Abstract. A weak T-cell immune response to the hepatitis B virus (HBV) is hypothesized to be the primary cause of chronic HBV infection. Emerging evidence suggests that long-term effective antiviral therapy restores the HBV-specific T-cell response from exhaustion. However, the extent to which the cellular immune response can be restored following the persistent suppression of HBV replication by antiviral therapy remains unclear. In order to investigate this question, 46 patients with chronic hepatitis B (CHB) treated with nucleos(t)ide analogues who demonstrated persistent suppression of HBV replication [defined as undetectable HBV DNA, hepatitis B e antigen (HBeAg) negative and adherence to antiviral therapy], 22 untreated CHB patients, 15 patients with acute hepatitis B (AHB) and 10 healthy adults were recruited. HBV-specific interferon-γ enzyme-linked immunospot (IFN-γ ELISPOT) assay and HBV-specific T-cell proliferation analysis were performed with a panel of overlapping peptides covering the envelope and core antigens. Data from this study showed that the HBV-specific immune responses to the peptide pools of the envelope and core protein in the treated patients were stronger than those in the untreated CHB patients, but significantly weaker than those in the AHB patients and healthy adults. A higher frequency of response to S than C peptide pools was confirmed by the IFN-γ ELISPOT assay in the treated CHB patients. The restoration of antiviral immunity was clearly associated with a reduction in HBV DNA and the duration of HBV DNA suppression. In conclusion, the HBV-specific immune responses in the CHB patients can be significantly restored from exhaustion following the persistent suppression of HBV replication as a result of antiviral treatment with nucleos(t)ide analogues.

Introduction

Worldwide, an estimated two billion people have been infected with hepatitis B virus (HBV), and >240 million suffer from chronic HBV (CHB) infection. CHB can result in a wide spectrum of liver diseases, including CHB, cirrhosis and hepatocellular carcinoma (1). Although HBV is noncytopathic, it is generally accepted that the outcome of HBV infection is mediated by the host immune response to HBV rather than the virus itself (2).

In CHB, cellular immune responses, particularly HBV-specific T-cell responses, are weak, oligoclonal and may be exhausted. These suppressed immune responses result in persistent HBV infection, and the fluctuating balance between virus replication and immune reactivity results in chronic liver inflammation, in which HBV replication is the driving force behind disease progression. The aim of CHB antiviral treatment is to inhibit HBV replication before irreversible damage occurs. During the past ten years, major advances have been made in CHB treatment. Current antiviral therapy by nucleos(t)ide analogues (NUC) or interferon (IFN)-α can alleviate liver inflammation, normalize serum alanine aminotransferase (ALT) levels, effectively suppress HBV replication and increase the rate of HBV envelope antigen (HBeAg) loss. It may even result in the HBV surface antigen (HBsAg) seroconversion in CHB patients (3,4). However, these treatments do not permanently eradicate the infection and there is a risk of HBV reactivation at withdrawal. The permanent suppression of HBV replication following treatment requires a robust acquired immune response against the HBV core and envelope antigens (5). There is evidence that antiviral therapy alters the balance between host immunity and viral replication, enabling weakened virus-specific immune responses to strengthen, broaden, and possibly control the infection, probably due to the decreased HBV antigen levels allowing the recovery of the T-cell response (6-9). This mechanism may be important in contributing to complete recovery from CHB.
Finding answers to these questions is vital to the future development of novel therapeutic strategies and immunomonitoring strategies to enable the earlier withdrawal of NUCs.

The present study examined changes in HBV-specific cytotoxic T lymphocytes (CTLs) and HBV-specific T-cell proliferation (against HBsAg and HBeAg) in the CHB patients with persistent suppression of HBV replication following antiviral therapy. The effects of antiviral therapy on the cellular immune responses and the association between the immune responses and the HBV virus load during antiviral therapy were investigated.

**Patients and methods**

**Study population.** A total of 83 patients with HBV infection, recruited between May 2012 and January 2013 from the Infection and Immunity Center at Beijing You'an Hospital at the Capital Medical University (Beijing, China), were enrolled in the studies. The following patient categories were used: NUC-treated CHB patients who presented with persistent suppression of HBV replication and undetectable HBV DNA, were HBeAg negative and who continued to use antiviral therapy (n=46); untreated CHB patients who were HBV DNA positive and HBeAg positive (n=22); and patients who were in convalescence from acute HBV infection (n=15). Ten healthy adults who were HBsAb-HBcAb-positive were enrolled as a healthy control (HC) group. A summary of the demographic, clinical and laboratory data from each group is shown in Table I. The study was approved by the Ethics Committee of the Beijing You'an Hospital in Capital Medical University, and all subjects provided written informed consent.

**Synthetic HBV peptides.** To investigate the HBV-specific T-cell responses, 16 to 20-mer peptides overlapping by 10 residues were used, which correspond to genotype C HBV (the most prevalent genotype in northern China). A panel of 55 overlapping peptides covering the full S open reading frame (ORF) and 28 overlapping peptides covering the full C ORF of HBV were obtained from Sigma-Aldrich (St. Louis, MO, USA). The two panels of overlapping peptides were dissolved in dimethyl sulfoxide and were placed into two mixtures, the S peptide pool (S-pool) and the C peptide pool (C-pool), respectively. The purity of these peptides exceeded 95%.

**Separation of peripheral blood mononuclear cells (PBMCs).** PBMCs were isolated from fresh blood using Ficoll-Hypaque density gradient centrifugation with Lymphoprep™ (Axis-Shield, Oslo, Norway) as previously described (8). Peripheral venous blood samples (20 ml) were collected in heparinized test tubes and diluted to a final volume of 40 ml with phosphate-buffered saline (PBS). This suspension was poured into a conical tube with 6 ml Lymphoprep. The PBMCs were isolated using density gradient centrifugation (800 × g, 20 min, without braking). Subsequently, the cells were washed twice with PBS and resuspended in R10 medium, which consisted of 90% complete RPMI-1640 medium (HyClone, Logan, UT, USA) and 10% heat-inactivated fetal bovine serum (FBS; HyClone). After isolation, the PBMCs were used for the in vitro experiment, and the rest were cryopreserved in 90% FBS and 10% dimethyl sulfoxide at -80°C for future use.

**Enzyme-linked immunospot (ELISPOT) assay for HBV-specific interferon-γ (IFN-γ) secretion.** To evaluate the HBV-specific reactivity, an IFN-γ ELISPOT assay was used to analyze PBMC IFN-γ secretion as previously described (8). A detailed description of the ELISPOT assay was published previously (8). The purity of these peptides exceeded 95%.
described (7,8). The antigens for the IFN-γ ELISPOT assays were the two pools of overlapping peptides (S-pool and C-pool). The positive controls were stimulated with 2 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich). The Multiscreen HTS 96-well filtration plates (Millipore, Billerica, MA, USA) were coated with 15 μg/ml anti-IFN-γ mouse monoclonal antibody 1-D1K (Mabtech, Nacka Strand, Sweden) overnight at 4°C, according to the manufacturer’s instructions. The plates were washed six times with PBS and blocked with R10 for 2 h at room temperature (RT). The freshly isolated PBMCs (2x10⁵ cells/well) were seeded and cultured in duplicate in R10 supplemented with CD28 monoclonal antibodies (Clone, CD28.2; eBioscience, San Diego, CA, USA) for 48 h at 37°C with 5% CO₂, and each specimen was respectively stimulated with the S-pool, C-pool and PHA at a final concentration of 2 μg/ml. Following washing, 50 μl of 1 μg/ml biotinylated mouse monoclonal antibody 7-B6-1 (Mabtech) was added and incubated for 2 h at RT. Then, 50 μl of 1,000-fold dilution streptavidin-alkaline phosphatase was added and incubated for 1 h at RT. Diluted BCIP/NBT (100 μl; Invitrogen, Carlsbad, CA, USA) was then added to each well and quenched with distilled water until distinct spots emerged. Following air-drying, the spot-forming cells (SFCs) were counted using an ELISPot reader (Sagecreation, Hangzhou, China). The quantity of SFCs was taken as the mean number of spots stimulated with antigen minus the spots in the absence of antigen per 1x10⁶ PBMCs.

Cell proliferation analysis. To assess the cell proliferation, CellTrace™ carboxyl fluorescein diacetate succinimimid ester (CFSE) was used as a cell proliferation-tracing reagent (Invitrogen). Freshly isolated PBMCs were diluted to ~20 million in/ml and labeled with a final concentration of 2 μmol/ml CFSE in the dark for 10 min at 37°C. Free CFSE was inactivated with FBS and washed away. The stained PBMCs were moved into a flat-bottom 96-well plate with a concentration of 200,000 cells per well. The PBMCs were cultured in R10 medium at 37°C and 5% CO₂. The HBV-specific T-cell proliferation was evaluated after seven days of incubation in the presence of HBV antigens (S-pool and C-pool) at a final concentration of 2 μg/ml. The positive control was in the presence of 1 mg/ml purified anti-CD3 and anti-CD28 mouse monoclonal antibodies (eBioscience), whilst the negative control was incubated in the R10 medium only. Cell proliferation was assessed by examining the dilution of CFSE by flow cytometry (FCM).

Flow cytometric analysis. Following incubation, PBMCs were harvested, washed with PBS and cell surface markers were stained with mouse monoclonal phycoerythrin-anti-CD3, mouse monoclonal allophycocyanin-anti-CD4 and mouse monoclonal peridinin-chlorophyll-protein-anti-CD8 antibodies (eBioscience, Inc.) for 20 min at RT. The stained cells were washed with PBS, fixed with 4% phosphate-buffered paraformaldehyde, and analyzed by FCAM. All results were collected using a BD FACSAvant II with corresponding antibodies (BD Biosciences, Franklin Lakes, NJ, USA), and at least 20,000 gated lymphocytes were collected for each sample. The proportion of proliferating cells was calculated using FlowJo software (Tree Star Inc., Ashland, OR, USA), and the results were expressed as the percentage of divided cells among gated lymphocytes.

HBV DNA assay and HBV marker assays. Serum HBV DNA was extracted from 850 μl plasma by the Cobas AmpliPrep® automated extractor and quantified using Roche COBAS® AmpliPrep/COBAS® TaqMan® HBV Test system and Roche original reagent (Roche China, Ltd., Shanghai, China) for the automated quantitative polymerase chain reaction amplification according to the manufacturer’s instructions. The HBV DNA load data were analyzed with AMPLILINK® software (Roche China, Ltd.) and expressed in IU/ml. The detection limit of the assay was 12 IU/ml. The quantification of the serum HBV markers (HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb) was determined by the Abbott Architect® i2000 system and the corresponding ARCHITECT assay (Abbott Laboratories, Chicago, IL, USA).

Statistical analysis. Continuous data was presented as the mean ± standard deviation or median (range). The cell proliferation data from the four groups were compared using one-way analysis of variance following the post hoc analysis of least significant difference. Data from the ELISPOT assays were evaluated using the Kruskal-Wallis and Mann-Whitney U-tests. The rates of positive response were compared using the χ² test. Correlations were examined using Pearson’s correlation. P<0.05 was considered to indicate a statistically significant difference.

Results

HBV-specific CTLs are suppressed in untreated patients and are restored by antiviral therapy. To confirm the effects of antiviral therapy on the frequency and functional changes of HBV-specific CTLs, an IFN-γ ELISPOT assay was performed on 22 untreated CHB patients, 46 NUC-treated CHB patients, 15 AHB patients and 10 healthy adults. There were no significant differences in age and gender between the four groups. The HBsAg and alanine transaminase (ALT) levels in the treated group were significantly lower than those in the untreated group (P<0.001). The antigens for the human IFN-γ ELISPOT assays were the S-pool peptides and the C-pool peptides.

The untreated CHB patients mounted weak HBV-specific CTL responses following stimulation with the S-pool and C-pool peptides. S-specific CTL responses were detectable in 63.6% of the untreated patients with a magnitude of 5.5 (range, 0-32) SFCs per 10⁶ PBMCs. C-specific CTL responses were detectable in 54.5% of the untreated patients with a magnitude of 6.0 (range, 0-39) SFCs per 10⁶ PBMCs. In the treated CHB patients who were undetectable for HBV-DNA following antiviral therapy, the positive rates of the S-specific and C-specific CTL responses increased to 93.5% and 91.3%, respectively. The magnitudes of the S-pool and C-pool specific CTLs increased to 16.5 (range, 0-175) and 13.5 (range, 0-163) SFCs per 10⁶ PBMCs, respectively. The S-pool and C-pool specific CTL responses were consistently and significantly higher than those in the untreated CHB patients. P<0.01 for the positive response to the S-pool, and P<0.001 for the positive response to the C-pool, as assessed by a χ² test. The P-values for the difference in the magnitude of response between the two groups for
the S‑pool and C‑pool were P<0.01 and P<0.05, respectively, as assessed by the Mann–Whitney test. The HBV‑specific CTL responses of the treated CHB patients were significantly weaker than those of the AHB patients, who exhibited a 100% positive CTL response and a higher frequency of SFCs. The S‑pool and C‑pool specific CTL responses were consistently higher in the HC group compared with the two groups of CHB patients, and were comparable to those in AHB patients. These results are shown in Fig. 1 and Table II.

HBV‑specific T‑cell proliferation, as determined by a proliferation‑tracing reagent, CFSE. The HBV‑specific T‑cell proliferation was also determined by CFSE staining in the presence of the HBV peptides (S‑pool and C‑pool). The untreated CHB patients showed significantly lower total (CD4+ and CD8+) HBV‑specific T‑cell proliferation (S‑pool, 2.1±0.8, C‑pool, 2.0±0.9). The treated CHB patients showed moderate proliferative responses (S‑pool, 3.0±2.0; C‑pool, 2.8±2.1). The AHB patients showed vigorous total HBV‑specific T‑cell proliferation (S‑pool, 6.9±2.1; C‑pool, 7.5±2.9), which were significantly higher than that of the two CHB patients groups (P<0.05). The HBV‑specific T‑cell proliferation of the HC group (S‑pool, 5.7±2.1; C‑pool, 6.2±2.5) was comparable to that of the AHB patients (P>0.05), and was significantly higher than those of the two CHB patient groups (P<0.001). These results are shown in Fig. 2.
The total T-cell numbers were further divided into subpopulations of CD8+ and CD4+ T-cells. The proliferative responses of the CD4+ and CD8+ T-cells among the three groups were in accordance with the data pertaining to the total HBV-specific T-cell proliferation. CD4+ and CD8+ T-cells contributed to the overall HBV-specific T-cell response observed in the four groups, although CD4+ responses were significantly greater in treated CHB patients, AHB patients and healthy adults than in untreated patients (P<0.05), as shown in Fig. 3.

**Antiviral therapy primarily improves the S-peptide-specific CTL response.** The S-specific and C-specific CTL responses were observed in a proportion of the untreated CHB patients (S pool: 14 patients, 63.6%; C pool: 12 patients, 54.5%), but were weak in these individuals. There was no significant difference between the magnitude of the S-specific CTLs and that of the C-specific CTLs in this group. The treated CHB patients showed vigorous HBV-specific CTL responses. The HBV-specific CTL responses in the treated CHB patients mainly reacted with the S-pool peptides, and the number of the S-specific CTLs (median, 16.5 SFCs per 10⁶ PBMCs; range, 0-175) was significantly higher than that of the C-specific CTLs (median, 13.5 SFCs per 10⁶ PBMCs; range, 0-163; P<0.05; Fig. 4A). No significant difference was identified.
between the S-specific T-cell proliferation and C-specific T-cell proliferation in treated and untreated CHB patients.

**HBV-specific immune responses in relation to the time duration of HBV DNA suppression.** All the treated CHB patients were undetectable for HBV-DNA following antiviral therapy, and the time duration of HBV DNA suppression varied at the time of analysis (median, 15 months; range, 4-40). The correlation between the immune responses and the duration of HBV DNA suppression was assessed. There was a significant positive correlation between the magnitude of HBV-specific CTLs and the duration of HBV DNA suppression (S-pool, r=0.458, P<0.01; C-pool, r=0.294, P<0.05), although only S-specific T-cell proliferation was significantly correlated with the duration of HBV DNA suppression (r=0.390, P<0.01; Fig. 5).

Correlation between the immune response and HBV markers. In the untreated CHB patient group, the association between the immune responses and the viral load was assessed. A significant inverse correlation was detected between the number of S-specific CTLs and the HBV DNA levels (r=-0.409, P<0.05; Fig. 6A). No correlation was found between the number of C-specific cells and HBV DNA levels (r=0.35, P<0.05; Fig. 6B). No correlation was detected between the immune responses and the HBsAg, HBeAg, and ALT levels.

**Discussion**

The antiviral therapy currently available has become more effective against CHB. The majority of CHB patients can achieve persistent suppression of HBV replication following
NUC treatment. Persistent suppression of HBV replication may alleviate liver inflammation and result in a decreased risk of liver cirrhosis or cancer in these patients (4). The mechanisms that may contribute to complete eradication of HBV infection include the innate immune responses and the acquired CD4+ and CD8+ T-cell responses, particularly the HBV-specific T-cell responses (8). These mechanisms have been shown to be of paramount importance in evaluating the future direction of antiviral treatment for HBV-infected patients (12-14). It may be beneficial to identify any immune function alterations in the patients with persistent suppression of HBV replication following antiviral therapy that may aid in predicting the long-term efficacy of antiviral therapy and provide immunotherapeutic targets. To quantify the level of functional T-cell restoration, four well-defined groups were selected. The untreated CHB patients acted as a negative control group, defining the basal level of impaired HBV-specific immunity prior to antiviral therapy. The AHB patients were defined as a positive control group who had the ability to eradicate HBV from the body by mounting a vigorous HBV-specific immune response. The healthy adults were defined as a positive control group who had previously contracted HBV, but had subsequently cleared the virus and now expressed antibodies against HBsAg and HBeAg.

The data from the ELISPOT assay provided important information. A proportion of the untreated patients exhibited HBV-specific CTL responses that were weak. These findings are consistent with the majority of previous studies (7,10,14). In the treated CHB patients, the frequency and magnitude of the antigen-specific T cell responses were significantly higher than those in the untreated CHB patient group. However, they were still significantly weaker than those in the AHB patients and healthy adults. These findings are consistent with the results from Boni et al (10), which showed that the T-cell responses in the NUC-treated patients with HBV DNA suppression but HBsAg persistence were markedly stronger than in the untreated patients with CHB, but were significantly weaker than in the patients with HBsAg clearance and anti-HBsAg antibody generation. As a result of the wide variability of responses among the treated groups, certain treated patients with persistent HBV-DNA negative status exhibited a less efficient immune response than those of particular untreated patients.

The results from the HBV-specific T-cell proliferation experiment were similar to the data for HBV-specific CTL responses. The untreated CHB patients also exhibited poor proliferative capacity of total T-cells, CD4+ T-cells and CD8+ T-cells in response to HBV antigens. The treated CHB patients showed an enhancement of HBV-specific proliferation compared with the untreated patients, although the difference between the two groups was not statistically significant. Emerging evidence indicates that a robust, early CD4+ T-cell response is critical in the induction of sustained CD8+ T-cell activity (15). The present study showed that the CD4+ T-cell made the primary contribution to the total T-cell proliferation in the treated CHB patients and AHB patients, who also simultaneously showed a significant enhancement of HBV-specific CTL activity. These results suggested that the immune status of the treated CHB patients was different from that of the untreated CHB patients, and the enhanced CD4+ T-cell proliferation may be important for the establishment of sustained CD8+ T-cell activity.

The results showed that the S-specific and C-specific CTL responses of the treated CHB patients were partly restored, and that the HBV-specific CTL responses were predominantly to the S-pool peptides. This finding is inconsistent with previous study results (8), which suggested that the T cells almost exclusively responded to the core antigens in HBsAg seroclearance patients. The differences among the results discussed above may be attributed to variations among research samples with different levels of HBV control.

In the treated CHB patients, it was found that the longer the duration of HBV DNA suppression, the stronger the HBV-specific T-cell response was. The results demonstrate a positive correlation between the magnitude of the HBV-specific T-cell responses and the duration of the HBV DNA suppression. The independent effect of the length of HBV DNA suppression on specific immune response, found in this study, was partly in accordance with previous studies (16,17). It is widely hypothesized that the T-cell function exhaustion of CHB patients occurs because of the prolonged exposure of T cells to high quantities of viral antigens and that T-cell resting from antigenic stimulation is a crucial requirement for restoration of a functional antiviral T-cell response (3,5,9,18). In this study, the results demonstrated a negative correlation between HBV-DNA levels and HBV-specific CTL responses in untreated CHB patients. These findings are in agreement with previous studies (14,17) and strengthen the evidence for an independent effect of viral load on the cellular immune response.
The liver is the main target organ for HBV infection, and the intrahepatic immune responses induced by HBV are crucial for viral clearance as well as disease pathogenesis. Previous studies in humans, chimpanzees and HBV transgenic mice reveal that intrahepatic HBV-specific T cells, as well as natural killer (NK) and NKT cells, are important in viral clearance and disease pathogenesis during HBV infection. A vigorous HBV-specific T cell response is readily detectable in the liver of AHB patients, but due to functional or quantitative differences in this response, chronically infected patients are unable to terminate the infection (19,20). Our results, based on peripheral blood samples, are in agreement with the studies already mentioned. However, the restoration of HBV-specific immune responses detected in the circulation only partially reflect the responses in the liver.

In conclusion, the data indicates that the exhausted HBV-specific immune responses are significantly restored following the persistent suppression of HBV replication as a result of antiviral therapy. The restoration of antiviral immunity is clearly associated with reduced HBV DNA levels and the duration of HBV DNA suppression, suggesting that there is a correlation between HBV viremia and HBV-specific immune function. These findings may be important in improving the current understanding of antiviral therapy and for developing appropriate therapeutic strategies against CHB.

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References