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Therapeutic potential of dendritic cell-based immunization against HBV in transgenic mice

Wen-Zheng Jiang [a,*], Yan Fan [a], Xia Liu [b], Ya-Li Zhang [a], Jie-Jun Wen [a], Wen-Li Hao [a], Min Qian [a]

[a] *School of Life Science, East China Normal University, Shanghai 200062, People's Republic of China*
[b] *College of Pharmacy, Second Military Medical University, Shanghai 200433, People's Republic of China*

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Abstract

Hepatitis B virus (HBV) transgenic mice that express HBV envelope proteins represent a model of chronic HBV infection suitable for the development of therapeutic immunization strategies. To address immunologically therapeutic effects induced by peptide-pulsed DCs, HBV transgenic mice were immunized with peptide-pulsed DCs, and the mice were killed after three times of immunization and the splenocytes were stimulated in vitro and detected by IFN-γ ELISPOT and cytotoxic T lymphocyte (CTL) activity. The data demonstrated that HBV-specific CD8+ T cell response could be induced and CD8+ T cells had specific CTL activity. Furthermore, ELISA and fluorescent quantitative PCR were performed to detect the level of serum HBsAg and HBV DNA and the results demonstrated that HBV-specific peptide-pulsed DCs could significantly reduce the concentration of serum HBsAg and HBV DNA. The serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured and no significant differences were observed between the different groups, which indicated that no hepatocellular injury occurred. Taken together, the data strongly demonstrated that CD8+ T cell responses and antiviral immunity were elicited in HBV transgenic mice, suggesting that peptide-pulsed DCs could elicit an effective antiviral immunity.

Keywords: Hepatitis B virus; Dentritic cell; Epitope; Cytotoxic T lymphocyte; Transgenic mice

1. Introduction

There are more than 370 millions carriers of HBV all over the world now. Among these, approximately 5–10% of adults and 80–90% of children become chronic carriers of HBV. Unfortunately, chronic hepatitis B virus infection may cause significant incidence of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Arbuthnot and Kew, 2001; Birrer et al., 2003). Currently, the most effective drugs used for treatment of chronic hepatitis B are interferon alpha (IFN-α) and nucleotide analogues, lamivudine, and adefovir.

Vaccine-based strategies to boost or to broaden the weak virus-specific T cell response of patients with chronic hepatitis B are proposed as a means of terminating this persistent infection. Many existing vaccines, except live, attenuated vaccines, lack an efficient Ag presentation mechanism to induce potent CD8+ CTL and CD4+ Th responses. DCs are antigen-presenting cells (APC) that are distributed throughout the body and play a central role in Ag presentation to CD4+ and CD8+ T cells (Steinman, 1991; Banchereau and Steinman, 1998). The ability of DCs to act as potent APC for the induction of T cell responses is attributed to the high expression of MHC molecules and adhesion and/or costimulatory molecules as well as their ability to produce cytokines essential for the activation and proliferation of the T cells. DCs can be expanded from murine bone marrow cells with granulocyte–macrophage CSF (GM-CSF) and IL-4, and, when pulsed with Ag protein or peptide, can induce specific Ab and CTLs in vivo (Inaba et al., 1990; Sornasse et al., 1992). Accordingly, activated DC immunotherapy has recently been studied in tumor or chronic virus infection (Sheng et al., 2005; Steinman, 1996).

It is difficult to study the experimental approaches to HBV pathogenesis because the host range of HBV is limited to man and chimpanzees, and in vitro systems for the propagation of HBV do not exist. The availability of transgenic mice that express HBV envelope proteins affords the opportunity to study

* Corresponding author. Tel.: +86 21 62233574; fax: +86 21 62233295.
E-mail address: wzjiang@bio.ecnu.edu.cn (W.-Z. Jiang).

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the mechanisms responsible for neonatal tolerance to HBV and to develop immunotherapeutic approaches that have the potential to terminate persistent infection (Chisari, 1996; Guidotti et al., 1995). These animals are immunologically tolerant to HBV-encoded antigens and represent a model of chronic HBV infection suitable for the development of therapeutic immunization strategies before testing in humans.

Many studies show that major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTLs) can play a central role in the prevention, control, and clearance of HBV infections (Guidotti et al., 1994; Thimme et al., 2003). Lysis of infected hepatocytes is unlikely to be the only mechanism through which CD8+ T cells exert their anti-viral effect. Indeed, CTL mediate not only the down-regulation of viral gene expression (Mancini et al., 1996) but also the control of viral replication (Guidotti et al., 1996b) in the transgenic mouse models. This phenomenon is probably due to the antiviral effects of Th1 cytokines such as IFN-γ, IFN-α, IFN-β, and TNF-α secreted by CD8+ T cells after antigenic stimulation or following a concomitant viral infection (Guidotti et al., 1996a). To induce specific CTL against HBV, many HBV antigens like hepatitis B surface antigen (HBsAg) or hepatitis B core antigen (HBcAg) have been used to prepare prophylactic or therapeutic vaccines. However, natural HBV antigens contain some inappropriate epitopes and conserved amino acid sequences which might induce inappropriate immune responses and result in hepatic pathology and lesions. Therefore, new generations of vaccines should be designed on the basis of immunodominant epitopes which could induce CTL responses different from that induced by natural virus infection. In the present study, the HBV immunodominant CTL epitope was chosen and introduced into the universal T helper epitope of tetanus toxoid to enhance the helper T cell responses. DCs pulsed with the synthetic peptide were used as the "therapeutic" vaccine and HBV-specific CTL and antiviral immunity were investigated in HBV transgenic mice.

2. Materials and methods

2.1. Synthetic peptides

Peptides with a purity of >95% were synthesized by an automatic solid-phase peptide synthesizer (Applied Biosystems, Foster City, CA) by GL Biochem Co. Ltd. (Shanghai, China) and purified by reversed-phase HPLC. The TT–HBsAg28–39 peptide consists of the 830–843 amino acid positions of the universal T helper epitope of tetanus toxoid (QYIKANSKFIGITE) and immunodominant, Ld restricted CTL epitope located between residues 28 and 39 in hepatitis B surface antigen (IPQSLDSWWTSL) (Moriyama et al., 1990) with the linker of "-Ala-Ala-Ala-". HIV-1 IIIB gp120 CTL epitope peptide (RGPGRAFVTI, 311–320) was used as control. Each amino acid coupling was followed by a capping cycle with 10% acetic anhydride:dimethylformamide (v/v) to facilitate subsequent purification. Peptides were dissolved in DMSO at a concentration of 20 mg/ml and stored at −20 °C.

2.2. Animals

Female BALB/c (H-2K^d) mice, 6–8 weeks old, were purchased from SIPPR/BK Animal Co. Ltd. (Shanghai, China). The HBV transgenic mice, 8–10 weeks old, were provided by infectious disease center of 458 hospital of People's Liberation Army (PLA), PR China, and kept under standard pathogen-free conditions. The HBV transgenic mouse lineage was initially produced on a BALB/c background. The transgene in these mice consists of 1.3 copies of the complete genome of HBV subtype ayw. High level of HBsAg and HBV DNA in the sera could be detected in the HBV transgenic mice.

2.3. Generation of bone marrow-derived dendritic cells

The procedure used in this study was previously described (Inaba et al., 1992). Briefly, bone marrow cells prepared from the femurs and tibias of normal BALB/c mice (SIPPR/BK Animal Co. Ltd., Shanghai, China) were depleted of red blood cells with 0.84% ammonium chloride and cultured in 6-well plates (1×10^6 cells/ml) in 3 ml of complete medium (RPMI1640 supplemented with 2 mM L-glutamine, 10 mM HEPES (pH 7.4), 100 U/ml penicillin, 100 μg of streptomycin, 50 μM 2-ME, and 10% FCS) supplemented with recombinant mouse GM-CSF (10 ng/ml; PeproTech, Rocky Hill, NJ) and recombinant mouse IL-4 (1 ng/ml; PeproTech). All cultures were incubated at 37 °C in 5% humidified CO_2. Non-adherent granulocytes were removed after 48 h of culture and fresh medium was added. On day 6, the non-adherent cells were harvested by gentle pipetting. After washing, DCs were collected and the purity was measured by FACS. The purity of DCs in the study was above 90%.

2.4. Immunization of mice with peptide-pulsed DCs

Bone marrow-derived DCs were pulsed with 10 μg/ml tetanus toxoid (TT)-HBsAg28–39 peptide for 3 h. DCs were then activated with LPS for 24 h, washed extensively, and used for subsequent immunization experiments. Peptide-pulsed DCs (5×10^5/mouse) were injected i.p. into HBV transgenic mice for 3 times at 7-day intervals. Mice were sacrificed after 7 days and the splenocytes were isolated for CTL induction in vitro.

2.5. CTL

Single cell suspensions were prepared from spleens of mice under germ-free condition. The fresh splenocytes were plated in 24-well microplates in RPMI1640 medium supplemented with 10% FCS and 50 U/ml murine IL-2 and in the presence of peptide-pulsed stimulator at the ratio of 10:1. Five days after stimulation, cells were harvested and used as fresh effector cells. The target cells were HBsAg28–39 peptide-pulsed P815 cells, plasmid pCMV-S-transfected P815 cells or unpulsed P815 cells. Plasmid pCMV-S was constructed by inserting HBV surface Ag gene (1–681) into the downstream of CMV promotor of eukaryotic expression vector

pcDNA3.1 as the routine method. The 1×10^6 target cells were labeled with 3.7×10^6 Bq $Na_2{}^{51}CrO_4$ (Amersham, UK) in 1 ml RPMI1640 medium supplemented with 20% FCS for 90 min at 37 °C, and then washed 3 times before the addition of effectors. Various concentrations of effector cells were mixed with 1×10^4 targets at effector/target (E/T) ratios of 12.5, 25, and 50 in 200 μl of culture medium in 96-well round-bottomed microplate in triplicates. The microplate was centrifuged for 3 min at 500 r/min, and then incubated for 4 h at 37 °C in 5% CO_2. After the incubation terminated, 100 μl/well of supernatants was harvested and counted on a γ-counter. Percentage of target cell-specific lysis was determined as: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100%. Spontaneous release and maximal release were determined in the presence of either medium alone or 1% SDS, respectively. Spontaneously released counts were always <15% of the total counts. Data shown are the mean of triplicate cultures.

2.6. IFN-γ enzyme-linked immunospot (ELISPOT) assay

IFN-γ ELISPOT assays were performed using a commercially available kit (R&D Systems, Minneapolis, MN). Peptide-pulsed P815 cells were used as stimulator cells. Effector cells (1×10^5) and stimulator cells (1×10^5) were seeded into 96-well polyvinylidene fluoride (PVDF)-backed microplates coated with monoclonal antibody specific for mouse IFN-γ. After incubation at 37 °C for 16 h, cells were removed and plates were processed according to the manufacturer's instructions. Resulting spots in each well were counted under a dissection microscope, and the values were expressed as spot-forming cells (SFCs) per 10^6 splenocytes.

2.7. Serological and biochemical analysis

Sera were examined for HBsAg by ELISA according to the manufacturer's instruction (Sino-American Biotech Co., Shanghai, China). Hepatocellular injury was monitored by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities with an auto-biochemical analyzer (Guidotti et al., 1999).

2.8. Quantitation of HBV DNA in serum

Sera from HBV transgenic mice were subjected to detection of HBV DNA by the fluorescent quantitative PCR method using a commercial PCR kit (Fosun High Technology Co. Ltd., Shanghai, China).

2.9. Statistical analysis

Data are expressed as the mean values ± S.D. of triplicate samples. The statistical significance of the differences was determined by the unpaired two-tailed Student's *t*-test. Differences were considered statistically significant if $p < 0.05$.

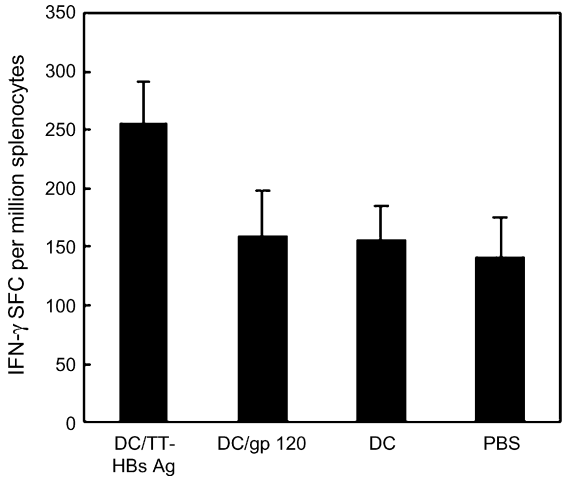

Fig. 1. IFN-γ ELISPOT assay. The splenocytes were cocultured with the stimulator in vitro, and the frequency of IFN-γ secreting cells was assayed with the mouse IFN-γ ELISPOT kit according to the manufacturer's instructions. The spots in each well were counted under a dissection microscope, and the values are expressed as spot-forming cells (SFCs) per 10^6 splenocytes. The data are the mean ± S.D. from five mice.

3. Results

3.1. The frequency of IFN-γ secretory cells determined by ELISPOT

The frequency of IFN-γ secreting cells in splenocytes reflected the number of CTL and cellular immune response. To detect the spot-forming cells relative to the number spleen cells, the splenocytes were stimulated with specific peptides in vitro, IFN-γ ELISPOT kit was used and the spots were counted under a dissection microscope. As shown in Fig. 1, synthetic TT–HBsAg28–39 peptide-pulsed DCs could induce the mice to produced more IFN-γ secreting cells than irrelevant peptide-pulsed DC or PBS ($p < 0.05$).

3.2. HBsAg-specific CTL induction

To examine CTL activities, HBV transgenic mice were vaccinated with HBV epitope peptide-pulsed DCs. The splenocytes

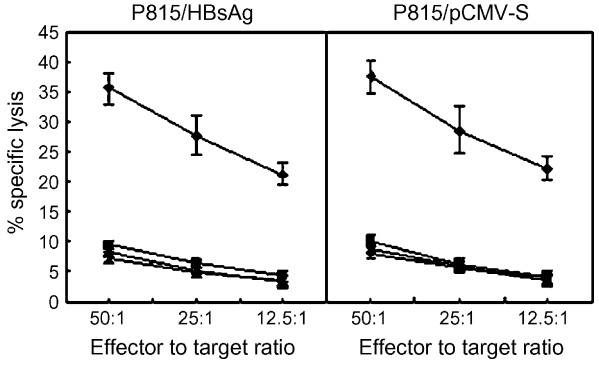

Fig. 2. CTL induction in vitro. CTL assay. Splenocytes were taken from mice 7 days after the final injection of TT–HBsAg28–39 peptide-pulsed DCs (♦), gp120 peptide-pulsed DCs (■), untreated DCs (▲), or PBS (×). After the splenocytes were stimulated in vitro for 5 days, HBsAg28–39 peptide-pulsed or plasmid pCMV-S transfected P815 cells was used as the target cells. Each point represents the mean ± S.D. of measurements from five different individuals.

from the immunized mice were cocultured with peptide-pulsed stimulators for 5 days and used as the effector cells. Peptide-pulsed, plasmid pCMV-S-transfected or untreated P815 cells were used as the target cells. Specific cytolytic activity of cells was tested in ^{51}Cr release assays. As shown in Fig. 2, HBV-specific peptide-pulsed DCs could induce HBV-specific CTL response, whereas there was no specific killing activities for the spleen lymphocytes from irrelevant peptide control mice. The cytolytic activity was specific because splenocytes did not kill untreated P815 cells (data not shown). These results demonstrated that the immunization of HBV specific peptide-pulsed DCs could induce cytolytic activity of lymphocytes against target cells.

3.3. Reduction of serum HBsAg level and HBV DNA titer in HBV transgenic mice

There are numerous hepatitis B surface antigen and HBV DNA in the sera in HBV transgenic mice, which are an important animal model for studying the immunotherapeutic effects of antiviral drugs or vaccines. To investigate the effect of antiviral immunity elicited by peptide-pulsed DCs in HBV transgenic mice, serum HBsAg level were detected by ELISA using HBsAg ELISA kit. As shown in Fig. 3A, serum HBsAg level from the mice immunized with TT–HBsAg28–39 peptide-pulsed DCs decreased markedly as compared with the mice immunized with irrelevant peptide control and negative control ($p < 0.05$). The titer of HBV DNA in sera were also detected by fluorescent quantitative PCR (Fig. 3B), the results demonstrated that the titer of HBV DNA in sera was significantly reduced from the mice immunized with HBV eptitope peptide-pulsed DCs compared with irrelevant peptide-pulsed DCs and negative control ($p < 0.05$). To detect whether the specific CTL elicited by HBV epitope peptide-pulsed DCs could cause the hepatocellular injury, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assayed. As shown in Fig. 3C, no significant difference was noted between the mice immunized with HBV epitope peptide-pulsed DCs with irrelevant peptide control or negative control ($p > 0.05$), which revealed that the inhibition of virus replication was primarily mediated by a nonlytic, lymphokine-based mechanism.

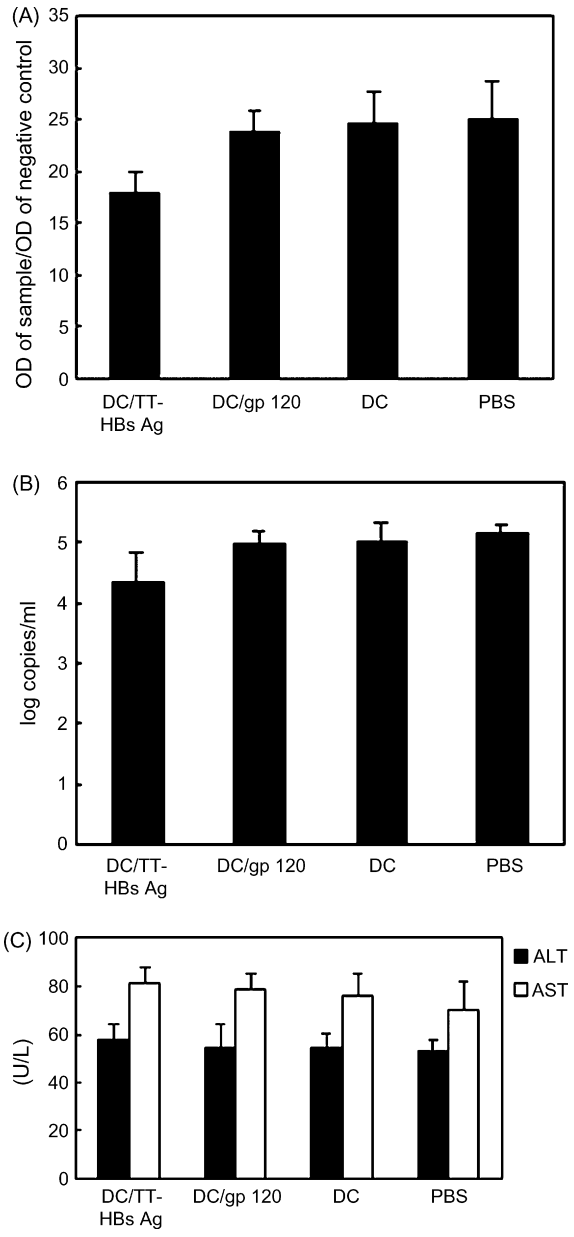

Fig. 3. Serum and biochemical analysis. (A) Detection of serum HBsAg level. The mice were immunized with peptide-pulsed DCs for three times at 7-day intervals and killed after 7 days of final vaccination. The sera were isolated and measured with HBsAg ELISA kit according to the manufacturer's instructions, the results are expressed with OD value of sample/OD value of negative control. The data are the mean ± S.D. from five mice. (B) Detection of the titer of HBV DNA in sera. HBV DNA in sera was extracted and analyzed by fluorescent quantitative PCR method using HBV DNA FQ-PCR kit. The results are expressed as log of copies/ml. The data are the mean ± S.D. from five mice. (C) Biochemical analysis of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. The mean ALT and AST activities were measured and expressed in units per liter. The data are the mean ± S.D. from five mice.

4. Discussion

The prospects for controlling new HBV infection depend on the availability of safe, effective, and affordable vaccines (Bloom et al., 1993). Recently, antigen-based vaccines have been used to treat human HBV carriers (Pol and Michel, 2006) and have been studied in HBV-transgenic mice, a murine model of HBV carriage (Akbar et al., 1997; Roh and Kim, 2003; Sette et al., 2001). DCs are the most potent APCs for initiating primary and secondary immune responses (Guermonprez et al., 2002). Thus, for vaccines or immunotherapies to be effective, antigens must be acquired and displayed by DCs. Many investigators have tried to use the potential efficacy of DCs to develop effective immunotherapies and vaccines against chronic viral infection including HBV. However, chronic HBV infection, like many other chronic viral diseases and cancers, is associated with T cell hyporesponsiveness or tolerance. The exact molecular mechanisms associated with this phenomenon are not entirely clear, but negative selection, peripheral anergy, and imbalances in lymphokine production all appear to contribute to maintaining the hyporesponsive

state of the host chronically exposed to viral or cancer antigens.

Several investigators have reported that DC-based immunization could break the tolerance in HBV transgenic mice and elicit effective antiviral immunity (Shimizu et al., 1998; Löhr et al., 2002). In HBV transgenic mice, DC function was normal, and there were HbsAg specific CTLs, which were functionally quiescent. When cytokine-activated DCs were administered to these transgenic mice, tolerance was broken, and antiviral responses were induced (Hart, 1997). Unfortunately, natural HBV antigens contain generally inappropriate epitopes which could elicit TH1/TH2 disequilibrium, immune deviation or immune deficiency, and the conserved amino acid sequences might also interfere with intercellular communication. Accordingly, effective protection relies on the appropriate match of a set of epitopes (Chaiken and Williams, 1996), natural antigens should be redesigned or modified on the basis of immunodominant epitopes. In present study, we introduced the common T helper epitope of tetanus toxoid into the immunodominant CTL epitope of HBsAg28–39 to strengthen the Th response, bone marrow-derived DCs were pulsed with the synthetic peptide and used as the therapeutic vaccine. The data showed that peptide-pulsed DCs had the ability to break the immunological tolerance and induce specific T cell response and antiviral immunity in HBV transgenic mice, which was consistent with the previous report. Shimizu et al. (1998) reported that the HBV transgenic mice failed to mount a CTL response to HBsAg after DNA immunization but they produced normal numbers of HBsAg-specific CTLs after the infusion of ex vivo activated DCs (Shimizu et al., 1998). Additional experiments are clearly required to understand why CTL tolerance in these transgenic mice could be broken by DC immunization but not by plasmid DNA or recombinant vaccinia virus (Wirth et al., 1995), both of which very efficiently induce HBsAg-specific CTLs in syngeneic nontransgenic animals (Schirmbeck et al., 1995).

Virus clearance crucially depends on the presence of activated peptide-specific CD8+ cytotoxic T lymphocytes (CTL). Through their T cell receptors (TCR) these CTL specifically recognize viral peptides that are presented by specific MHC class I molecules and cause lysis of the infected cells. Thus, CD8+ T lymphocytes are an important component of host defense mechanisms responsible for successful HBV clearance. In this study, chromium release assay and IFN-γ ELISPOT assay were used to detect the function of the effectors. HBV peptide-pulsed DCs induced potent CTL which could lyze peptide-pulsed or HBsAg-expressing target cells at different effector:target ratio. IFN-γ ELISPOT analysis demonstrated that TT–HBsAg28–39 peptide-pulsed DCs could generate more number of IFN-γ secreting cells than irrelevant peptide control or negative control. These results strongly revealed that HBV peptide-pulsed DC could induce potent CD8+ T cell response.

It has been proposed that elimination of virus is due to CTL-mediated lysis of infected hepatocytes and/or antiviral effects of CTL-derived cytokines, such as IFN-γ and TNF-α (Guidotti et al., 1994). CTL can inhibit HBV gene expression and replication in the liver of transgenic mice by secreting antiviral cytokines that interrupt the HBV life cycle. These cytokines activate two functionally independent virocidal pathways: an early pathway that eliminates HBV nucleocapsid particles and their cargo of replicating viral genomes from the hepatocyte (Wieland et al., 2000), and a later pathway that post-transcriptionally downregulates the viral RNA (Tsui et al., 1995). Because this potentially "curative" process is much more efficient than killing, CTL-induced intracellular inactivation of HBV could be the principal mechanism of viral clearance during HBV infection. Thus, noncytopathic mechanisms that eliminate replicative HBV DNA intermediates from the cytoplasm and covalently closed circular DNA from the nucleus appear to be crucial for clearance of HBV from the hepatocyte (Guidotti et al., 1999). To address whether the specific CTLs brought the hepatocellular injury, ALT and AST activities were measured by biochemical analysis and the results demonstrated that there was no significant difference in the immunized mice. These data support the concept that HBV-specific CTLs can abolish viral replication in HBV transgenic mice by noncytopathic mechanisms.

The detection of the level of HBsAg and HBV DNA in the sera in HBV transgenic mice reflects the immunotherapeutic effects of antiviral drugs or vaccines. In the study, serum HBsAg level and HBV DNA titer from the mice immunized with TT–HBsAg28–39 peptide-pulsed DCs decreased markedly as compared with the mice immunized with irrelevant peptide control and negative control, which showed that HBV epitope peptide-pulsed DCs could elicit anti-HBV immunity in HBV transgenic mice. Akbar et al. reported that HBsAg-pulsed DCs which were isolated from single cell suspensions of murine spleen cells resulted in negative HBsAg in 2 of 20 HBV-Tg mice (Akbar et al., 2004).

In summary, the present data show that vaccination therapy using DCs pulsed with immunodominant epitope peptides can elicit specific CTL response against HBV. The level of HBsAg and HBV DNA in the sera decreased significantly after immunological therapy. This report provides an additional rationale for the future development of DC-based strategies for the prevention and treatment of chronic virus infections.

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