Multiple Hepatitis B Virus (HBV) Quasispecies and Immune-Escape Mutations Are Present in HBV Surface Antigen and Reverse Transcriptase of Patients With Acute Hepatitis B

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Background. This study characterizes and defines the clinical value of hepatitis B virus (HBV) quasispecies with reverse transcriptase and HBV surface antigen (HBsAg) heterogeneity in patients with acute HBV infection.

Methods. Sixty-two patients with acute HBV infection (44 with genotype D infection and 18 with genotype A infection) were enrolled from 2000 to 2010. Plasma samples obtained at the time of the first examination were analyzed by ultradeep pyrosequencing. The extent of HBsAg amino acid variability was measured by Shannon entropy.

Results. Median alanine aminotransferase and serum HBV DNA levels were 2544 U/L (interquartile range, 1938–3078 U/L) and $5.88 \log_{10}$ IU/mL (interquartile range, 4.47–$7.37 \log_{10}$ IU/mL), respectively. Although most patients serologically resolved acute HBV infection, only 54.1% developed antibody to HBsAg (anti-HBs). A viral population with ≥ 1 immune-escape mutation was found in 53.2% of patients (intrapatient prevalence range, 0.16%–100%). Notably, by Shannon entropy, higher genetic variability at HBsAg amino acid positions 130, 133, and 157 significantly correlated with no production of anti-HBs in individuals infected with genotype D ($P < .05$). Stop codons were detected in 19.3% of patients (intrapatient prevalence range, 1.6%–47.5%) and occurred at 11 HBsAg amino acid positions, including 172 and 182, which are known to increase the oncogenic potential of HBV.

Finally, ≥ 1 drug resistance mutation was detected in 8.1% of patients (intrapatient prevalence range, 0.11%–47.5% for primary mutations and 10.5%–99.9% for compensatory mutations).

Conclusions. Acute HBV infection is characterized by complex array of viral quasispecies with reduced antigenicity/immunogenicity and enhanced oncogenic potential. These viral variants may induce difficult-to-treat HBV forms; favor HBV reactivation upon iatrogenic immunosuppression, even years after infection; and potentially affect the efficacy of the current HBV vaccination strategy.

Keywords. HBV; acute infection; HBsAg; reverse transcriptase; quasispecies.

At least 2 billion people have been exposed to hepatitis B virus (HBV), and around 250 million are chronically infected. Around 1 million deaths annually are due to end-stage liver disease or hepatocellular cancer (HCC) [1].

The launch of the vaccine against HBV significantly reduced the number of new HBV infections worldwide. Despite the dramatic success of HBV vaccination, cases of acute HBV infection continue to be registered, and HBV remains a major agent of viral hepatitis. Although the vast majority of patients resolve this acute infection and develop long-lasting immunity, the serological resolution of acute HBV infection does not imply the eradication of the virus but indicates the persistence of a stable HBV DNA minichromosome, referred to as covalently closed circular DNA (cccDNA), in most infected patients [2–5]. The persistence of cccDNA despite the resolution of infection can potentially predispose the patient to HBV reactivation during immunosuppressive therapy [5–7] and can represent an important risk factor for HCC development [6, 8, 9].

So far, several studies have provided a pretty complete epidemiological characterization of patients with acute HBV infection [10–14]. However, an in-depth evaluation of genetic

variability throughout the genes encoding HBV surface antigen (HBsAg) and reverse transcriptase (RT; target of the currently available anti-HBV drugs) in patients with acute HBV infection is still missing.

HBsAg contains the major hydrophilic region that is a dominant epitope crucial for binding with neutralizing antibodies, including those induced by the vaccine. So far, around 20 immune-escape mutations in HBsAg, favoring HBV evasion from humoral responses, have been identified [15]. Immune-escape mutations have relevant pathobiological implications, particularly at the time of immunosuppression-driven HBV reactivation in patients with onco-hematological, rheumatological, and dermatological diseases [19–23]. Information about the genesis of these mutations is lacking (ie, are they generated and selected at the time of immunosuppression, or are they selected at the time of immunosuppression but generated during acute infection?) but may be relevant to improving knowledge about the pathogenesis of HBV reactivation.

Beyond immune-escape mutations, HBsAg sequences can also carry stop codons, which can determine the production of truncated HBsAg that, when retained inside the hepatocytes, can promote metabolic alterations associated with neoplastic transformation of these cells, thus contributing to HCC onset [24–27]. Detection of stop codons at the time of acute HBV infection (which, thus far, has not been investigated) may have implications for the persistence of HCC risk (which is approximately 3-fold greater than that in uninfected populations) despite serologically resolved HBV infection [28].

A paucity of information is available on the circulation of drug-resistant strains (particularly when present as minor variants) during acute HBV infection [29, 30]. Understanding this issue may be important in light of the recently proposed treatment for severe forms of acute hepatitis B [31, 32].

In this light, this study aims at investigating, by ultradeep sequencing, the extent of genetic heterogeneity in HBsAg and RT (in terms of immune-escape mutations, stop codons, and drug resistance mutations) in a well-characterized cohort of patients with an acute HBV genotype D or A infection.

MATERIALS AND METHODS

Patients

This study included 62 consecutive individuals enrolled at 2 units of infectious diseases in the Campania region of Italy from September 1999 to April 2010 (median enrollment year, 2006 [interquartile range {IQR}, 2002–2009]). All subjects had acute HBV infection at enrollment; 44 were infected with HBV genotype D, and 18 were infected with HBV genotype A. The 2 infectious diseases units had been using the same clinical and laboratory approach to detection HBV infection for years and had cooperated in several clinical investigations [11, 30, 33].

The definition of acute HBV infection was based on positivity for HBsAg and serum HBV DNA, positivity for anti-HBV core antigen (HBc) immunoglobulin M, and alanine aminotransferase levels >10 times the upper normal value. All patients had also a clinical history compatible with acute HBV infection. Demographic and clinical data, including age, race, sex, HBV serological findings, alanine aminotransferase level, aspartate aminotransferase level, bilirubin level, and serum HBV DNA level, were collected for each patient at the time of diagnosis and stored in an anonymized database. The assays used for HBV serological analysis and HBV DNA testing are reported in the Supplementary Text.

The study was approved by the Ethics Committee of the Azienda Ospedaliera Universitaria of the Second University of Naples. In addition, at the time of the first examination, all patients provided written informed consent for the collection and storage of biological samples and for the anonymous use of their data in clinical research. The research was conducted on viral DNA samples (used for clinical routine) and previously anonymized data, according to the requirements set by Italian data protection code (legislative decree 196/2003).

Ultradeep Pyrosequencing of HBV RT and HBsAg

The extent of genetic heterogeneity in RT (amino acid positions 1–345) and in HBsAg (amino acid positions 1–226) was investigated by ultradeep pyrosequencing (performed using the Roche 454 Junior system). The procedure was previously described [19] and is reported in the Supplementary Text.

Association Between HBsAg Variability and HBsAg Seroconversion

The correlation between the extent of genetic variability at each position in the major hydrophilic region of HBsAg (amino acid positions 99–169) and anti–HBV surface antigen (HBs) antibody production was evaluated by stratifying HBV genotype D–infected patients according to the serological outcome of acute HBV infection: one group comprised patients who, after acute infection, became HBsAg negative without developing anti-HBs (ie, patients with anti-HBc positivity alone), and the other comprised patients who became HBsAg negative and anti-HBs positive.

For each patient, the Shannon entropy weighted for the number of viral haplotypes detected (also defined as information content) was estimated for each HBsAg amino acid position. The formula is reported in the Supplementary Text. The Mann–Whitney test was used to verify whether the information content at a specific HBsAg amino acid position was significantly different between the 2 groups of patients.

Statistical Analysis

Data were analyzed using the statistical software package SPSS (version 17.0; SPSS, Chicago, Illinois) and the open-source R environment.

RESULTS

Patients' Characteristics

The characteristics of the 62 patients at the time of diagnosis of acute HBV infection are shown in Table 1. Patients were

Table 1. Patient Characteristics, Overall and by Infecting Hepatitis B Virus (HBV) Genotype

Characteristic	Overall Population (n = 62)	HBV Genotype D (n = 44)	HBV Genotype A (n = 18)	P Value[a]
Age, y, median (IQR)	36 (29–46)	34 (27–46)	37 (30–46)	NS
Male sex	47 (75.8)	30 (68.2)	17 (94.4)	.03
Risk factor				
Sexual intercourse	28 (45.2)	19 (43.2)	9 (50.0)	NS
Drug addiction	18 (29.0)	17 (38.6)	1 (5.6)	.01
Healthcare interventions	11 (17.7)	4 (9.1)	7 (38.9)	.01
Unknown	5 (8.0)	4 (9.1)	1 (5.6)	NS
Italian nationality	53 (85.5)	36 (81.8)	17 (94.4)	NS
Year of examination, median (IQR)	2006 (2002–2009)	2005 (2002–2008)	2006 (2002–2010)	NS
AST level, IU/dL, median (IQR)	1653 (1216–2253)	1777 (1334–2499)	1528 (1098–2006)	NS
ALT level, IU/dL, median (IQR)	2544 (1938–3078)	2716 (2035–3319)	2372 (1841–2837)	NS
Log HBV DNA level, IU/mL, median (IQR)	5.88 (4.47–7.37)	5.24 (4.42–6.91)	6.53 (4.53–7.84)	NS
Severe acute hepatitis B	4 (6.5)	4 (9.1)	0 (0.0)	NS
Coinfection, by antibody detected				
Anti-HDV	0 (0.0)	0 (0.0)	0 (0.0)	NS
Anti-HCV	8 (12.9)	8 (18.2)	0 (0.0)	NS
Anti-HIV	0 (0.0)	0 (0.0)	0 (0.0)	NS
Outcome				
HBsAg negative, anti-HBs positive	33 (53.2)	24 (54.5)	9 (50.0)	NS
HBsAg negative, anti-HBs negative	28 (45.2)	19 (43.2)	9 (50.0)	NS
Chronic HBV infection	1 (1.6)	1 (2.3)	0 (0.0)	NS

Data are no. (%) of subjects, unless otherwise indicated.

Abbreviations: ALT, alanine aminotransferase; anti-HBs, antibody to hepatitis B virus surface antigen; anti-HCV, antibody to hepatitis C virus; anti-HDV, antibody to hepatitis delta virus; anti-HIV, antibody to human immunodeficiency virus; AST, aspartate aminotransferase; HBsAg, hepatitis B virus surface antigen; IQR, interquartile range; NS, not significant.

[a] Values were calculated by the Mann–Whitney test, for continuous variables, and the Fisher exact test, for dichotomous variables and reflect the statistical significance of differences between the group of patients infected with HBV genotype D and the group infected with HBV genotype A.

monitored for a median follow-up of 12 months (range, 9–52 months). All patients were naive to therapy.

Most patients (85.5%) were Italian (median age, 36 years [IQR, 29–46 years]; Table 1). The population was mainly composed of males (75.8%), and this prevalence was higher in patients infected with genotype A than in those infected with genotype D (94.4% vs 68.2%; $P = .03$; Table 1). The median log serum HBV DNA load was 5.88 IU/mL (IQR, 4.47–7.37 IU/mL), while the median alanine aminotransferase and aspartate aminotransferase levels were 2544 IU/mL (IQR, 1938–3078 IU/L) and 1653 IU/mL (IQR, 1216–2253 IU/L), respectively (Table 1). Unsafe sexual intercourse was the main risk factor for HBV acquisition in both populations (43.2% among those infected with genotype D and 50.0% among the infected with genotype A; Table 1).

Regarding the serological outcome, HBsAg loss was observed in 98.4% of patients (61 of 62). Nevertheless, HBsAg seroconversion (ie, development of an anti-HBs titer of >10 IU/mL) was observed in only 33 patients (53.2%).

The only patient who developed a chronic infection harbored genotype D. This patient was an injection drug user and had acute HBV infection in January 2001. She was observed as an outpatient every 4 months up to June 2009 and had a virological and biochemical profile compatible with an inactive carrier state (defined as a serum HBV DNA level of <20 IU/mL and persistently normal transaminase levels).

Characterization of HBsAg Genetic Heterogeneity in Terms of Immune-Escape Mutations and Stop Codons

The median number of HBsAg sequences obtained by ultradeep pyrosequencing was 4530 (IQR, 2821–6449).

As first step, the extent of genetic heterogeneity between patients infected with genotype D and A was compared by estimating the mean evolutionary divergence. Greater mean HBsAg evolutionary divergence (±SD) was found in genotype D than A (0.027 ± 0.011 and 0.003 ± 0.002 nucleotide substitutions/site for genotypes D and A, respectively; $P < .001$). This prompted us to investigate the distribution of HBsAg immune-escape mutations and stop codons in genotype D and A.

Detection of Immune-Escape Mutations

Among 44 patients with HBV genotype D infection, at least 1 immune-escape mutation was detected in 15 (34.1%; Figure 1). In 9 of 15 (60.0%), the immune-escape mutations sP120S (n = 2), sP120T (n = 2), sA128V (n = 2), sT118A (n = 2), and sG145R (n = 1) occurred, with an intrapatient prevalence of 80%–100% (Figure 1), thus supporting their fixation in the viral population as predominant species. These mutations occurred as a single immune-escape mutation in the majority of patients (7 of 9; (Figure 1). In the remaining 6 patients, a single immune-escape mutation (sT123A, sM133I/L/T, sC138Y, or sG145R) was detected, with an intrapatient prevalence of <5% (Figure 1). Despite their
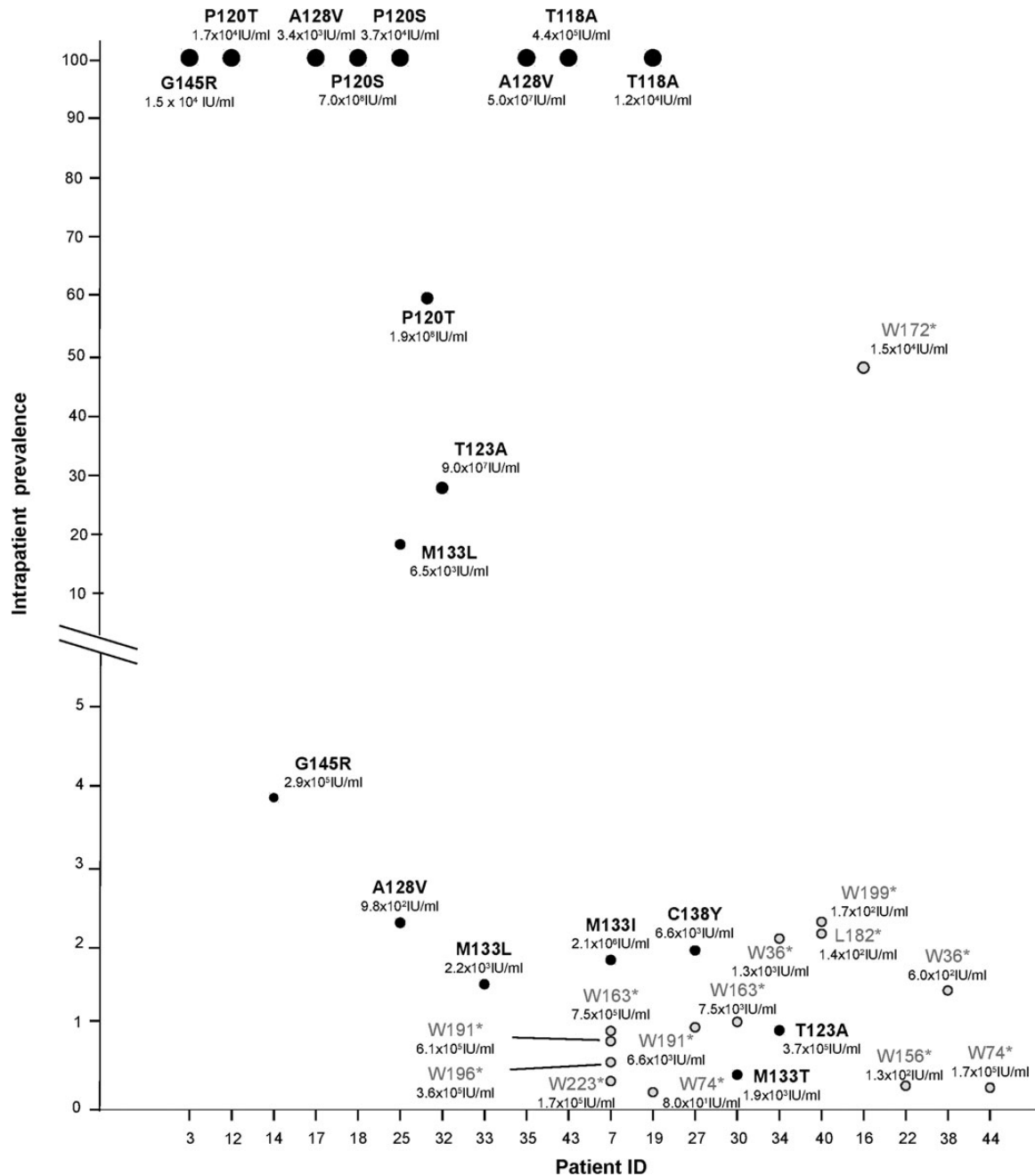
Figure 1. Intrapatient prevalence of immune-escape mutations (black dots) and stop codons (gray dots) in hepatitis B virus (HBV) surface antigen (HBsAg) detected in the 44 patients infected with HBV genotype D. Immune-escape mutations were defined as mutations known to affect HBsAg recognition by antibodies and are available online (http://hbv.bioinf.mpi-inf.mpg.de/index.php) and reported elsewhere [16]. The value reported below the mutations refers to the mutational load of each mutation, calculated according to the following formula: [percentage of sequences containing each mutation] × [contextual viral load] (Supplementary Text).

low prevalence, sG145R and sM133I were characterized by a high mutational load (295 115 IU/mL and 2 193 600 IU/mL, respectively; Figure 1). Notably, sT123A and sM133L were detected in 2 of 4 patients who developed severe acute hepatitis B. Also, sP120S, sM133L, and sG145R are known to act as vaccine-escape mutations [18]. The majority of patients (4 of 5) with these vaccine-escape mutations did not develop anti-HBs (see also the findings reported in the paragraph entitled Correlation between

the extent of genetic variability at specific HBsAg positions and the achievement of HBsAg seroconversion). Most immune-escape mutations resided in the first loop of the major hydrophilic HBsAg region (amino acid positions 99–137), with the exception of sC138Y, and sG145R, which resided in the second loop (amino acid positions 138–169).

Among 18 patients infected with HBV genotype A, the lysine and asparagine at HBsAg positions 122 and 131 (which are

known to hamper HBsAg recognition by antibodies in genotype D) were found in 17 and 18, respectively; they represent the wild-type amino acids at HBsAg positions 122 and 131 in HBV genotype A (Figure 2). Whether they are a sign of the higher intrinsic propensity of HBV genotype A to an easier escape from the immune pressure and, therefore, help in evading from humoral response needs to be further investigated. Beyond sT131N and sR122K, 4 patients (22.2%) carried other immune-escape mutations, such as sA159G, sC138Y, and sT123A (Figure 2). All of these mutations were present only in minority species (intrapatient prevalence, 0.16%–9.8%; Figure 2). In line with the in vivo ability of A159G to hamper anti-HBs production [18], the 2 patients with A159G did not develop anti-HBs.

No mutations associated with the acquisition of a new N-linked glycosylation site were detected.

Detection of Stop Codons

Stop codons were detected at 10 HBsAg positions in 22.7% of HBV genotype D–infected patients (10 of 44; Figure 1). They were localized in the C-terminal HBsAg domain and were characterized by a median intrapatient prevalence significantly lower than that observed for immune-escape mutations (0.9% [IQR, 0.2%–2.7%] vs 43.3% [IQR, 3.0%–100%]; $P = .0005$).

Similarly, a trend toward a decreased median mutational load in the presence of stop codons, compared with immune-escape mutations, was observed (3.6 log IU/mL [IQR, 2.4–5.2 log IU/mL] vs 4.2 log IU/mL [IQR, 3.6–6.1 log IU/mL]). Only sW172* (detected in patient 16) was observed, with an intrapatient prevalence of 43.3%. Owing to the overlap between the genes encoding RT and HBsAg, this stop codon corresponded to the drug resistance mutation rtA181T (Table 2).

The stop codons sW172* and sL182* are known to increase the oncogenic potential of HBV and occurred with an intrapatient prevalence of 3.72% and 47.5%, respectively (corresponding to a mutational load of 15 012 IU/mL and 149 IU/mL, respectively; Figure 1). Furthermore, the stop codons sW156*, sW191*, and sW223* reside in epitopes crucial for the HBsAg-specific cytotoxic response during the acute phase of HBV infection [32]. These stop signals were characterized by an intrapatient prevalence of 0.13%–0.89%, corresponding to a mutational load of 139–616 872 IU/mL (Figure 1).

In the single HBV genotype A–infected patient, 2 stop codons were detected, at HBsAg positions 16 and 35, both localized in the N-terminal part of HBsAg (Figure 2). Their intrapatient prevalence was 6.76% and 0.54%, respectively,
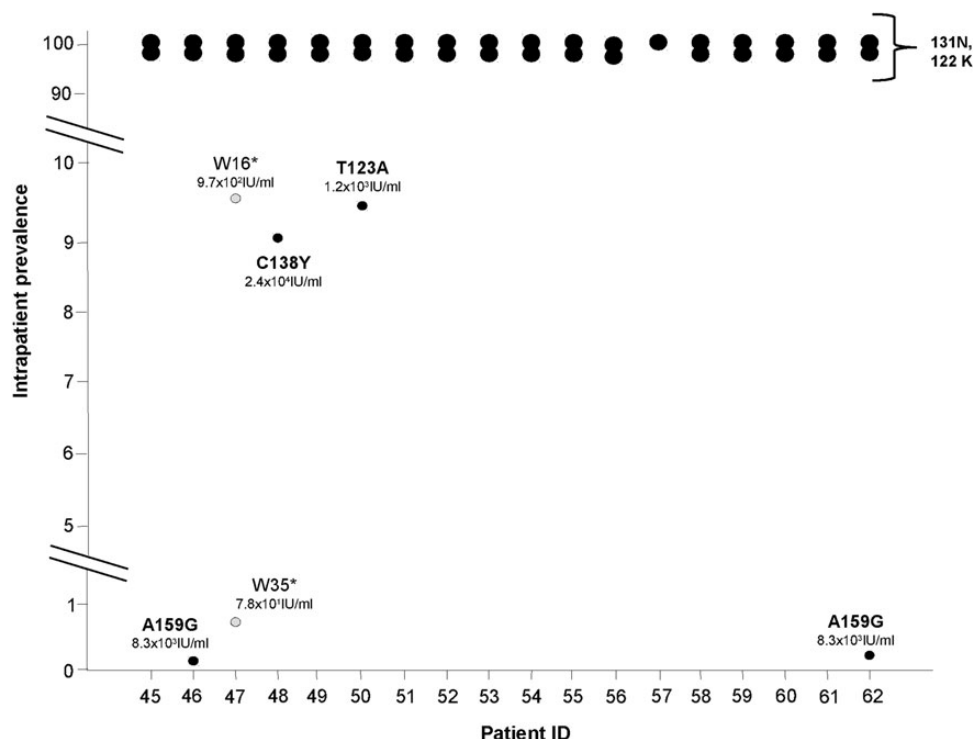
Figure 2. Intrapatient prevalence of immune-escape mutations (black dots) and stop codons (gray dots) in hepatitis B virus (HBV) surface antigen (HBsAg) detected in 18 patients infected with HBV genotype A. Immune-escape mutations were defined as mutations known to affect HBsAg recognition by antibodies and are available online (http://hbv.bioinf.mpi-inf.mpg.de/index.php) and reported elsewhere [16]. The value reported below the mutations refers to the mutational load of each mutation, calculated according to the following formula: [percentage of sequences containing each mutation] × [contextual viral load] (Supplementary Text).

Table 2. Drug Resistance Mutations Detected by Ultradeep Pyrosequencing in Patients With Acute Hepatitis B Virus (HBV) Infection

Patient	Log HBV DNA Load, IU/mL[a]	HBV Genotype	Drug Resistance Mutation in RT[b]	Intrapatient Prevalence, %[c]	Mutational Load, IU/mL[c]
7	7.96	D	rtA181T	0.11%	1.1×10^5
7	7.96	D	rtA194T	0.23%	2.1×10^5
7	7.96	D	rtM204I	0.23%	2.1×10^5
16	4.49	D	rtA181T	47.50%	1.5×10^4
47	4.16	A	rtV173L	10.50%	1.5×10^3
51	6.66	A	rtA194T	1.13%	5.2×10^4
53	6.85	A	rtV173L	63.48%	4.5×10^6
53	6.85	A	rtL180M	99.98%	7.1×10^6

[a] Data are from serum samples collected at the time of the first examination.

[b] Data denote drug resistance mutations that were detected in reverse transcriptase (RT) sequences obtained by ultradeep pyrosequencing. We considered for the definition of drug resistance mutations only the mutations detected in ≥4 sequences obtained by both forward and reverse primers. The list of drug resistance mutations is available online (http://hbv.bioinf.mpi-inf.mpg.de/index.php; updated on October 2013). In the list of drug resistance mutations, rtA194T was also included because a previous study potentially associated this mutation with reduced HBV susceptibility to tenofovir (Supplementary Text).

[c] Data are expressed as the percentage of reads with the specific mutation. Mutational load was calculated by the following formula: [percentage of sequences containing each mutation] × [contextual viral load].

corresponding to a mutational load of 973 IU/mL and 78 IU/mL, respectively (Figure 2).

Correlation Between the Extent of Genetic Variability at Specific HBsAg Positions and the Achievement of HBsAg Seroconversion

Anti-HBs development (a marker of full immunity against HBV) was observed in 53.2% of patients with acute HBV infection. Thus, the correlation of HBsAg genetic variability with the development of anti-HBs was investigated in patients infected with HBV genotype D (due to larger sample size). These patients were stratified in 2 groups according to the clinical outcome of acute HBV infection (Supplementary Table 1), as described in "Materials and Methods" section. The clinical and virological characteristics were comparable between the 2 groups, with the exception of male sex, which was more common among patients who developed anti-HBs, and positivity for anti-HCV, which was found only in patients who did not develop anti-HBs (Supplementary Table 1). The distribution of immune-escape mutations in patients with or without anti-HBs is reported in Supplementary Figure 1 and 2. A more heterogeneous distribution of immune-escape mutations (in terms of type and intrapatient prevalence) was observed in the group of patients who did not develop anti-HBs. In this group of patients, at least 1 immune-escape mutation was observed in 8 (42.1%), with an intrapatient prevalence of 1.7%–100% (Supplementary Figure 1). In 3 of 8 patients (37.5%), ≥2 immune-escape mutations were detected.

We then investigated whether the extent of genetic variability at HBsAg amino acid positions in the major hydrophilic HBsAg region (positions 99–169) may correlate with anti-HBs development. Thus, for each patient, the Shannon entropy weighted for the intrapatient prevalence of viral haplotypes (ie, information content) was used to measure the degree of genetic variability at each amino acid position.

In this analysis, a higher extent of genetic variability at 3 HBsAg positions (130, 133, and 157) significantly correlated with no anti-HBs production. In particular, these 3 positions were mutated in 0 of 24 patients who developed anti-HBs (Table 3). At least 1 amino acid mutation at position 130 (G130E), 133 (M133I/L/T), and 157 (A157T/V) was detected in 3 of 19 patients (15.8%), 4 of 19 (21.0%), and 3 of 19

Table 3. Correlation Between the Extent of Hepatitis B Virus Surface Antigen (HBsAg) Amino Acid Variability at Specific HBsAg Amino Acid Positions and the Production of Antibody to HBsAg (Anti-HBs)

HBsAg Amino Acid Position[a]	Anti-HBs Positive, No. (%) (n = 24)	Anti-HBs Negative, No. (%) (n = 19)	Information Content, Range[b]	P Value[c]	Mutations Detected by UDPS
130	0 (0)	3 (15.8)	4.28–4.15	.050	G130E
133	0 (0)	4 (21)	4.30–3.66	.02	M133L/I/T
157	0 (0)	3 (15.8)	4.31–4.13	.050	A157T/V

Abbreviation: UDPS, ultradeep pyrosequencing.

[a] All amino acid positions in the major hydrophilic region of HBsAg (ie, positions 99–169) were analyzed. The table reports only the HBsAg amino acid positions (130, 133, and 157) significantly correlated with no anti-HBs production.

[b] The information content was used to measure the extent of amino acid variability at each HBsAg position and was reported for patients with at least 1 mutation at position 130, 133, and 157. The formula for the calculation of the information content is reported in the Supplementary Text. This analysis was led in 2 groups of patients: one comprised patients who became HBsAg negative without developing anti-HBs (ie, patients with antibody to HBV core antigen alone), and the other comprised patients who became HBsAg negative and anti-HBs positive.

[c] Values were calculated by the Mann–Whitney test, for comparison of information content observed at positions 130, 133, and 157 of HBsAg in patients who developed and those who did not develop an anti-HBs titer.

(15.8%), respectively, who did not develop anti-HBsAg. This corresponds to an information content of 4.28–4.15 for position 130, 4.30–3.66 for position 133, and 4.31–4.13 for position 157 ($P \le .050$).

As previously mentioned, M133I/L/T are known to act as immune-escape mutations, while G130E and A157T/V reside at positions involved in reduced HBsAg recognition by antibodies [16]. In addition, HBsAg positions 130 and 133 are localized in an HLA class II epitope (positions 124–137) that is known to be important for the production of neutralizing antibodies [34].

Drug Resistance Detection

The median number of RT sequences detected by ultradeep pyrosequencing was 4296 (IQR, 2741–6473). A higher mean RT evolutionary divergence (±SD) was found in genotype D than A (0.045 ± 0.01 vs 0.007 ± 0.00 nucleotide substitutions/site; $P < .001$). By ultradeep pyrosequencing, at least 1 drug resistance mutation was detected in 8.1% of patients (5 of 62; 2 infected with genotype D, and 3 infected with genotype A; Table 2).

rtM204I was detected in a single HBV genotype D–infected patient (patient 7), in combination with rtA181T and rtA194 T, highlighting the presence of a viral quasispecies with an altered susceptibility to several anti-HBV drugs. These 3 mutations occurred with an intrapatient prevalence of <0.3%, corresponding to a mutational load of >100 000 IU/mL, owing to the high HBV DNA level in patients' sera (Table 2). rtA181T was also detected in patient 16 (infected with genotype D), with an intrapatient prevalence of 47.50% (corresponding to a mutational load of 14 769 IU/mL; Table 2).

The compensatory mutations rtV173L and rtL180M were detected in 2 HBV genotype A–infected patients (patient 47 and 53). In patient 53, these 2 mutations occurred with very high intrapatient prevalence values (63.48% and 99.98%, corresponding to mutational loads of 4 532 386 IU/mL and 7 138 310 IU/mL, respectively; Table 2).

DISCUSSION

In this study, we investigated the genesis of immune-escape mutations, stop codons in HBsAg, and drug resistance mutations in RT in the setting of acute HBV infection. To our knowledge, this is one of the first studies to address this issue by evaluating the extent of RT and HBsAg genetic heterogeneity among HBV quasispecies.

At least 1 immune-escape mutation was detected in 53.2% of patients. Of these mutations, sP120S, sM133L, and sG145R, known to act as vaccine-escape mutations, were detected in 11.4% of HBV genotype D–infected patients. sG145R is known not only to reduce the affinity of HBsAg for neutralizing antibodies, but also to abrogate their production [35]. In line with this finding, patients with G145R did not develop anti-HBs. Some immune-escape mutations, such as sM133L, sT123A, sC138Y, and sA159G, never occurred with an intrapatient prevalence of >30%. This is in line with previous in vitro studies showing the ability of these mutations to affect viral fitness [17, 36, 37].

Clarifying the issue of immune-escape mutations is critical since these mutations have important pathobiological implications. Recent studies have proposed that these mutations can favor immune-suppression–driven HBV reactivation in patients with serologically resolved HBV infection during the initial weakening of the immune system [19–23]. Immune-escape mutations (generated during acute infection) may thus predispose patients to HBV reactivation upon iatrogenic immunosuppression, even years after the serological resolution of HBV infection. These findings are potentially relevant to improving knowledge about the pathogenesis of HBV reactivation.

Moreover, we observed that an increased genetic variability at specific HBsAg positions (130, 133, and 157) significantly correlated with no anti-HBs production (a marker of full immunity against HBV that was present in only 53.2% of patients in our study). These 3 positions are involved in the lack of HBsAg recognition by antibodies, and 2 of them reside in an HLA class II epitope critical for neutralizing antibody production [34]. Further immunological studies are necessary to investigate whether mutations at these positions might interfere with anti-HBs titer production. This may be relevant in light of findings from a recent study that showed that the frequency of HBc-specific CD8$^+$ T cells continuously declines after HBsAg loss, supporting the role of humoral (rather than CD8$^+$ T-cell immune responses) in the prevention of HBV reactivation after HBsAg clearance [38]. In line with this finding, the absence of anti-HBs has been associated with an increased risk of HBV reactivation in patients with serologically resolved HBV infection receiving immunosuppressive therapy for onco-hematological diseases [39, 40].

Beyond immune-suppression driven HBV reactivation, immune-escape mutations can contribute to HBV transmission in vaccinated individuals and children born to HBsAg-positive mothers [41, 42]. They can also favor HBV reactivation in patients who received a liver transplant from an anti-HBc–positive donor [43].

Moreover, recent studies highlighted that enrichment of immune-escape mutations was negatively correlated with HBsAg loss during treatment with potent anti-HBV drugs in patients with chronic HBV infection [44, 45]. It has been postulated that increased HBsAg genetic diversity perturbs host immune control, resulting in survival of more viral variants in patients with persistently elevated HBsAg levels [45]. Although further studies are necessary to clarify this point, these mutations might interfere with the response to anti-HBV drugs, thus reducing the probability to achieve HBsAg loss also during acute infection.

Notably, at least 1 stop codon in HBsAg was detected in 22.7% of patients (10 of 44) with acute HBV genotype D

infection. Their intrapatient prevalence was significantly lower than that observed for immune-escape mutations, thus supporting findings of previous in vitro studies showing their ability to impair viral replication capacity [25]. The presence of stop codons can determine the production of truncated HBsAg that can accumulate in the endoplasmic reticulum, thus inducing oxidative stress and in turn favoring the neoplastic transformation of hepatocytes [24–27]. Stop codons might contribute to the risk of developing HCC despite the serological resolution of HBV infection, when the acute infection evolves toward an occult infection, or in the setting of HBV DNA integration in the host [2–6, 8, 9]. Their detection at the time of acute infection might be used as biomarker to identify patients who (despite the serological resolution of infection) might have a higher risk of developing HCC.

In addition, stop codons were detected mainly in genotype D. This might explain findings from epidemiological studies showing that genotype D is associated with faster progression to HCC than genotype A [46]. This concept can be also applied to other HBV genotypes associated with an increased HBV-associated oncogenic potential, such as genotype C. In this regard, a recent study showed that, in genotype C, the stop codon at position 182 was correlated with HCC onset, with increased cell proliferation observed in in vitro experiments [26]. Another recent study detected stop codons at positions 95, 182, and 216 in HCC tumor tissues and showed their ability to promote cell cycle advancement [47]. Further studies are necessary to investigate the circulation of stop codons in other HBV genotypes associated with increased oncogenic potential, including genotype E.

Finally, by analyzing RT sequences, ≥1 drug resistance mutation was detected in 8.1% of patients with acute HBV infection, with intrapatient prevalence values of 0.11%–47.50% for primary mutations and 10.50%–99.98% for compensatory ones.

The issue of drug resistance mutations in acutely infected patients has been investigated in few studies. In particular, by using a population-based sequencing approach, no drug resistance mutations were detected in a cohort of patients with acute HBV infection from whom data were retrospectively collected in England between 1997 and 2001 [48]. Conversely, by using a cloning sequencing approach, Baxa et al showed the presence of rtA181T, rtM250V, and rtS202G mutations in 3 of 23 patients with acute HBV infection from the United States, with intrapatient prevalence values of 1.54%, 1.39%, and 1.67%, respectively [29]. Our results are in line with those of Baxa et al, highlighting the presence of drug resistance mutations, mainly as minority species. So far, treatment for acutely infected patients is recommended in cases of severe or fulminant forms of acute hepatitis [31, 32, 49, 50]. Treatment is also suggested for patients with extrahepatic manifestations and for those with a prolonged disease course and impaired immune system, to shorten the duration of illness and to prevent the progression to chronic HBV infection [50]. In this light, the identification of drug resistance mutations may be important to set up the most appropriate anti-HBV treatment and to prevent severe complications, including death. The detection of drug-resistant strains may serve as a basis to promote research on alternative therapeutic strategies that can also be used during acute infection.

In conclusions, acute hepatitis B is characterized by a complex array of viral quasispecies with reduced antigenicity/immunogenicity and enhanced oncogenic potential. These viral variants may induce difficult-to-treat forms of HBV infection, favor HBV reactivation upon iatrogenic immunosuppression even years after infection, and potentially affect the efficacy of the current HBV vaccination strategy.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

Potential conflicts of interest. C. F. P. has received grants or funding from Gilead, Bristol Myers Squibb, Roche Diagnostics, Abbvie, Janssen, and Merck Sharp and Dohme. V. S. has received grants or funding from Gilead, Bristol Myers Squibb, and Roche Diagnostics. E. S. has received grants or funding from Gilead, Bristol Myers Squibb, Roche Diagnostics, Abbvie, Janssen, and Merck Sharp and Dohme. N. C. has received grants or funding from Gilead and Bristol Myers Squibb. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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