Original article

Immune modulator and antiviral potential of dendritic cells pulsed with both hepatitis B surface antigen and core antigen for treating chronic HBV infection

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Background: Commercially available prophylactic vaccines containing hepatitis B surface antigen (HBsAg), which are used to prevent HBV infections, are not as effective as a therapeutic immune modulator for treating patients with chronic hepatitis B (CHB). In this study, the immunogenicity of dendritic cells (DC) loaded with both HBsAg and hepatitis B core antigen (HBcAg) was tested in HBV transgenic mice (TM; 1.2HB-BS10) *in vivo* and in patients with CHB *in vitro*.

Methods: Spleen DC from HBV TM were cultured with a vaccine containing both HBsAg and HBcAg to produce HBsAg/HBcAg-pulsed DC. HBV TM were immunized twice at an interval of 4 weeks with HBsAg/HBcAg-pulsed DC and other immune modulators. Antibody titres to HBsAg (anti-HBs) were measured in sera. Antigen-specific T-cells and cytotoxic T-lymphocytes (CTLs) in the spleen and liver were detected by lymphoproliferative and ELISPOT assays, respectively. HBsAg/HBcAg-pulsed human blood DC were cultured with autologous T-cells from CHB patients to assess their antigen-specific immune modulatory capacities.

Results: Significantly higher levels of anti-HBs, HBsAg-specific and HBcAg-specific T-cells and CTLs were detected in the spleen and liver of HBV TM immunized with HBsAg/HBcAg-pulsed DC compared with those immunized with other vaccine formulations ($P<0.05$). HBsAg/HBcAg-pulsed human blood DC also induced HBsAg- and HBcAg-specific proliferation of autologous T-cells from CHB patients.

Conclusions: The immune modulatory capacities of HBsAg/HBcAg-pulsed DC in HBV TM *in vivo*, and in patients with CHB *in vitro*, inspire optimism about a clinical trial with this cell-based vaccine in patients with CHB.

Introduction

Important insights about epidemiology, virology, immunology and pathogenesis of HBV have been documented during the past four decades. However, no curative therapy against chronic hepatitis B (CHB) has been developed. Clinical trials with different antiviral drugs (type-I interferon [IFN] and nucleoside/nucleotide analogues) have inspired considerable optimism about their use in CHB patients on the basis of intermediate outcomes [1]. However, a systemic review of a National Institutes of Health Consensus Development Conference that analysed all randomized clinical trials (RCTs) with antiviral drugs in CHB patients from 1989 to 2008 showed that drug treatment did not improve ultimate clinical outcomes or all intermediate outcomes in any RCT [2]. Low and moderate quality RCTs suggested improvement of some intermediate parameters by using antiviral drugs in CHB patients [2]. However, these drugs are costly, might require prolonged use, and are associated with several side effects, including the emergence of treatment-induced mutant HBV. These findings indicate that alternative therapeutic approaches should be developed to treat CHB.

The concept of immune therapy as an alternative therapeutic approach for treating CHB patients has emerged for several reasons. First, patients with CHB exhibit distorted immune responses to various HBV-related antigens [3]. In addition, sustained control of HBV replication and liver damage in CHB patients is usually associated with restoration of host immunity [4]. However, the therapeutic efficacy of polyclonal immune

modulators, such as immune IFN, growth factors and cytokines, was not satisfactory in CHB patients [5]. Subsequently, therapeutic vaccines containing hepatitis B surface antigen (HBsAg) have been used since the early 1990s. However, it is unlikely that the present vaccines will be able to stand the test of time [6].

We found some limitations to the current therapeutic vaccines for CHB patients. First, HBsAg is administered to CHB patients with the assumption that it will be internalized, processed and presented by antigen-presenting cells to induce HBsAg-specific immune responses [7]. However, because the phenotypes and functions of dendritic cells (DC), the most potent antigen-presenting cells, are distorted in CHB [8], it is unlikely that the DC of CHB patients would be able to properly process and present HBsAg for restoration of HBV-specific immunity. Second, vaccine therapies in CHB patients have been accomplished with HBsAg-based vaccines only. However, both HBsAg and hepatitis B core antigen (HBcAg)-specific immune responses are essential for sustained control of HBV replication and containment of liver damage [3]. Third, almost all patients with CHB harbour considerable levels of HBsAg; thus, μg levels of HBsAg in commercial vaccines might not be sufficient to overcome the immune tolerance state of these patients. In addition, clonal deletion of HBV-specific T-cells and the exhaustion of antiviral cytotoxic T-lymphocytes (CTLs) by high doses of antigen might also have a role in the minimal effect of HBsAg-based vaccine therapy [9]. Taken together, these factors indicate that a better regimen of immune therapy against HBV might be created by delivering HBV-related antigens with adequately activated antigen-presenting DC in CHB patients.

The immune modulator effects of HBsAg-pulsed DC have been evaluated in HBV transgenic mice (TM) by other investigators, as well as our group [10,11]. Jiang *et al.* [10] have shown that immunization with peptide-pulsed DC could elicit antiviral immunity in HBV TM. However, they used a murine model of HBV that expressed only HBsAg; therefore, the clinical implications of their study for translation research in patients with CHB are limited [10]. We found that HBsAg-pulsed DC induced antibody to HBsAg (anti-HBs) production, but not HBsAg-specific cellular immunity, in HBV TM [11].

Availability of a human consumable vaccine containing both HBsAg and HBcAg led us to perform the present study. First, we loaded spleen DC from normal C57BL/6J mice with this vaccine to optimize culture conditions. Then, we assessed the specificity and immune modulatory functions of HBsAg/HBcAg-pulsed DC *in vitro*. Subsequently, a preclinical study was conducted in HBV TM with HBsAg/HBcAg-pulsed DC and several other immune modulators. Finally, we extended this study to evaluate whether HBsAg/HBcAg-pulsed human blood DC from CHB patients were capable of activating autologous immunocytes in an antigen-specific manner *in vitro*. The research potential of this study will be discussed for developing antigen-specific immune therapy against chronic HBV infection in humans.

Methods

Mice

HBV TM (official designation 1.2HB-BS10) were prepared by microinjecting the complete genome of HBV plus 619 base pairs (bp) of HBV DNA into the fertilized eggs of C57BL/6 mice. The HBV TM expressed HBV DNA and messenger RNA of 3.5, 2.1 and 0.8 kbp of HBV in the liver. HBV DNA were also detected in the liver and sera of HBV TM. HBsAg was found in the sera of all HBV TM [12]. Male C57BL/6J mice that were 8 weeks old were purchased from Nihon Clea Co. (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 (Ehime, Japan).

Patients with CHB

Peripheral blood mononuclear cells and DC were isolated from five patients with clinical, biochemical and histological evidence of CHB. They were attending Ehime University Hospital (Ehime, Japan) for regular follow-up. The mean ±SD age of the patients was 35 ±12 years and the level of alanine aminotransferase was 127 ±32 IU/l (normal range 5–48 IU/l). Liver biopsy revealed a moderate degree of activity of hepatitis and moderate levels of fibrosis. Patients had not taken any antiviral or immune modulator drugs during the previous 6 months. Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Ehime University Hospital human research committee.

Detection of HBV-related markers

Levels of HBsAg and anti-HBs in sera were estimated using a chemiluminescence enzyme immunoassay (Special Reference Laboratory, Tokyo, Japan) and were expressed as IU/ml and mIU/ml, respectively [13]. Antibodies to HBcAg in sera were estimated by the passive hemagglutination method (Tokyo Institute of Immunology, Tokyo, Japan). HBV DNA in the sera of HBV TM was assessed by the PCR method (Special Reference Laboratory).

Isolation of T-cells, B-cells and DC

The methodologies for isolating spleen cells, T-cells, B-cells and DC are described in detail elsewhere [14]. In brief, spleens were removed aseptically, cut into pieces and incubated at 37°C in 5% CO_2 for 30 min in RPMI 1640 (Nipro, Osaka, Japan) supplemented with 1 μg/ml collagenase (type IV; Sigma Aldrich Corporation, St Louis, MO, USA), and a single-cell suspension of spleen was produced. T-cells were purified from single-cell suspensions of spleen by a negative-selection column method (Mouse Pan T Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany). $CD8^+$ T-cells were purified from T-cells by a column method (Mouse CD8 Isolation Kit; Miltenyi Biotec).

To isolate spleen DC, single-cell suspension of spleen was centrifuged at 10,000×*g* for 30 min on a dense albumin column (specific gravity 1.082) at 4°C and then cultured on a plastic surface for 90 min at 37°C. The adherent cells were cultured for an additional 18 h in culture medium containing RPMI 1640 plus 10% fetal calf serum (Filtron PTY Ltd, Brooklyn, Australia). Macrophages were discarded from DC populations by two additional adherent steps on plastic dishes at 37°C.

Liver non-parenchymal cells (NPC) were isolated as described previously [15]. In brief, liver tissues were homogenized, suspended in 35% percoll (Sigma Aldrich Corporation), and centrifuged to get liver NPC. Liver NPC were suspended in RPMI 1640 plus 10% fetal calf serum.

Human blood DC were enriched from peripheral blood of CHB patients by culturing them with granulocyte macrophage colony-stimulating factor and interleukin (IL)-4 for 6 days, as described previously [16].

Preliminary experiments to optimize culture conditions for preparing immunogenic antigen-pulsed DC

Normal C57BL/6J mice were immunized with HBsAg (10 μg; Tokyo Institute of Immunology), HBcAg (10 μg; Tokyo Institute of Immunology), HBsAg/HBcAg (10 μg Center for Genetic Engineering and Biotechnology [CIGB], Havana, Cuba) and pyruvate dehydrogenase complex (PDC; 10 μg; Sigma Aldrich Corporation) [17] in phosphate-buffered saline (PBS) twice at an interval of 4 weeks. Lymphocytes from immunized mice were cultured with antigen-pulsed DC from non-immunized mice to evaluate if antigen-pulsed DC could induce proliferation of antigen-specific lymphocytes *in vitro*.

Preparation of antigen-pulsed DC for immunization of HBV TM

HBsAg, HBcAg and HBsAg/HBcAg, used for the assessment of therapeutic efficacy of antigen-pulsed DC in HBV TM, were provided by the CIGB [18]. *Pichia-pastoris*-derived recombinant HBsAg was used. HBcAg were derived from *Escherichia coli* purified recombinant full-length HBcAg (GenBank accession number X02763). HBsAg/HBcAg consisted of equal amounts of HBsAg and HBcAg. HBsAg was produced as a 22 nm particle to >95% purity at the CIGB production facilities as a component of the commercial anti-HBV vaccine, Heberbiovac-HB1. HBcAg was purified from *E. coli* strain W3110, which had been transformed previously with a plasmid containing the entire core antigen gene under the control of a tryptophan promoter. The resulting HBcAg had a purity >95% [18].

Murine antigen-pulsed DC were prepared based on data from preliminary studies and also according to our previous report [19]. Briefly, spleen DC were cultured with PDC (10 μg), HBsAg (10 μg), HBcAg (10 μg) and HBsAg/HBcAg vaccine (HBsAg 5 μg and HBcAg 5 μg) in culture medium for 48 h. DC were recovered from the cultures and washed 5× with PBS. The viability of DC was assessed by the trypan blue exclusion test. The T-cell stimulatory capacity of antigen-pulsed DC were confirmed by lymphoproliferative assays. Human blood DC was cultured with HBsAg/HBcAg vaccine (HBsAg 5 μg and HBcAg 5 μg) or PDC (10 μg) for 48 h to prepare antigen-pulsed DC, as described elsewhere [16].

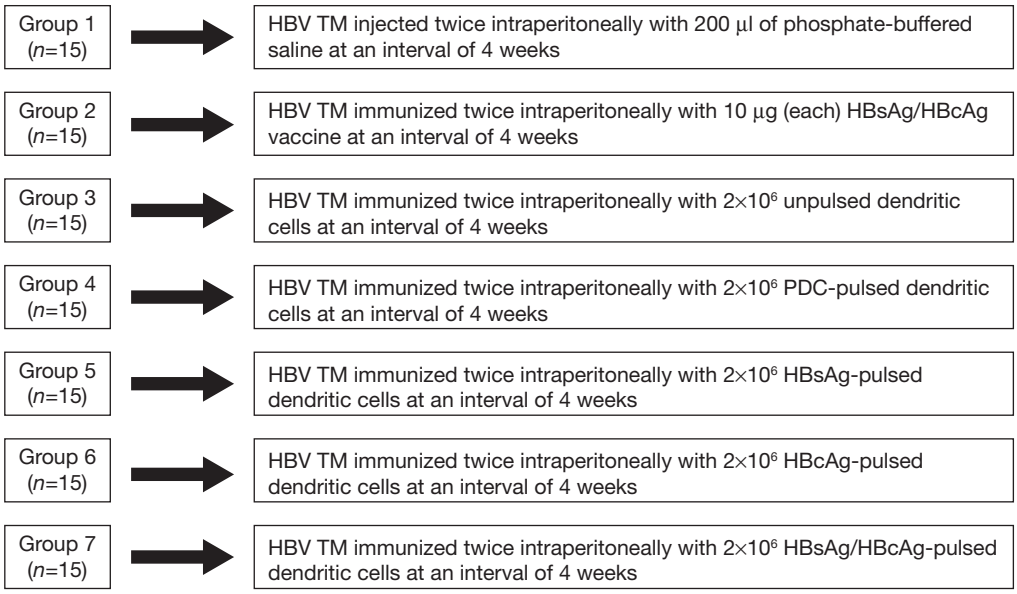
Immunization schedule

The immunization schedule is shown in Figure 1. Seven groups of HBV TM with comparable levels of HBsAg in the sera were used for this preclinical study. One group of HBV TM was injected with PBS (n=15). A second and third group of HBV TM received the HBsAg/HBcAg vaccine (10 μg; n=15) and 2×10^6 unpulsed DC (n=15), respectively. A fourth, fifth and sixth group of HBV TM received 2×10^6 PDC-pulsed DC (n=5), 2×10^6 HBsAg-pulsed DC (n=15) and 2×10^6 HBcAg-pulsed DC (n=15). Finally, a seventh group of HBV TM received HBsAg/HBcAg-pulsed DC (n=15). All vaccinations were done by the intraperitoneal route, twice at an interval of 4 weeks. HBV TM were bled from the tail vein at different intervals to assess different immunological parameters. The mice were finally euthanized to estimate vaccine-induced cellular immune responses in the spleen and liver.

Lymphoproliferative assays

As described previously, murine lymphocytes, murine liver NPC and human peripheral blood mononuclear cells were cultured in the absence or presence of different immune modulators for 120 h to evaluate antigen-specific cellular immune responses [14–17,19,20]. All cultures were performed in 96-well U-bottom plates (Corning Inc., 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 by a multiple cell harvester (Labo Mash;

Figure 1. Immunization schedule for assessment of antigen-specific humoral and cellular immune responses in HBV TM



HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; PDC, pyruvate dehydrogenase complex; TM, transgenic mice.

Futaba Medical, Osaka, Japan) onto a filter paper (LM 101–10; Futaba Medical). The levels of incorporation of [^{3}H]-thymidine were determined in a liquid scintillation counter (Beckman LS 6500; Beckman Instruments, Inc., Fullerton, CA, USA) from the level of blastogenesis. Triplicate cultures were assayed routinely and the results were expressed as 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. A stimulation index >3.0 was considered significant.

ELISPOT assays

Different types of immunocytes (1×10^5) were stimulated with antigen in presence of mitomycin-C-treated spleen adherent cells in an IFN-γ-coated ELISPOT plate (Mabtech, Nacka Strand, Sweden) for 24 h. After removing the cells, biotinylated antibody (2A5-biotin) was added into the wells. After 2 h incubation, the plates were further 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 were counted using an ELISPOT reader (KS ELISPOT; Carl Zeiss, Thornwood, NY, USA). Different types of immunocytes were stimulated with concanavalin A as a positive control.

Statistical analyses

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

Results

Features of antigen-pulsed DC and their functional capacities

We first isolated DC from normal C57BL/6 mice spleen and human blood. Murine spleen DC expressed major histocompatibility complex (MHC) class II antigen and CD86 antigens. Human monocyte-derived DC expressed human leukocyte antigen DR and CD86 antigens. A functional study showed that both murine spleen DC and human blood DC induced proliferation of allogenic T-cells in a dose-dependent manner (SMFA *et al.,* data not shown).

Antigen-pulsed DC from normal C57BL/6 mice produced significantly higher levels of IL-12 (HBsAg-pulsed DC 154.3 ±12.3 pg/ml and HBcAg-pulsed DC 213.2 ±23.6 pg/ml; $n=3$) compared with unpulsed DC (35.3 ±9.6 pg/ml; $n=3$; $P<0.05$). Also, HBsAg-pulsed and HBcAg-pulsed DC induced proliferation of lymphocytes from HBsAg- and HBcAg-immunized normal C57BL/6J mice (SMFA *et al.,* data not shown). After

optimizing culture conditions for preparing immunogenic HBsAg-pulsed DC and HBcAg-pulsed DC, we prepared HBsAg/HBcAg-pulsed DC from normal C57BL/6J mice. HBsAg/HBcAg-pulsed DC expressed significantly higher levels of MHC class II and CD86 compared to unpulsed DC ($P<0.05$). Also, HBsAg/HBcAg-pulsed DC produced significantly higher levels of IL-12 and IFN-γ compared with unpulsed DC ($P<0.05$). As shown in Table 1, HBsAg/HBcAg-pulsed DC did not induce significant proliferation of lymphocytes from PDC-immunized normal C57BL/6J mice (stimulation index 1.0), but induced vigorous proliferation of lymphocytes from HBsAg/HBcAg-immunized normal C57BL/6J mice (stimulation index 17.3 ±3.2; $n=3$). By contrast, PDC-pulsed DC induced significant proliferation of lymphocytes from PDC-immunized normal C57BL/6J mice (stimulation index 9.6 ±2.2; $n=3$). However, PDC-pulsed DC did not induce proliferation of HBsAg/HBcAg-immunized normal C57BL/6 mice (stimulation index 1.0). After assessment of immunogenecity of antigen-pulsed DC in normal C57BL/6 mice *in vitro*, we next evaluated immunogenecity of antigen-pulsed DC in HBV TM *in vivo*.

HBsAg and anti-HBs in HBV TM immunized with antigens and antigen-pulsed DC

When the levels of HBsAg were estimated 4 weeks after the second immunization, the levels of HBsAg in the sera were decreased or became undetectable in HBV TM immunized with HBsAg-pulsed DC and HBsAg/HBcAg-pulsed DC. However, there was no significant alteration in HBsAg levels in HBV TM immunized with other formulations (Figure 2A).

Anti-HBs were not detected in PBS-injected HBV TM ($n=15$). In addition, anti-HBs were not detected in HBV TM injected twice with vaccine containing HBsAg/HBcAg ($n=15$) or unpulsed DC ($n=15$), PDC-pulsed DC ($n=5$) or HBcAg-pulsed DC ($n=15$). However, anti-HBs were detected in all HBV TM after two injections with HBsAg/HBcAg-pulsed DC (Figure 2B). Anti-HBs were also detected in HBV TM immunized with HBsAg-pulsed DC, but the levels of anti-HBs in these HBV TM were significantly lower than those in HBV TM immunized with HBsAg/HBcAg-pulsed DC (Figure 2B).

Antigen-specific cellular immune responses caused by vaccination with HBsAg/HBcAg-pulsed DC

We checked antigen-specific cellular immune responses in different groups of HBV TM. Lymphocytes from HBV TM immunized with HBsAg/HBcAg-pulsed DC proliferated in response to both HBsAg and HBcAg (Figure 3). However, lymphocytes from PBS-injected HBV TM, HBsAg/HBcAg vaccine-immunized HBV TM, unpulsed DC-injected HBV TM and PDC-pulsed DC-immunized HBV TM did not proliferate because of stimulation with HBsAg or HBcAg. Lymphocytes from HBV TM immunized with HBsAg-pulsed DC and HBcAg-pulsed DC proliferated in response to respective antigens only, but not to both HBsAg and HBcAg (Figure 3).

These data revealed that immunization with HBsAg/HBcAg-pulsed DC was capable of inducing HBsAg-specific humoral, HBsAg-specific cellular and HBcAg-specific cellular immune responses in HBV TM. Next, we checked if antigen-specific CTLs were produced in the spleen as a result of immunization of HBV TM with HBsAg/HBcAg-pulsed DC.

Detection of IFN-γ producing CTL in HBV TM immunized with HBsAg/HBcAg-pulsed DC

Spleen T-cells from HBV TM injected with HBsAg/HBcAg-pulsed DC, but not those from HBV TM immunized with HBsAg/HBcAg vaccine, unpulsed DC or PBS, or PDC-pulsed DC, produced significant numbers of IFN-γ-secreting T-cells in response to stimulation with HBsAg and HBcAg in the ELISPOT assay (Table 2). Also, abundant numbers of IFN-γ-secreting T-cells were detected in ELISPOT assays when spleen T-cells were stimulated with concanavalin A (positive control; SMFA *et al.* data not shown).

Antigen-specific immunocytes in the liver resulting from immunization with HBsAg/HBcAg-pulsed DC

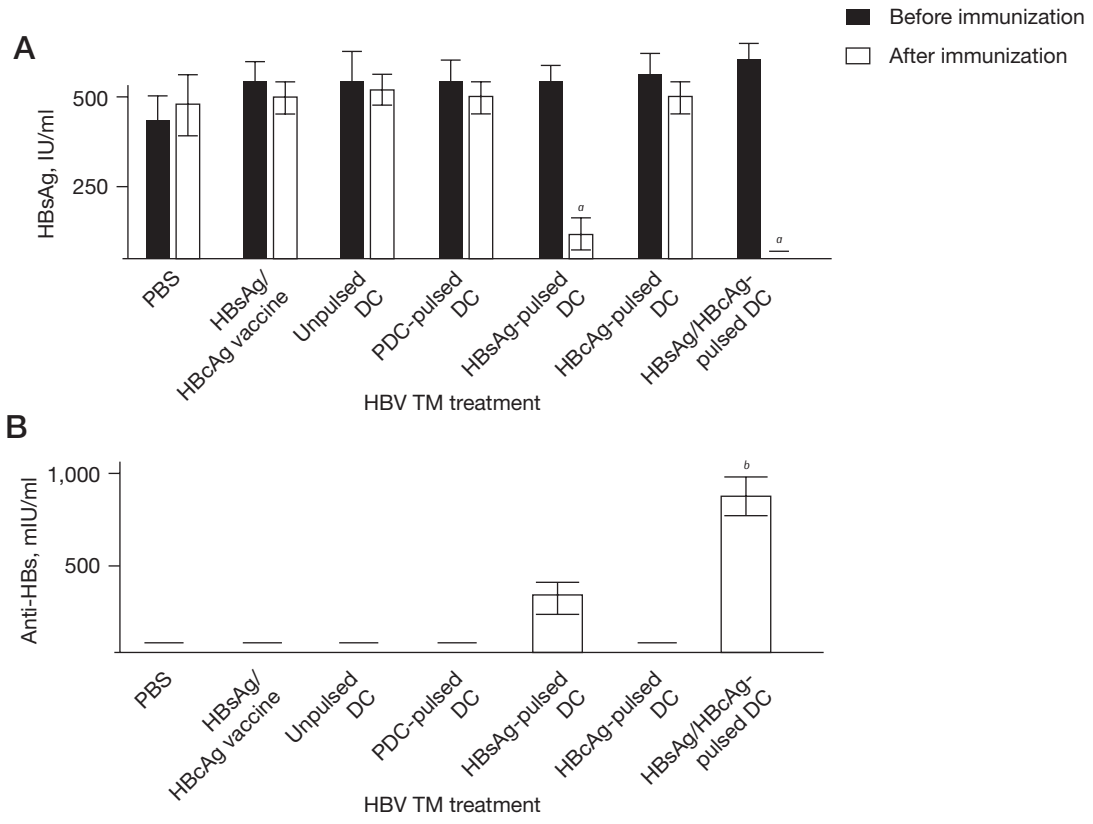
Although antigen-specific lymphocytes and CTLs were detected in the spleen, it was important to assess if antigen-specific lymphocytes and CTLs were induced

Table 1. Antigen-specific proliferative capacities of antigen-pulsed dendritic cells

Lymphocyte	DC	Stimulation index
HBsAg/HBcAg-immunized mice	PDC-pulsed	1.0
HBsAg/HBcAg-immunized mice	HBsAg/HBcAg-pulsed	17.3 ±3.2
PDC-immunized mice	HBsAg/HBcAg-pulsed	1.0
PDC-immunized mice	PDC-pulsed	9.6 ±2.2

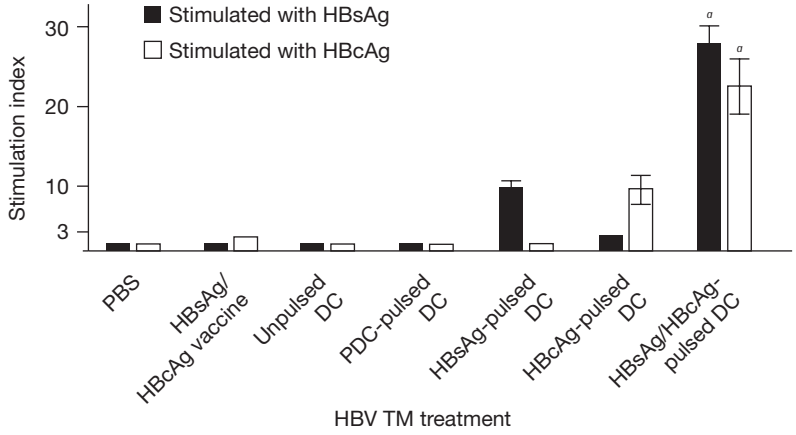
Normal C57BL/6J mice were immunized with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg) or pyruvate dehydrogenase complex (PDC)-pulsed dendritic cells (DC), as described in *Methods*. Mice were euthanized 4 weeks after the second immunization, and spleen cells were injected with different stimulants. The levels of blastogenesis in cultures containing T-cells and irreverent antigen-pulsed DC were regarded as a stimulation index of 1.0. Data for stimulation indices are mean ±SD of three separate experiments.

Figure 2. Levels of HBsAg and anti-HBs in HBV TM before and after immunization with vaccines and antigen-pulsed DC



(A) HBV transgenic mice (TM) were immunized with different immunization regimens, as described in Figure 1. The levels of hepatitis B surface antigen (HBsAg) in the sera of different groups of mice before immunization and 4 weeks after second immunization are shown. **(B)** Antibody to HBsAg (anti-HBs) was not detected in HBV TM 4 weeks after injection with phosphate-buffered saline (PBS), vaccine containing HBsAg/hepatitis B core Antigen (HBcAg), unpulsed dendritic cells (DC), pyruvate dehydrogenase complex (PDC)-pulsed DC or HBcAg-pulsed DC. However, anti-HBs was detected in all HBV TM 1 month after immunization with HBsAg/HBcAg-pulsed DC. Data are mean ±SD of the levels of anti-HBs in sera. [a]$P<0.05$ compared with the levels of HBsAg before immunization. [b]$P<0.05$ compared with other groups.

Figure 3. Antigen-specific cellular immune responses caused by vaccination with HBsAg/HBcAg-pulsed DC



Hepatitis B surface antigen (HBsAg)- and hepatitis B core antigen (HBcAg)-specific proliferation was observed in T-cells from HBV transgenic mice (TM) immunized with HBsAg/HBcAg-pulsed dendritic cells (DC); however, proliferation did not result from immunization with other vaccine or DC formulations. Data are mean ±SD of levels of proliferation. [a]$P<0.05$ compared with other groups. PBS, phosphate-buffered saline; PDC, pyruvate dehydrogenase complex.

in the liver of HBV TM as a result of vaccination with HBsAg/HBcAg-pulsed DC. Liver NPC from HBV TM immunized with HBsAg/HBcAg-pulsed DC proliferated in response to stimulation with HBsAg (stimulation index 12.8 ±3.2; *n*=5) and HBcAg (stimulation index 16.4 ±4.5; *n*=5). However, liver NPC from HBV TM injected with PBS, HBsAg/HBcAg vaccine, unpulsed DC and PDC-pulsed DC did not proliferate in response to HBsAg or HBcAg (stimulation index <3.0). In addition, significantly higher numbers of IFN-γ-secreting $CD8^+$ T-cells were detected among liver NPC only from HBV TM immunized with HBsAg/HBcAg-pulsed DC, but not from other HBV TM (Figure 4). However, we did not observe an increase of alanine aminotransferase (before vaccination 29 ±4 IU/l versus 4 weeks after vaccination 27 ±3 IU/l) or evidence of liver damage in liver biopsy specimens in any HBV TM immunized with HBsAg/HBcAg-pulsed DC (SMFA *et al.*, data not shown).

Induction of antigen-specific lymphocytes from CHB patients by HBsAg/HBcAg-pulsed DC *in vitro*

To assess a clinical implication of this study regarding immunogenecity of HBsAg/HBcAg-pulsed DC in HBV TM, we performed an *in vitro* study using lymphocytes from patients with CHB. Autologous T-cells from patients with CHB were cultured with HBsAg/HBcAg-pulsed DC, unpulsed DC, PDC-pulsed DC or HBsAg/HBcAg vaccine. T-cells from CHB patients did not exhibit significant proliferation in response to unpulsed DC or PDC-pulsed DC. However, T-cells from CHB patients proliferated in the presence of HBsAg/HBcAg-pulsed autologous DC (Figure 5). Low levels of proliferation of autologous T-cells from one of five patients with CHB were also detected when these were cultured with HBsAg/HBcAg (Figure 5).

Antiviral capacity of HBsAg/HBcAg-pulsed DC in HBV TM

All HBV TM expressed HBsAg; however, free HBV DNA could be detected in some but not all HBV TM. To assess the antiviral capacity of HBsAg/HBcAg-pulsed DC, we immunized five HBV TM with detectable levels of HBV DNA using HBsAg/HBcAg-pulsed DC, unpulsed DC or PDC-pulsed DC. Levels of HBV DNA in the sera were decreased in all HBV TM as a result of immunization with HBsAg/HBcAg-pulsed DC. HBV TM expressed a mean ±SD level of 354 ±14 copies/ml of HBV DNA in the sera before vaccination. At 4 weeks after two vaccinations with HBsAg/HBcAg-pulsed DC, HBV DNA could not be detected in any HBV TM (level of detection; 200 copies/ml). However, no significant changes of HBV DNA levels were seen in HBV TM immunized with two injections of HBsAg/HBcAg vaccine, unpulsed DC or PDC-pulsed DC.

Discussion

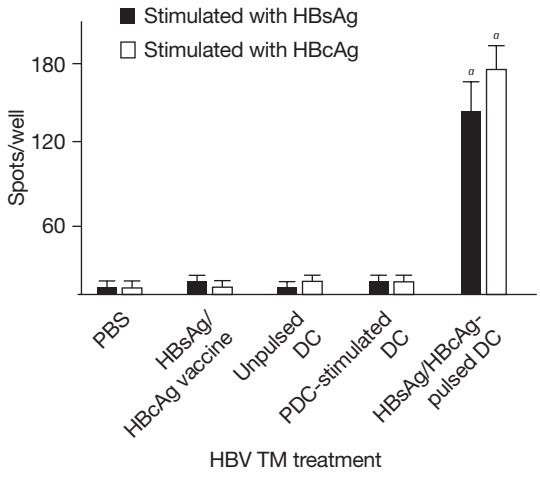
Several RCTs have documented the low therapeutic efficacy and considerable side effects of antiviral drugs used for CHB patients [2]. However, these drugs will remain the most useful and powerful tools for management of CHB patients [21] until more effective therapeutic regimens with fewer side effects can be developed. As an alternative therapeutic approach, polyclonal immune modulators have been used for

Table 2. IFN-γ-secreting T-cells in the spleen of HBV TM resulting from immunization with HBsAg/HBcAg-pulsed DC

HBV TM treatment	HBsAg-specific ELISPOT	HBcAg-specific ELISPOT
PBS	7 ±2	9 ±2
HBsAg/HBcAg vaccine	13 ±3	5 ±1
Unpulsed DC	11 ±4	12 ±3
PDC-pulsed DC	16 ±6	19 ±7
HBsAg/HBcAg-pulsed DC	198 ±23[a]	365 ±34[a]

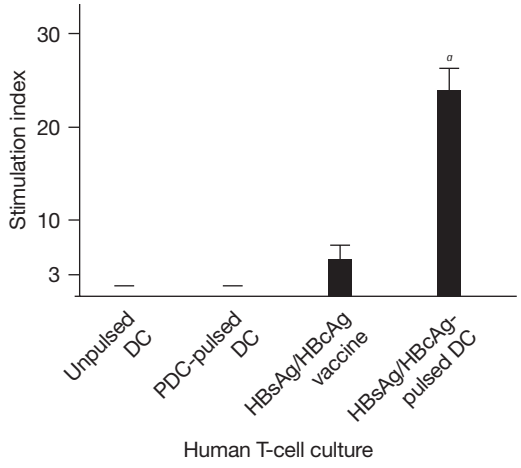
HBV transgenic mice (TM) were injected with phosphate-buffered saline (PBS) or immunized with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg)-based vaccines, unpulsed dendritic cells (DC), pyruvate dehydrogenase complex (PDC)-pulsed DC or HBsAg/HBcAg-pulsed DC, twice at an interval of 4 weeks. HBV TM were euthanized 4 weeks after the second immunization, and T-cells were stimulated with HBsAg or HBcAg on an ELISPOT plate to assay the production of inteferon-γ spot. The spots were counted after deducting spots from control plates. Data are mean ±SD of three separate experiments. [a]$P<0.05$ compared with other groups.

Figure 4. IFN-γ-producing CTL in HBV TM immunized with HBsAg/HBcAg-pulsed DC



Significant numbers of interferon (IFN)-γ producing cytotoxic T-lymphocytes (CTL) were detected among liver non-parenchymal cells from HBV transgenic mice (TM) immunized with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg)-pulsed dendritic cells (DC). Data are mean ±SE of four separate experiments. [a]$P<0.05$ compared to HBV TM immunized with other vaccine or DC formulations. PBS, phosphate-buffered saline; PDC, pyruvate dehydrogenase complex.

Figure 5. Antigen-specific lymphocytes from CHB patients by HBsAg/HBcAg-pulses DC *in vitro*



Antigen-specific proliferation of human peripheral blood T-cells from patients with chronic hepatitis B (CHB) resulting from stimulation with hepatitis B surface antigen (HBsAg)/hepatitis B core antigen (HBcAg)-pulsed dendritic cells (DC). The levels of blastogenesis in cultures containing T-cells and unpulsed DC were regarded as a stimulation index of 1.0. Data are mean and ±SD of five separate experiments. $^{a}P<0.05$ compared with HBV transgenic mice (TM) immunized with other vaccine or DC formulations. PDC, pyruvate dehydrogenase complex.

more than three decades in CHB patients; however, the clinical outcome with these agents has not been satisfactory [5]. To develop more effective immune therapy against HBV, vaccine therapy in which vaccines containing HBsAg were administered alone as well as in combination with other antiviral drugs in CHB patients [22,23]. Although some intermediate outcomes of CHB patients has been improved by this vaccine therapy, the present regimen of vaccine therapy still has limitations [6], as described in the *Introduction*.

The immune modulator effects of antigen-pulsed DC have been evaluated in HBV TM by other investigators, as well as our group [10,11]. Jiang *et al.* [10] used a murine model of HBV that expressed only HBsAg; the clinical implications of their study for translation research in patients with CHB are limited. We found that HBsAg-pulsed DC induced anti-HBs, but almost no HBsAg-specific cellular immunity, in HBV TM [11]. Also, we did not find therapeutic potentiality of HBsAg pulsed in CHB patients [24].

The present study has some noteworthy features. First, we used a vaccine that contains both HBsAg and HBcAg. We also prepared HBsAg/HBcAg-pulsed DC vaccine using a technique that we have been working with for more than a decade [14–16,19,20]. Next, an *in vitro* study revealed that HBsAg/HBcAg-pulsed DC could induce proliferation of both HBsAg- and HBcAg-specific lymphocytes from normal C57BL/6 mice. Subsequently, a preclinical trial using HBsAg/HBcAg-pulsed DC was accomplished in HBV TM. The data conclusively showed that HBsAg/HBcAg-pulsed DC induced anti-HBs in the sera, HBsAg and HBcAg-specific lymphocytes in the spleen, and HBsAg and HBcAg-specific CTLs in the spleen and the liver. Also, HBsAg/HBcAg-pulsed DC were able to reduce HBV DNA levels in HBV TM. Despite extensive immune modulating capacities of such preparations in HBV TM, we did not find any biochemical or histological evidence of liver injury with this approach, suggesting the viral clearance was mediated by non-cytotoxic effects of DC-based vaccine.

Our main target was to develop an effective immune therapeutic strategy for patients with CHB. Accordingly, we used a vaccine preparation that was a human-grade HBsAg and HBcAg. Indeed, clinical trials with this vaccine have already been performed in normal volunteers [18]. Accordingly, data from this study can be used to support the need for clinical trials using this vaccine. Further support of this concept was accumulated from data that showed that HBsAg/HBcAg-pulsed DC induced proliferation of both HBsAg- and HBcAg-specific T-cells from CHB patients (Figure 5).

The synergistic effect on the resulting immune response of HBsAg/HBcAg stimulation could be explained by the simultaneous stimulation of diverse Toll-like receptor (TLR) on DC. The nuclear content inside the *E.-coli*-derived recombinant HBcAg has been characterized as RNA (TLR3 and TLR7 ligands) [25–27]. Also, there is a recognized interaction between HBsAg and CD14, a component of TLR4 [28]. Finally, the aggregation of HBsAg and HBcAg in the liquid combined HBsAg/HBcAg formulation has been previously reported [25,29]. When these antigens were loaded on DC, antigen-pulsed DC were able to induce or activate HBsAg- and HBcAg-specific immune responses in HBV TM.

In conclusion, we have shown an improved immune therapeutic approach against chronic HBV infection. The antigens that we used are safe for human consumption and have been used in normal volunteers [18]. We prepared immunogenic antigen-pulsed DC with these antigens. HBsAg/HBcAg-pulsed DC induced both humoral and cellular immune responses in HBV TM *in vivo*; in addition, these DC induced proliferation of lymphocytes from CHB patients *in vitro*. We have already used HBsAg-pulsed DC in patients with CHB and confirmed its safety; however, only HBsAg-pulsed DC was not sufficiently effective to contain HBV replication and liver damages [24]. The next challenge will be to assess if HBsAg/HBcAg-pulsed DC can cause sustained control of HBV replication and reduction of liver damage in CHB, a finding that could not be completely assessed in HBV TM. The answer to that question will depend

on results of clinical trials in CHB patients, which are warranted based on the findings of this study.

Disclosure statement

The authors declare no competing interests.

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