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CTL escape mutations of core protein are more frequent in strains of HBeAg negative patients with low levels of HBV DNA

Hossein Sendi[a,b,c,d,*], Marjan Mehrab-Mohseni[a,d], Saeid Shahraz[e], Helene Norder[a,b], Seyed-Moayed Alavian[f], Babak Noorinayer[g], Mohammad R. Zali[g], Paul Pumpens[h], Herbert L. Bonkovsky[c,d], Lars O. Magnius[a,b]

[a] Department of Virology, Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden
[b] Microbiology and Tumor Biology Center, Karolinska Institute, SE-171 77 Stockholm, Sweden
[c] Biology Department, University of North Carolina, Charlotte, NC 28223, USA
[d] Liver Center, Cannon Research Center, Carolinas Medical Center, Charlotte, NC 28203, USA
[e] Harvard Initiative for Global Health, Harvard University, 104 Mount Auburn Street, 3rd Floor, Cambridge, MA 02138, USA
[f] Baqiyatallah Research Center for Gastroenterology and Liver Disease, Tehran, Iran
[g] Research Center for Gastroenterology and Liver Diseases, Shaheed Beheshti University of Medical Sciences, Tehran, Iran
[h] Latvian Biomedical Research and Study Centre, Riga, Latvia

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ABSTRACT

Background: Recent studies have suggested that Cytotoxic T lymphocytes (CTL) play a key role in eliminating hepatitis B virus (HBV).
Objectives: We aimed to investigate the role of mutations in different immune epitopes of hepatitis B core antigen (HBcAg) among Iranians with hepatitis B e antigen negative chronic hepatitis B (e-CHB), and asymptomatic carriers (ASCs).
Study design: Amino acids 1–150 of HBcAg were characterized for HBV strains from 29 e-CHB patients and 48 ASCs from Iran. All patients were infected with HBV genotype D and had previously been investigated for the presence of pre-core and basic core promoter (BCP) mutants.
Results: Amino acid mutations of core protein were observed more frequently in HBV strains from ASCs than e-CHB patients ($p = 0.014$). Asn^{67} mutation was mutually exclusive to the combination Ile^{66} and Ser^{69} ($P < 0.001$). Substitutions for Ser^{21} and Thr12Ser were associated with lower serum levels of HBV DNA ($p < 0.001$). None of the patients with mutations in HLA-A2 CTL epitope, 18–27, had serum HBV DNA more than 10^5 copies/mL ($p < 0.001$). By multivariate analysis, high level (>10^5 copies/mL) of serum HBV DNA was inversely associated with the presence of mutations in CTL epitopes of HBc (OR: 0.11, $p = 0.015$), while it was directly associated with the presence of promoter double $T^{1762}A^{1764}$ mutations together with G^{1757} (OR: 16.87, $p = 0.004$).
Conclusion: The inverse correlation between serum levels of HBV DNA and CTL escape mutations of the core protein in HBeAg seroconverted patients, supports the notion that selection of CTL escape mutations consolidates the persistence of HBV infection despite reducing viral fitness.

1. Background

More than 300 million individuals worldwide are chronically infected with hepatitis B virus (HBV). Hepatitis B e antigen (HBeAg) negative chronic hepatitis B represents the predominant form of chronic hepatitis B (CHB) in several parts of the world including Iran. This form of CHB, defined as HBeAg negative CHB (e-CHB),[1] is mostly associated with mutations in the basal core promoter (BCP) and pre-core (preC) regions of HBV.

The HBV genome has four open reading frames, one of which is the core gene encoding the 183 or 185 amino acid (aa) long nucleocapsid, or core (HBc) protein which is preceded by the BCP and preC regions.[2]

The major B-cell epitopes of HBV are localized around the most protruding HBc region (aa 71–87[3] involving the tip of the spike [residues 76–82]).[4,5] The other B-cell epitope lies around aa 129–132.[5,6] Another epitope, corresponding to residues 107–118,

Abbreviations: aa, amino acid(s); ASCs, asymptomatic carriers; BCP, basal core promoter; cc, correlation coefficient; CHB, chronic hepatitis B; CTL, Cytotoxic T lymphocyte; e-CHB, HBeAg-negative CHB; HBc, hepatitis B core; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; preC, pre-core; Th, T helper.

* Corresponding author at: Liver Center, Cannon Research Center, Carolinas Medical Center, Charlotte, NC 28203, USA. Tel.: +1 704 355 7226; fax: +1 704 355 7648.
E-mail address: hsendi@uncc.edu (H. Sendi).

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was also suggested to be a B-cell epitope, although it does not seem to be on the surface of the complete virion.[7]

HLA-class-II-restricted, T helper cell epitopes of the HBc protein have been mapped to peptides corresponding to aa positions 1–20, 28–47, 50–69, 72–90, 81–105, 90–99, 108–122, 111–125, 117–131, 120–139, 126–146, and 141–165.[6,8–11]

Special attention has been devoted to the search for HLA class I-restricted Cytotoxic T lymphocyte (CTL) epitopes within the HBc molecule. A single HLA-A2-restricted epitope HBc aa (18–27) has been identified, containing the predicted HLA-A2 binding motif with Leu at position 2 and Val at the C-terminus.[8,12]

Although, the immunological basis for HBV persistence is not fully understood, CTLs are known as the main actors of the immune system in viral clearance.[13] It is well known that the CTL response is less vigorous in chronically infected patients,[11] and a robust T-cell response against residues 18–27 of HBc protein[13] has been observed in patients who spontaneously clear the infection, but not in patients with CHB.[8,14–16] However, the differences in immune responses among subgroups of patients with CHB patient have been less thoroughly investigated.

2. Objectives

In a recent study, we characterized the BCP and preC regions of HBV isolates of Iranian patients with e-CHB, and asymptomatic carriers (ASCs).[17] The present study investigates the prevalence of mutations in different HBc immune epitopes of HBV strains in the previously studied Iranian patients and its association to serum levels of HBV DNA, aminotransferase levels, and also BCP and preC mutational patterns.

3. Study design

3.1. Patients

The study included 97 patients negative for HBeAg, and positive for anti-HBe referred to the Hepatology Clinic of Taleghani General Hospital, Tehran, Iran between March 2000 and April 2003. Based on clinical, biochemistry tests, and imaging findings, the patients were divided into two groups: thirty-five patients were categorized as e-CHB while 62 were classified as ASCs. All patients were enrolled in the study after giving informed consent. Clinical characteristics of patients were previously described,[17] and all patients were shown to be infected with HBV genotype D.[17]

3.2. Methods

For core gene sequencing, HBV DNA was extracted from 200 μL of serum with QIAamp DNA mini kit (Qiagen Inc., Valencia, CA). Amplification was carried out with the primers hepA and hep66, and was nested with the primers hepA and hep68.[18] The PCR amplicons were purified with GFX PCR DNA and gel band purification kit (Amersham, Uppsala, Sweden). Purified products were used as templates in the sequencing reaction using the dideoxynucleotide chain termination method with ABI PRISM Big dye TM terminator cycle sequencing reaction kit (Applied Biosystems, a Division of PerkinElmer, Version 3) and the primers used in the PCR were used as sequencing primers. All amplificates were sequenced bi-directionally. The ABI PRISM 3100 Genetic Analyser (Applied Biosystems) was used for electrophoresis and data collection. The sequences obtained were edited using the SeqMan program in the LASERGENE package (DNA STAR Inc., Madison, WI). Three-dimensional predictions of the mutated HBc structures were performed on the basis of the X-ray structure of the HBc, genotype A[3], by a comparative modeling program 3D-JIGSAW[19] (http://bmm.cancerresearchuk.org/~3djigsaw/) and presented by Chimera software.[20]

3.3. Statistical analysis

Statistical analysis was performed with Chi-square and Fisher's exact tests for the absence or presence of mutations within different regions. Independent *t* test was used to compare the average mutation numbers within the same regions (SPSS Inc., Chicago, IL). Pearson's correlation coefficient (cc) was used to calculate the correlation between each pair of regional mutations. We employed a multivariate logistic regression analysis in order to find the association between the serum HBV DNA, and a set of epitope mutations as independent variables. Correlation and regression analyses were done with the aid of Stata 11 (StataCorp, USA).

4. Results

The core region of the infecting strain could be amplified from serum of 77 (79%) patients. Two e-CHB patients had strains with major deletions in core region and were excluded from statistical comparison. Sequencing data between amino acids 1–150 of core region in 48 ASCs and 27 e-CHB patients are presented in Fig. 1. Data regarding HBV DNA and serum aminotransferase levels, preC, and core promoter mutations are also available.[17]

4.1. Amino acid divergence in different regions of HBc protein

Ser^{21} and Thr^{80} were the most variable amino acid residues and could be substituted by seven and five different amino acids, respectively. Substitutions of Ser^{87} were found to be associated with substitutions of the Ser^{21} in most cases ($p < 0.002$), while the latter were associated with Thr^{12} ($p < 0.02$). Substitutions of Ser^{87} were also associated with Glu^{64} in most cases ($p < 0.01$), while substitutions of Met^{93} were always accompanied with Asp^{64} ($p < 0.001$). Asp^{64} was never accompanied with Thr^{49} ($p < 0.01$), and was more frequently associated with substitutions of Thr^{67} ($p = 0.010$), Tyr^{38} ($p = 0.010$), and Thr^{80} ($p = 0.018$). Double amino acid mutations Ile^{66} and Ser^{69} were always accompanied with Glu64Asp and never accompanied with Asn^{67} ($p < 0.001$).

Seven regions were found to be more variable within the HBV core protein and corresponded to residues 18–27, 35–45, 49–69, 76–87, 91–95, 105–116, and 130–135. It was also possible to localize each of these regions within respective immune epitope (Th, CTL, and B-cell; Table 1). Region 49–69, which is a Th epitope, was the most variable, followed by region 105–116 which is a B-cell epitope. There were significant positive correlations among the amino acid substitutions when comparing different regions. Specifically, correlations were found significant between regions 18–27 and 91–95 (cc = 0.43, $p = 0.0001$), 18–27 and 76–87 (cc = 0.41, $p = 0.0001$). The Thr12Ser mutation was also found to be correlated with substitutions within CTL epitopes 18–27 (cc = 0.43, $p = 0.0001$), and 91–95 (cc = 0.40, $p = 0.0002$).

"Hot spot" amino acid exchanges were localized on the three-dimensional structure of HBc tetramers in the context of major CTL and B epitopes, and most variable HBc regions (Fig. 2).

The overall frequency of amino acid mutations of HBc protein is higher in strains of ASCs than e-CHB patients (4.81 vs 3.22; $p = 0.014$). HBV isolates of ASCs also had a higher frequency of mutation in their B-cell epitopes than isolates from e-CHB patients (2.08 vs 1.22; $p = 0.026$, Table 1). This difference was most significant in region aa 105–116 ($p = 0.013$).