic HBV carriers (table 2). The magnitude of necroinflammation (assessed by HAI-NI score) was HAI-NI ≥7 in 22% (56 of 248) of the HBeAg-positive and 16% (71 of 454) of the HBeAg-negative chronic HBV carriers. In addition, severe hepatic fibrosis (HAI-F ≥3) was detected in 25% (62 of 248) of the HBeAg-positive and 20% (90 of 454) of the HBeAg-negative patients.

Comprehensive Analyses of Virological, Biochemical and Histopathological Parameters of Incidentally Detected Chronic HBV Carriers

The AASLD, EASL, and APASL guidelines recommend antiviral treatment for patients with chronic HBV infection with high HBV load and features of liver damages [4–6]. To evaluate the need of antiviral therapy in this cohort, we made a comprehensive assessment of their biochemical, virological and histopathological features. As shown in table 3, among 248 HBeAg-positive patients, 229 had HBV DNA of $>10^5$ copies/ml. Some 126 patients had both HBV DNA $>10^5$ copies/ml and serum ALT >42 U/l. Among them, 32 patients had HBV DNA $>10^5$ copies/ml, serum ALT >42 U/l, moderate levels of hepatic necroinflammation (HAI-NI ≥7) and severe hepatic fibrosis (HAI-F ≥3).

Among HBeAg-negative patients, 67 had both HBV DNA $\geq 10^5$ copies/ml and serum ALT >42 U/l. Among them, 28 patients had HBV DNA $\geq 10^5$ copies/ml, serum ALT >42 U/l, moderate hepatic necroinflammation (HAI N1≥7) and severe hepatic fibrosis (HAI-F ≥3) (table 3).

Discussion

Considerable numbers of chronic HBV carriers in Bangladesh were not aware of their HBV infection. Similar situations in terms of knowledge, attitude and practice regarding HBV prevail in most developing countries of

Table 1. Baseline data of the 702 patients

Number of patients	702
Age, years	27.3 ± 8.8
Male, n	534
Female, n	168
HBeAg-positive, n	248
HBeAg-negative, n	454
ALT levels	52 ± 55 U/l (range 4–948 U/l)
HBV DNA levels	300–5.3 $\times 10^{12}$ copies/ml

Table 2. Serum HBV DNA, ALT, and liver histology of study population

	HBeAg-positive (n = 248)	HBeAg-negative (n = 454)
HBV DNA		
HBV DNA <1 $\times 10^5$ copies/ml	19 (7.7%)	325 (71.6%)
HBV DNA >1 $\times 10^5$ copies/ml	229 (92.3%)	129 (28.4%)
ALT		
≤42 U/l	107 (43.1%)	248 (54.6%)
43–84 U/l (twice of ULN)	104 (41.9%)	172 (37.9%)
85–126 U/l (thrice of ULN)	25 (10.1%)	23 (5%)
>126 U/l	12 (4.8%)	11 (2.4%)
Liver histology		
Extent of hepatitis		
HAI-N1 ≤3	76 (30.6%)	216 (47.6%)
HAI-N1 4–6	116 (46.8%)	167 (36.8%)
HAI-N1 ≥7	56 (22.6%)	71 (15.6%)
Extent of fibrosis		
HAI-F0	20 (8.1%)	49 (10.8%)
HAI-F1	166 (66.9%)	315 (69.3%)
HAI-F3	54 (21.8%)	79 (17.4%)
HAI-F4	8 (3.2%)	11 (2.4%)

ALT: normal range <42 U/l.

Table 3. Incidentally detected chronic HBV carriers with high levels of HBV DNA, increased ALT, moderate necroinflammation and significant fibrosis

	HBeAg-positive	HBeAg-negative
Number of patients	248	454
HBV DNA $>10^5$ copies/ml	229 (92.3%)	129 (28.4%)
HBV DNA $>10^5$ copies/ml plus ALT >42 U/l	126 (50.8%)	67 (14.8%)
HBV DNA $>10^5$ copies/ml plus ALT >42 U/l plus HAI-NI ≥7 plus HAI-F ≥3	32 (12.9%)	28 (6.2%)

Asia and Africa. As they are asymptomatic and unaware of their HBV infection, they act as a permanent reservoir of HBV infection and transmit the virus to healthy persons in developing countries.

Although all patients in this study cohort were unaware of their HBV infectivity, many of them are on the way to developing severe complications like liver cirrhosis. A total of 60 patients (~8.5%) had high levels of HBV DNA, ALT above the ULN, a moderate degree of hepatic inflammation and severe liver fibrosis. All professional liver organizations recommend treatment for these patients to block further progression of liver damage [4–6]. Similar studies have been conducted in different countries to assess the real extent of problem regarding management of chronic HBV carriers. Kumar et al. [7] from India assessed virological, biochemical and histological parameters of liver functions in 1,387 incidentally detected chronic HBV carriers. They showed that more than 50% of these patients had evidence of progressive liver diseases for which treatment may be indicated. A similar outcome has also been reported from Pakistan, Egypt, and other countries [9, 10, 14–17].

However, these patients are not considered for antiviral therapy owing to complex socio-economic problems and the medical delivery system in developing countries. The majority of HBV-infected subjects have not been identified in developing countries owing to lack of an effective HBV monitoring system. In addition, both patients and physicians are reluctant to further assess the virological, biochemical and histological status because they assume that no curative therapy can be offered to patients. Finally, because of a lack of health insurance in these countries, patients are usually incapable of bearing the costs of expensive investigations and therapy.

Although there is a paucity of information about data of a nationwide survey regarding HBV prevalence in Bangladesh, published data show that about 5–6% apparently healthy individuals seem to be infected with HBV. Rudra et al. [18] have shown that about 6.2% of apparently health blood donors of Mymensingh, Bangladesh were infected with HBV. Ashraf et al. [19] have reported that 5.8% of the population of a semiurban area of Dhaka was expressing HBsAg. Mahtab et al. [20] also found almost similar data by checking HBsAg among 1,018 apparently healthy individuals. Taken together, there may be about 6–8 million chronic HBV carriers in Bangladesh (population of Bangladesh 160 million) and most of them are unaware of their infective state with the HBV. Although Bangladesh harbors millions of chronic HBV carriers, biochemical (ALT levels), immunological (HBeAg status), virological (HBV DNA levels), and histopathological (liver biopsy assessment) tests for such patients have not been conducted. In fact, this is the first study to assess biochemical, virological, and histopathological parameters of liver functions in considerable numbers of chronic HBV carriers in Bangladesh. This study clearly states that mechanisms should be developed to diagnose and manage these patients for containment of HBV and HBV-related complications.

One of the apparent limitations of this study is that the subjects were checked for HBV DNA, ALT and liver histology only once. Serial assessment of virological, biochemical and histological parameters would provide more insight into the natural disease course in these patients. However, a national policy would be required to accomplish this. Our main aim was to gain an insight into the pathogenesis of these patients to design a strategy for their management. We found that considerable numbers of asymptomatic HBV carriers have been harboring severe liver damage, so our study should convince physicians and policy makers in Bangladesh and other developing countries to develop a management strategy for such patients.

Disclosure Statement

All authors do hereby declare that there is no conflict of interest about this article.

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