The Characteristics of the Cell-Mediated Immune Response Identify Different Profiles of Occult Hepatitis B Virus Infection

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Background& Aims: **Hepatitis B virus (HBV) DNA detection in serum and/or in the liver of hepatitis B surface antigen (HBsAg)-negative patients with or without serologic markers of previous viral exposure is defined as occult HBV infection. Because the role of the adaptive response in keeping HBV replication under control in occult infection still is undefined, this study was performed to characterize the features of the HBV-specific T-cell response in this condition.** ***Methods:*** **HBV-specific T-cell frequency and function were tested ex vivo and after in vitro expansion in 32 HBsAg-negative patients undergoing diagnostic liver biopsy for chronic hepatitis C: 18 with occult HBV infection (11 anti-HBc–negative and 7 anti-HBc–positive patients) defined by the detection of intrahepatic HBV DNA by polymerase chain reaction; 14 without detectable intrahepatic HBV DNA (5 anti-HBc–positive and 9 anti-HBc–negative patients). Six patients with chronic hepatitis B and 7 HBsAg-inactive carriers were studied for comparison.** ***Results:*** **The presence or absence of serologic HBV markers defined 2 profiles of HBV-specific T-cell responses in occult infection. Anti-HBc–positive patients showed a T-cell response typical of protective memory, suggesting that this condition represents a resolved infection with immune-mediated virus control. In contrast, HBV-specific T cells in anti-HBc–negative patients did not readily expand and produce interferon-γ in vitro, suggesting the possibility of a low-dose infection insufficient to allow maturation of protective memory.** ***Conclusions:*** **Our results suggest different mechanisms of control of viral replication in seropositive and seronegative occult infections. Additional studies aimed at understanding possible different clinical implications are needed.**

One of the fundamental steps of the hepatitis B virus (HBV) life cycle is the conversion of the 3.2-Kb relaxed circular DNA in a covalently closed-circular DNA in the nucleus of the infected hepatocyte, where it then is conjugated with nuclear proteins forming a minichromosome. Covalently closed-circular DNA is the template for transcription leading to the production of new infectious virions in the infected cell. The highly stable covalently closed-circular DNA, resistant to cell enzymes digestion, is probably the basis for life persistence of HBV infection, even after complete clinical recovery from acute hepatitis B.[1] Thus, an overt HBV infection can persist in association with a chronic active or inactive disease with the presence of hepatitis B surface antigen (HBsAg) in the serum, but HBV also can persist decades after acute hepatitis along with a readily detectable memory T-cell response,[2,3] despite a profound down-regulation of HBV gene expression[4–6] under the effect of the protective antiviral immune response. This type of condition with persistence of minute amounts of virus in the liver and/or serum and with possible detection of HBV DNA also in peripheral blood mononuclear cells (PBMCs) likely can be identified with the so-called occult HBV infection and it generally is characterized by the presence of serum anti-HBV antibodies. However, occult infection with negative HBsAg can be present also in completely seronegative patients.[7]

The lack of HBsAg detection in occult infections may depend on mutations in the "a" determinant, but this condition only accounts for a minority of cases in the Mediterranean area and recent studies tend to exclude that HBV genetic mutations are responsible for the strong suppression of viral replication typical of occult HBV infection.[8] Several other mechanisms could be involved, such as viral interference by other viruses, including hepatitis C virus (HCV), down-regulation of HBV gene expression by an undefined cellular mechanism, and virus control by the adaptive T-cell response.

In the clinical setting, occult HBV infection represents not only a condition at risk of HBV reactivation, but it is also a cofactor of liver disease progression and hepato-

Abbreviations used in this paper: CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; ELISPOT, enzyme-linked immunosorbent spot; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction.

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carcinogenesis in patients with chronic HCV infection.[9–12] Moreover, outcome of interferon (IFN) treatment for chronic HCV infection could be influenced by the concomitant presence of occult HBV infection, even if definitive conclusions have not been achieved.[9,10,13–17]

To elucidate the possible pathogenetic mechanisms responsible for occult HBV infection, we have analyzed the features of the HBV-specific cell-mediated immune response in completely seronegative and in anti-HBc–positive patients with occult HBV infection. Results show different profiles of T-cell responses according to the serologic status of occult infection.

Materials and Methods

Patients

A total of 78 patients with chronic HCV infection undergoing liver biopsy before IFN treatment and without evidence of chronic HBV infection (HBsAg negative) were enrolled at the Unit of Infectious Diseases and Hepatology of the Azienda Ospedaliero-Universitaria of Parma (Italy).

Among these 78 patients, 18 subjects were positive for intrahepatic HBV DNA (Table 1, patients 1–18). HBV-specific HLA class I and class II restricted T-cell responses were studied in these 18 subjects and also in 5 anti-HBc–positive patients negative for intrahepatic HBV DNA (Table 1, patients 19–23) and in 9 chronic hepatitis C (CH-C) patients negative for HBV serum markers and for intrahepatic HBV DNA (Table 1, patients 37–45). The latter were selected randomly (first available patients) from the overall group of the 60 CH-C patients negative for intrahepatic HBV DNA. Liver histology of CH-C patients with and without occult HBV infection also is shown in Table 1.

Six anti-HCV–negative, highly viremic (HBV-DNA level, $>10^5$ copies/mL), anti-HBe–positive patients and 7 anti-HCV–negative, HBsAg-inactive carriers (HBV-DNA level, $<10^4$ copies/mL) were studied for comparison. All patients were anti–human immunodeficiency virus negative.

This study was approved by the Ethical Committee of the Azienda Ospedaliero-Universitaria of Parma, and all subjects gave written informed consent.

HBV-DNA Analyses

DNA was extracted from the frozen liver specimens and the PBMCs by standard procedures, as described in the supplementary material (see supplementary materials and methods online at www.gastrojournal.org).

Occult HBV infection was identified as previously described with slight modification.[11] All liver and PBMC DNA extracts were analyzed for the presence of HBV genomes by performing 4 different in-house single-step or nested polymerase chain reaction (PCR) amplification assays to detect preS-S, precore-core, Pol, and X regions. A sample was scored as HBV-DNA positive when amplification products were detected using at least 2 different sets of primers in 2 or more independent experiments. Moreover, direct sequencing of all amplified HBV products confirmed the specificity of the reactions. All negative cases were tested twice. In each PCR experiment, the following were included as negative controls: (1) serum and tissue DNA extracts from subjects known to be negative for HBV infection, (2) DNA-free reaction buffer, and (3) water. The limit of sensitivity of our single-step and nested PCR methods was in the range of 10^3 and 10 genome equivalents/mL, respectively, which approximately correspond to 2–200 IU/mL.

Serum HBV DNA was quantified by the COBAS TaqMan Hepatitis B Virus Test (Roche Diagnostics, Mannheim, Germany).

Detection of Amplification Products by Southern Blotting

The PCR products were separated on a 1% agarose gel and transferred onto a nylon Hybond N^+ membrane (Amersham, Buckinghamshire, England). The membrane was hybridized at 65°C overnight with a ^{32}P random prime-labeled (Amersham) full-length HBV genome probe and then was washed and exposed to X-Omat film (Kodak, Rochester, NY) at −80°C.

Analysis of the Entire PreC-C Genomic Region of HBV DNA by PCR and Direct Sequencing

Liver DNA extracts from a selected number of subjects were amplified by a nested PCR technique using oligonucleotide primers specific for HBV-DNA sequences flanking the entire preC-C genomic region and the Expand High Fidelity PCR System (Roche Diagnostics) according to the manufacturer's instructions. Technical details are reported in the supplementary material (see supplementary materials and methods online at www.gastrojournal.org).

Synthetic Peptides, Peptide-HLA Class I Tetramers, and Antibodies

A panel of 315 peptides (15-mer) overlapping by 10 residues and covering the overall sequence of HBV genotype D were purchased from Chiron Mimotopes (Victoria, Australia). Fifteen-mer peptides were pooled in 16 mixtures (supplementary Table 1; see supplementary material online at www.gastrojournal.org). Recombinant S protein (Glaxo Smith Kline, Uxbridge, UK) and pre-S1 and pre-S2 polypeptides (Merck Sharp & Dohme, Whitehouse Station, NJ) also were used for the ex vivo enzyme-linked immunosorbent spot (ELISPOT) assay. Phycoerythrin-labeled tetrameric peptide-HLA class I complexes, representing the HLA-A2 restricted epitopes HBV core 18–27 (FLPSDFFPSV),