Hepatitis B Surface Antigen Serum Levels Help to Distinguish Active From Inactive Hepatitis B Virus Genotype D Carriers

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BACKGROUND & AIMS: The accurate identification of inactive (serum HBV-DNA persistently ≤2000 IU/mL) hepatitis B virus (HBV) carriers (IC) is difficult because of wide and frequent HBV-DNA fluctuations. We studied whether hepatitis B surface antigen (HBsAg) serum levels (HBsAgsl) quantification may contribute to diagnosis of HBV phases in untreated hepatitis B e antigen-negative genotype D asymptomatic carriers. **METHODS:** HBsAgsl were measured at baseline and end of follow-up and correlated with virologic and biochemical profiles of 209 consecutive carriers followed-up prospectively (median, 29; range, 12–110 months). HBV phases were defined after 1-year monthly monitoring of HBV-DNA and transaminases. **RESULTS:** HBsAgsl were significantly lower in 56 inactive carriers (IC) than 153 active carriers (AC): median, 62.12 (range, 0.1–4068) vs median, 3029 (range, 0.5–82,480) IU/mL; $P < .001$. Among AC, HBsAgsl were lower in 31 AC whose viremia remained persistently <20,000 IU/mL (AC1) than in 122 AC with fluctuations ≥20,000 IU/mL (AC2): 883 (0.5–7838) vs 4233 (164–82,480) IU/mL, $P = .002$. HBV infection was less productive in IC and AC1 than AC2 (log_{10} HBV-DNA/HBsAgsl ratios 0.25 and 0.49 vs 2.06, respectively, $P < .001$) and in chronic hepatitis than cirrhosis (1.97 vs 2.34, respectively; $P = .023$). The combined single point quantification of HBsAg (<1000 IU/mL) and HBV-DNA (≤2000 IU/mL) identified IC with 94.3% diagnostic accuracy, 91.1% sensitivity, 95.4% specificity, 87.9% positive predictive value, 96.7% negative predictive value. During follow-up, HBsAgsl were stable in AC but declined in IC (yearly median decline, −0.0120 vs −0.0768 log_{10} IU/mL, respectively, $P < .001$), 10 of whom cleared HBsAg. **CONCLUSIONS: HBsAgsl vary during chronic hepatitis B e antigen-negative genotype D infection and are significantly lower in IC. Single-point combined HBsAg and HBV-DNA quantification provides the most accurate identification of IC, comparable with that of long-term tight monitoring.**

Keywords: HBsAg Quantification; Inactive HBV Infection; HBeAg Negative Chronic Hepatitis B.

Currently, hepatitis B e antigen (HBeAg)-negative hepatitis B virus (HBV) carriers represent the great majority of cases of HBV infection in many geographical areas, including Europe, having become more prevalent during the last decade because of the aging of the HBV-infected population.[1–4] Clinical conditions associated with chronic HBeAg-negative HBV infection are variable, ranging from inactive carrier (IC) status to active chronic HBV infection (CHB).[5] The survival of those in the IC stage is comparable with the noninfected population, at least in Western countries.[6,7] By contrast, the rate of progression to cirrhosis among those with HBeAg-negative CHB ranges from 2.8 to 9.7 × 100 per year.[8–13] Current antiviral treatments may slow disease progression in cirrhotic patients and lead to cure of CHB if identified before the development of cirrhosis.[14] Thus, an early diagnosis of CHB may allow earlier consideration of initiating antiviral therapy. However, differential diagnosis between active CHB and IC status is problematic because HBeAg-negative CHB is characterized by wide fluctuations in viral replication and biochemical activity, with intermittent reductions of HBV-DNA serum levels below the inactive carrier cut-off ≤2000 IU/mL and spontaneous normalization of alanine aminotransferase (ALT) lasting from a few weeks to several months.[10,15,16] Therefore, to warrant an accurate differential diagnosis between CHB and IC, it is mandatory to monitor serum HBV-DNA by sensitive and quantitative polymerase chain reaction assays and ALT over a period of at least 1 year.[1,16,17] In addition, ALT levels are not a specific marker of viral-induced liver damage, and their fluctuation in an inactive carrier may be caused by nonviral liver disease cofactors.[18]

Abbreviations used in this paper: AC, active carriers; AC1, active carriers with HBV-DNA persistently <20,000 IU/mL; AC2, active carriers with HBV-DNA ≥20,000 IU/mL; ALT, alanine aminotransferase; anti-HBc, antibody to hepatitis B core antigen; anti-HBe, antibody to hepatitis B e antigen; AUROC, area under the receiver operating characteristic; cccDNA, covalently closed circular DNA; DA, diagnostic accuracy; CHB, chronic hepatitis B; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBsAgsl, HBsAg serum levels; HBV, hepatitis B virus; IC, inactive carriers; IgM, immunoglobulin M; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver operating characteristic.

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Thus, the identification of IC status is not always straightforward. Recently, hepatitis B surface antigen (HBsAg) serum levels were shown to correlate with intrahepatic covalently closed circular DNA (cccDNA) levels (the hallmark of infected hepatocytes),[19,20] to decline during therapy, and to be associated with long-term sustained virologic response.[21,22] These findings suggest that quantification of HBsAg might help to characterize the phase of HBV infection in HBeAg-negative carriers. We studied HBsAg serum levels and assessed their potential diagnostic value in a large cohort of untreated, HBeAg-negative/antibody to hepatitis B e antigen (anti-HBe)-positive, asymptomatic HBV genotype D carriers who had been followed up prospectively.

Patients and Methods

Patients

We studied 209 consecutive HBeAg-negative/anti-HBe-positive chronic HBsAg carriers infected with HBV genotype D (median age, 48 years; range, 18–77 years; 104 males and 105 females), admitted to the Liver Unit of the University Hospital of Pisa between 2000 to 2006. All the carriers were untreated and asymptomatic. The study was approved by the Ethical Committee of our hospital, and participants gave their written consent. Inclusion criteria were as follows: HBsAg/anti-HBe positive for at least 2 years; negative for HBeAg and antibodies against hepatitis C virus (HCV), hepatitis D virus, human immunodeficiency virus; absence of autoimmune disease and high alcohol intake (>40 g/day). They were part of a cohort of 314 HBeAg-negative/anti-HBe-positive HBsAg carriers followed up at our unit: 24 of them were excluded because they were symptomatic, 52 were infected with HBV genotypes other than D, 20 were already in treatment at the time of their first observation, 9 had missing clinical data.

To define accurately the infection profile of each carrier, serum tests were performed monthly during the first 12 months of the prospective follow-up and every 3 months thereafter. At the end of the 1-year monthly monitoring, HBV carriers were classified according to their biochemical and virologic profiles: (1) inactive carriers (IC) had persistently serum HBV-DNA levels ≤2000 IU/mL and normal ALT (<40 U/L); (2) active carriers (AC) had HBV-DNA serum levels >2000 IU/mL, with or without elevated ALT. Transient elastography became available in our unit on April 2004 and was performed in all newly enrolled carriers during the first year of follow-up from April 2004 on and at the first visit for carriers already in follow-up at that time. Liver biopsy specimens were obtained, within the first year of monitoring, from 109 of the 153 individuals in the AC phase; biopsy was not performed in the remaining 44 carriers because of persistently normal ALT and transient elastography values <6 kPa in 21; histologic diagnosis of cirrhosis already present in 11; signs of cirrhosis at ultrasound in 7; 5 refused to undergo biopsy.

Quantitative HBsAg was performed in all cases at baseline and end of the follow-up or at the time of initiation of antiviral therapy. Additional samples (median, n = 5; range, 3–16) were obtained in 67 HBV carriers to analyze the kinetics of HBsAg during the natural course of the infection.

Serology and Histology

Serum biochemistry included aspartate transferase (AST) and ALT, γ-glutamyl transpeptidase, alkaline phosphatase, albumin, globulins, total bilirubin, prothrombin time, and α-1 fetoprotein. HBsAg, antibody to hepatitis B surface antigen, antibody to hepatitis B core antigen (anti-HBc), HBeAg and anti-HBe, antibody to HCV, antibody to hepatitis D virus, and antibody to human immunodeficiency virus were detected by commercially available immunoassays (Abbott Laboratories, N Chicago, IL). Immunoglobulin M (IgM) anti-HBc level were determined by CORE-M-IMx (Abbott Laboratories), using 0.200 and 0.100–0.200 Index as cut-off and grey zone of chronic hepatitis respectively.[23]

HBsAg was quantified using the Architect HBsAg assay (Abbott Laboratories; dynamic range, 0.05–250.0 IU/mL) after 1:100 dilution. Samples with HBsAg levels >250.0 IU/mL at 1:100 dilution were retested at 1:1000 final dilution. Samples with HBsAg levels <0.05 IU/mL at 1:100 dilution were retested undiluted. Serum HBV-DNA levels were quantified by COBAS Amplicor Monitor 2.0 HBV assay (Roche Diagnostic Systems Inc, Mannheim, Germany) with a lower limit of detection of 200 copies/mL and linearity range from 200 to 20,000 copies/mL (conversion factor, 5.6 copies = 1 IU) until 2005 and thereafter by COBAS TaqMan assay, sensitivity 12 IU/mL, dynamic range 6–1.10×10^8 IU/mL. HBV genotyping was performed by direct sequencing of the region encoding for the small hepatitis B surface protein. Liver specimens were processed using standard criteria; grading of inflammation and staging of fibrosis were assessed by Ishak score.

Statistical Analysis

Data were expressed as median and range values. The logarithmic transformation was used for quantitative data without normal distribution. The Spearman correlation test was used to analyze the correlations between HBsAg serum levels and other continuous variables. Differences between subgroups were analyzed using Mann–Whitney rank sum test or Kruskal-Wallis test where appropriate. To identify factors independently correlated with HBsAg serum levels and HBsAg δ variations, variables with statistical associations ($P < .05$) or trends ($P < .10$) at univariate analysis were included in multiple regression analysis. Factors independently associated with HBsAg clearance were identified by using logistic regression analysis. The diagnostic performance of HBsAg serum levels was evaluated by receiver operating characteristic (ROC) curve. The cut-off value to discriminate chronic hepatitis patients

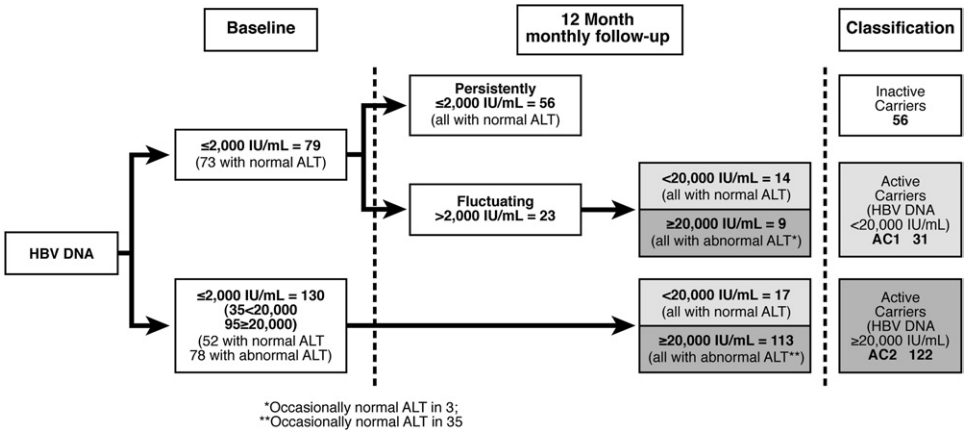

Figure 1. Classification of the 209 HBeAg-negative/anti-HBe-positive HBV carriers according to HBV-DNA and ALT serum levels during the 1-year monthly monitoring.

from inactive carriers was selected considering the highest sensitivity+specificity sum. To increase the specificity in the identification of IC, we selected an additional cut off combining a specificity of at least 90% with the highest sensitivity. Analysis of variance for repeated measures was performed to analyze the different profiles of HBsAg during follow-up. Statistical analysis was performed by SPSS (version 10.0; SPSS Inc, Chicago, IL) software package.

Results

Stringent Classification of the HBV Carriers

The 209 untreated HBeAg-negative/anti-HBe-positive carriers were followed up prospectively for a median time of 34.5 (range, 6–110) months, with monthly blood controls during the first year and every 3 months thereafter. In 18 patients with baseline evidence (previous histologic diagnosis or ultrasound signs) of cirrhosis, the monthly monitoring was reduced to 6 months: all of them had viremia ≥20,000 IU/mL more than once during the follow-up. To avoid the misclassifications caused by HBV-DNA fluctuations in case of cross-sectional evaluation,[1,10,16] the HBV infection profile (inactive vs active) of each carrier was defined after the 1 year of monthly serum HBV-DNA and ALT monitoring. Fifty-six of 79 (70.9%) carriers with baseline HBV-DNA levels ≤2000 IU/mL maintained these viremia levels and normal ALT during the 1-year monthly follow-up and were classified as IC (Figure 1). All of them had liver stiffness values <6 KPa (Table 1) and their virologic and biochemical profiles remained unchanged throughout the overall follow-up (median 38 months, range 24–104). The remaining 23 carriers with baseline HBV-DNA levels ≤2000 IU/mL showed fluctuations of HBV-DNA above 2000 IU/mL and were classified as AC together with 130 subjects who had HBV-DNA >2000 IU/mL at the baseline assessment. During the further follow-up (median, 21 months; range, 12–98), HBV-DNA levels remained persistently below 20,000 IU/mL and ALT normal in a subgroup of 31 AC (14 of them with baseline HBV-DNA <2000 IU/mL). They were classified as AC1 (Figure 1) and showed median liver stiffness values of 4.7 kPa (range,

Table 1. Demographic Features and HBsAg Serum Levels According to the Virologic Profile

	Inactive infection HBV DNA ≤2000 IU/mL (IC)	Active infection 1 HBV DNA >2000 and <20,000 IU/mL (AC1)	Active infection 2 HBV DNA ≥20,000 IU/mL (AC2)
Carriers, n	56	31	122
Age (range), n	49 (20–75)	43 (21–64)	47 (18–77)
Male/female	29/27	10/20	65/58
Follow-up (range), *mo*	38 (24–104)	33 (24–106)	33 (6–110)
Baseline HBsAg (range), *IU/mL*	62.12 (0.1–4068)	883 (0.5–7838)	4233 (164–82,480)
End of follow-up HBsAg (range), *IU/mL*	40.92 (n.d.–4143)	613 (0.41–7754)	3887 (172–65,160)
Baseline HBV DNA (range), *IU/mL*	49 (n.d.–1990)	2758 (n.d.–19524)	389,500 (98–166,000,000)
End of follow-up HBV DNA (range), *IU/mL*	30 (n.d.–1114)	1483 (n.d–14532)	396,450 (15–151,000,000)
Baseline ALT (range), *U/L*	21 (10–35)	22 (11–39)	68 (11–722)
End of follow-up ALT (range), *U/L*	20 (13–38)	23 (12–40)	98 (15–2056)
Liver elastometry by Fibroscan (range), *kPa*	4.3 (3.1–5.6)	4.7 (3.2–5.8)	11.2 (3.2–59.8)
Histology	Not performed	10 patients Grading 3/18: 6 pts 4/18: 4 pts Staging 0/6: 8 pts 1/6: 2 pts	79 Chronic hepatitis pts Grading: 7/18 (4–14/18) Staging: 2/6 (0–4/6) 20 Cirrhotic pts Grading: 10/18 (8–14/18) Staging 5/6 (5–6/6)

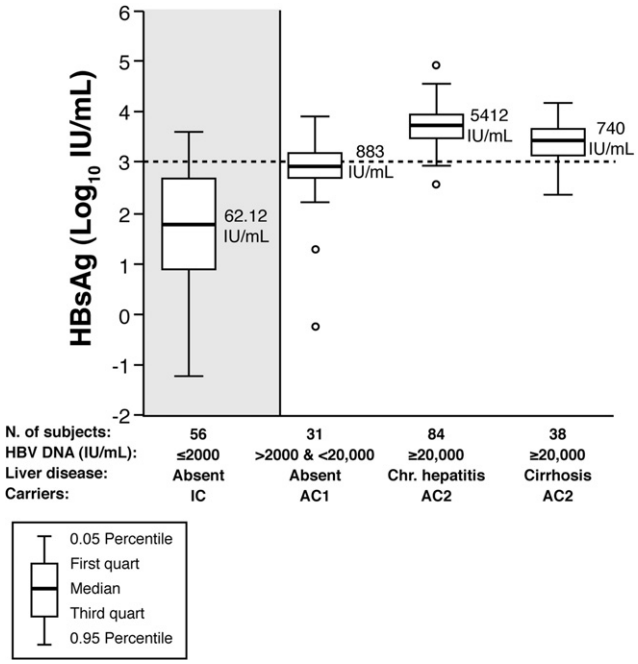

Figure 2. Baseline HBsAg levels according to phase of infection (inactive vs active) and in active carriers (*AC*) according to HBV-DNA levels (persistently <20,000 IU/mL vs ≥20,000 IU/mL) and the presence and the stage of liver disease (chronic hepatitis vs cirrhosis). HBsAg levels were significantly different in the following: IC vs AC: $P < .001$; IC vs AC1: $P < .001$; AC1 vs AC2: $P < .001$.

3.2–5.8) and minimal necroinflammation (grading <4/18) and fibrosis (staging: 0/6 in 8, and 1/6 in 2 patients) at liver biopsy. The overall clinicovirologic features of AC1 carriers were suggestive for the absence of significant liver disease. All the remaining 122 active carriers (AC2) had evidence of liver disease (Table 1): their viremia fluctuated above 20,000 IU/mL and fell below 2000 IU/mL occasionally in 15 (12%) patients. Fifty-two (42.6%) patients showed major ALT fluctuations with flares that were followed by temporary normalizations in 20 of them.

Baseline HBsAg Serum Levels and HBV Infection Profiles

Median baseline HBsAg levels in the IC group were 62.12 IU/mL (range, 0.1–4068), significantly lower than in the AC group (3029 IU/mL; range, 0.5–82,480; $P < .001$). Among the AC group, the 31 AC1 cases had HBsAg levels significantly lower than the 122 AC2 patients (883 IU/mL; range, 0.5–7838 vs 4233 IU/mL; range, 164–82,480 IU/mL, respectively; $P = .002$), (Table 1, Figure 2). Among the 79 carriers with baseline HBV-DNA serum levels ≤2000 IU/mL, the 23 carriers with increases of viremia >2000 IU/mL during the follow-up showed HBsAg levels significantly higher than the 56 true IC (1709 IU/mL; range, 0.52–16,969 vs 62.12 IU/mL; range, 0.1–4068 IU/mL, respectively, $P < .001$). Overall HBsAg and HBV-DNA serum levels correlated only weakly ($\rho = 0.638$, $P < .001$), with less no correlation in the AC group ($\rho = 0.321$, $P < .001$). The $\log_{10}$ HBV-DNA/HBsAg ratio was significantly lower in the IC group than in the AC group (0.25 vs 1.6, respectively, $P < .001$) but comparable for IC and AC1 (0.49). The $\log_{10}$ HBV-DNA/HBsAg ratio was significantly different among AC1 (0.49), AC2 with chronic hepatitis (1.97), and AC2 with cirrhosis (2.34; Figure 3).

Univariate analysis showed a correlation of HBsAg levels with age ($P < .001$), ALT ($P < .001$), HBV-DNA ($P < .001$), IgM anti-HBc ($P < .001$) serum levels, biochemical profile (flares vs no flares, $P < .001$), and phase of infection (active vs inactive, $P < .001$) but not with sex. In multivariate analysis age (B, −0.016; 95% confidence interval [CI]: −0.025 to −0.007, $P = .001$), HBV-DNA levels (B, 0.163; 95% CI: 0.061–0.265, $P = .002$) and phase of infection (B, 0.757; 95% CI: 0.531–0.982, $P < .001$) were independently associated with HBsAg levels that were lower in older carriers, with lower HBV-DNA levels, and inactive infection. A separate analysis, considering only AC2, showed that biochemical profile was the only independent factor associated with HBsAg levels, with lower HBsAg levels being observed in the 52 patients with ALT flares (median, 3097 [range, 234–15,510] vs median, 5657 [range, 749–82,480] IU/mL, respectively, $P = .031$). Correlation of HBsAg levels with stage of liver disease (cirrhosis/chronic hepatitis) showed a trend towards lower HBsAg levels in cirrhotic patients (median, 2740 [range, 234–14,732] vs median, 5412 [range, 373–82,480] IU/mL, respectively, $P = .056$).

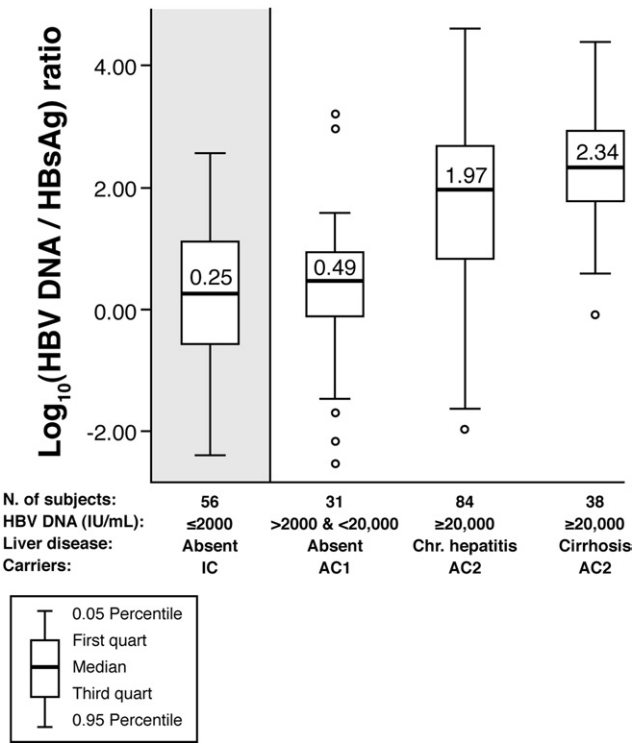

Figure 3. $\log_{10}$ HBV-DNA/HBsAg ratio by phase of infection and disease stage: IC vs AC: $P < .001$; IC vs AC1: P = NS; AC1 vs AC2: $P < .001$; chronic hepatitis vs cirrhotic patients: $P = .023$.

HBsAg Serum Levels During Follow-up

Baseline and end of follow-up HBsAg levels were compared in all carriers. HBsAg showed an overall median decline of −0.060 $\log_{10}$ IU/mL (range, −3.435 to +1.78). HBsAg decline was significantly higher in the IC (−0.212 $\log_{10}$ IU/mL; range, −3.435 to +1.781) than in the AC group (−0.016 $\log_{10}$ IU/mL; range, −0.917 to +0.878; $P <$ .001). Ten individuals of the IC group cleared serum HBsAg during follow-up (annual rate of HBsAg clearance, 0.055 per person-year); their median HBsAg decline was −0.471 $\log_{10}$ IU/mL (range, −0.176 to −3.453) compared with the median decline of −0.130 $\log_{10}$ IU/mL (range, −1.781 to +1.863) for IC who did not clear HBsAg. None of the AC group cleared HBsAg during the follow-up. The overall changes in HBsAg levels were independently associated with HBsAg clearance ($P <$ .0001) and follow-up duration ($P <$ .001). Baseline HBsAg levels ($P =$ .001) and yearly HBsAg log decline ($P =$ .016) were independently associated with HBsAg clearance. The yearly median HBsAg decline in the IC group was significantly higher than in the AC group (−0.0768 $\log_{10}$ IU/mL; range, +2.0509 to −1.3124 vs −0.0120 $\log_{10}$ IU/mL; range, +1.0426 to −0.8108; respectively, $P <$ .001); this held true for both the IC with (−0.2868 $\log_{10}$ IU/mL) and without (−0.0588 $\log_{10}$ IU/mL, $P =$ .021 vs AC) HBsAg clearance.

In 47 carriers, HBsAg levels fluctuations were monitored yearly during 3-year follow-up and showed different profiles for the IC, AC2, and AC1 groups (analysis of variance for repeated measures, $P <$ .001). Median HBsAg levels were stable in the AC2 group (3.52, 3.61, 3.52, and 3.49 $\log_{10}$ IU/mL at baseline and 1, 2, and 3 years, respectively) but showed moderate declines in AC1 (2.95, 3.01, 2.84, and 2.71 $\log_{10}$ IU/mL) and IC (2.2, 2.07, 1.66, and 1.48 $\log_{10}$ IU/mL), respectively.

Kinetics of HBsAg levels were analyzed in 20 AC2 patients (during ALT flares in 10) using a median of 9 time points (range, 5–16). Significant fluctuations of HBsAg levels were observed in patients with ALT flares; HBsAg levels achieved the nadir 1 month after the ALT peak (median δ variation of −0.31 $\log_{10}$ IU/mL; range, +0.06 to −1.55) but increased to preflare levels within 3–6 months thereafter (median change of −0.08 $\log_{10}$ IU/mL; range, +0.32 to −0.42; Figure 4*A* and *B*). On the contrary, HBsAg levels showed minor fluctuations in patients with persistent ALT elevations without flares: median, −0.01 $\log_{10}$ IU/mL (range, −0.17 to +0.04) and median, −0.02 $\log_{10}$ IU/mL (range, −0.08 to +0.14) at 3 and 6 months, respectively.

Identification of Inactive Carriers and CHB Patients Using HBsAg Serum Levels

In Table 2 we report the diagnostic performance for the identification of AC and IC by single point measurements of HBV-DNA and HBsAg or their combination. The area under the receiver operating characteristic (AUROC) of HBsAg to identify IC in the overall cohort was 0.94 (95% CI: 0.905–0.975, $P <$ .001, Figure 5*A*): the HBsAg cut off with the highest sensitivity and specificity was 844.5 IU/mL (sensitivity, 88.2%; specificity, 89.3%). A specificity of 90.2% was given by the 650 IU/mL HBsAg cut off, with 82.1% sensitivity, 75.4% positive predictive value (PPV), 93.2% negative predictive value (NPV), and 88% diagnostic accuracy (DA) (Table 2). In the attempt to identify a cut off able to distinguish patients with active infection and liver disease (AC2) from IC, we ran an additional AUROC excluding the AC1 group, who did not show evidence of liver disease, in spite of viremia occasionally >2000 IU/mL. The AUROC was 0.968 (95% CI: 0.942–0.993, $P <$ .001, Figure 5*B*): the HBsAg cut off with the highest sensitivity and specificity was 1000 IU/mL. The diagnostic performance of the 1000 IU/mL cut off to identify AC showed sensitivity, 94.3%; specificity, 91.1%; PPV, 95.8%; NPV, 87.9%; and DA, 93.3% (Table 2).

Combining HBsAg <1000 IU/mL and HBV-DNA ≤2000 IU/mL, the identification of IC by a single point evaluation achieved DA of 94.3%, 91.1% sensitivity, 95.4%

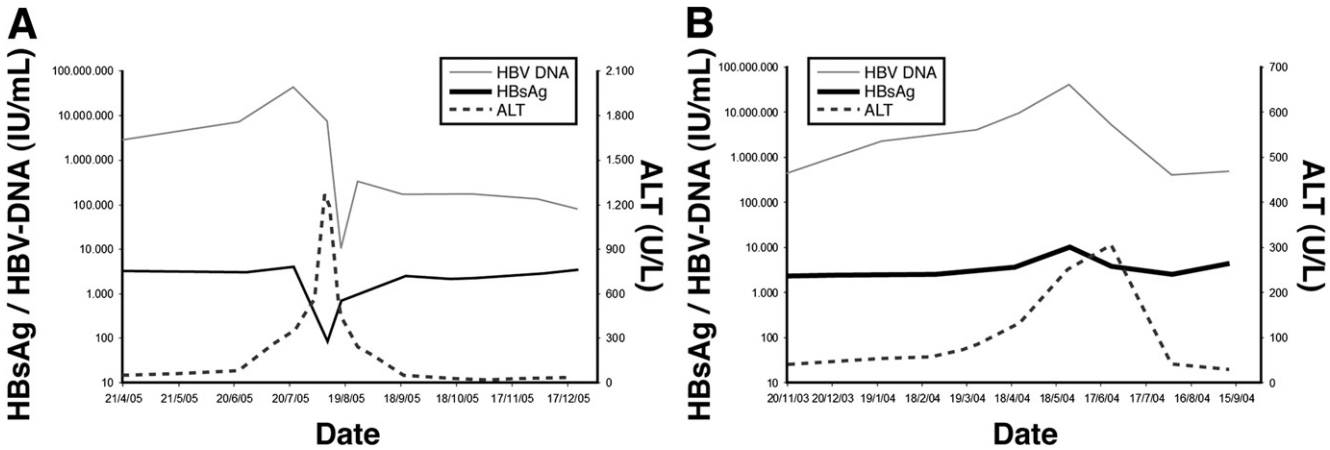

Figure 4. Individual patient profiles during ALT flare: (*A*) HBsAg fluctuations during ALT flare (case A) and (*B*) HBsAg fluctuations during ALT flare (case B).

Table 2. Prediction of HBV Infection Phase by HBV-DNA and HBsAg Serum Levels

Prediction of:	Active infection			Inactive infection		
By level IU/mL (cut off)	HBV-DNA >2000	HBsAg ≥1000	HBsAg ≥1000	HBV-DNA ≤2000	HBsAg ≤650	HBsAg ≤650
Population (No. carriers)	All 209	All 209	178[a]	All 209	All 209	178[a]
Sensitivity (%)	85.0	83.0	94.3	100.0	82.1	82.1
Specificity (%)	100.0	91.1	91.1	85.0	90.2	97.5
Positive predictive value (%)	100.0	96.2	95.8	70.9	75.4	93.9
Negative predictive value (%)	70.9	66.2	87.9	100.0	93.2	92.2
Diagnostic accuracy (%)	89.0	85.2	93.3	89.0	88.0	92.7
Likelihood ratio for positive test	∞	9.30	10.56	6.65	8.38	33.40
Likelihood ratio for negative test	0.15	0.19	0.06	0.00	0.20	0.18

Prediction of:	Inactive infection	
By level IU/mL (cut off)	HBsAg ≤650 and HBV-DNA ≤2000	HBsAg <1000 and HBV-DNA ≤2000
Population (No. carriers)	All 209	All 209
Sensitivity (%)	82.1	91.1
Specificity (%)	98.0	95.4
Positive predictive value (%)	93.9	87.9
Negative predictive value (%)	93.8	96.7
Diagnostic accuracy (%)	93.8	94.3
Likelihood ratio for positive test	41.89	19.91
Likelihood ratio for negative test	0.18	0.09

[a]Inactive carriers (IC) and active carriers with viremia ≥20000 IU/mL (AC2).

specificity, 87.9% PPV, and 96.7% NPV (Table 2). Actually, the 7 AC carriers who were erroneously classified as IC because of baseline HBsAg <1000 IU/mL and HBV-DNA <2000 IU/mL were AC1 (viremia persistently <20,000 IU/mL). The diagnostic performances of both HBsAg cut offs (≤650 UI/mL and ≥1000 IU/mL) were comparable when we applied them to the end of follow-up serum samples. Briefly, 2 IC cases with baseline HBsAg levels >650 IU/mL showed a decline below the threshold determining a slight increase in the assay sensitivity (85.7% vs 82.1%, respectively), whereas 2 AC2 cases showed a decline below 650 IU/mL with a small decrease in specificity for the 650 IU/mL cut off (95.9% vs 97.5%, respectively) and in sensitivity of the 1000 IU/mL cut off (81.7% vs 83%, respectively).

Discussion

Studying prospectively a large cohort of well-characterized, untreated HBeAg-negative, anti-HBe-positive, chronic asymptomatic HBV genotype D carriers, we found that HBsAg serum levels were significantly lower in inactive than active infection (median values, 62.12 IU/mL; range, 0.1–4068 IU/mL vs 3029 IU/mL; range, 0.5–82,480 IU/mL, respectively, $P < .001$, Figure 2). The stringent criteria used to classify the HBV carriers (1 year of monthly monitoring of both HBV-DNA and ALT) allowed us to characterize precisely their virologic profile avoiding the risk of misclassification caused by the fluctuations of viral load, which are common in chronic HBeAg-negative HBV infection (Figure 1).[10,15,16] Accord-

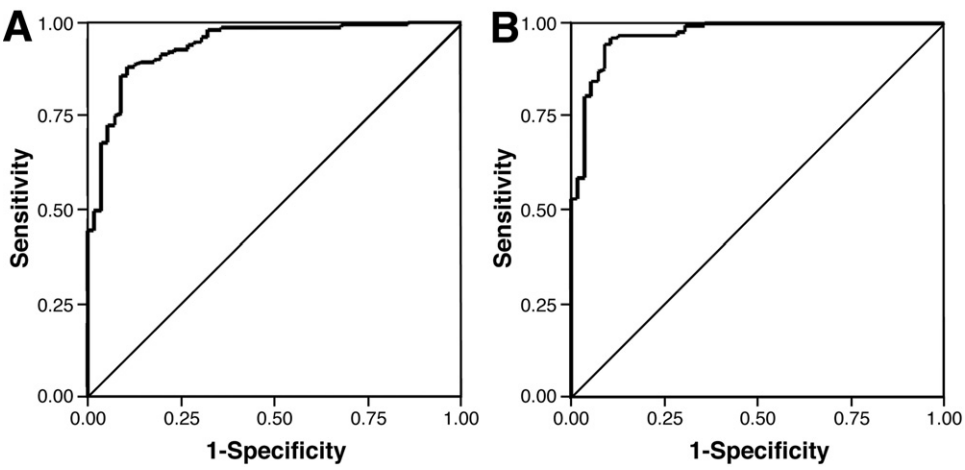

Figure 5. (*A*) Receiver operating characteristic curve for HBsAg levels in AC vs IC: AUROC = 0.94, 95% CI: 0.905–0.975, $P < .001$. (*B*) ROC curve for HBsAg levels in AC2 vs IC: AUROC = 0.968, 95% CI: 0.942–0.993, $P < .001$.

ingly, 23 (23/79, 29.1%) carriers with baseline viremia ≤2000 IU/mL showed evidence of active infection at 1-year follow-up, but their baseline HBsAg serum levels were significantly higher than in true IC (1709 vs 62.12 IU/mL, respectively, $P < .001$). Thus, HBsAg quantification appears to provide additional information for the characterization of the phase of HBV infection as compared with monitoring HBV-DNA and ALT only. Indeed, the single point quantitative HBsAg assessment was accurate and reliable for distinguishing IC from AC with specificity (90.2%) and PPV (75.4%) higher than single point HBV-DNA measure (specificity, 85%; and PPV, 70.9%). However, the most accurate identification of IC was achieved by combining HBsAg (<1000 IU/mL) and HBV-DNA (≤2000 IU/mL) quantification, which showed 94.3% DA, 91.1% sensitivity, 95.4% specificity, 87.9% PPV, and 96.7% NPV. Therefore, the combined single point testing of HBsAg and HBV-DNA provides a very accurate virologic characterization of the HBV carrier because it can be obtained only after a long-term stringent monthly monitoring of ALT and HBV-DNA. Our findings hold true in genotype D-infected carriers but need to be confirmed in larger and independent cohorts of carriers infected with other HBV genotypes, because recent reports suggest that secretion of HBsAg[24] and its serum levels and declines during antiviral therapy[21] are HBV genotype dependent.

The added diagnostic value of quantitative HBsAg stems from the fact that its serum levels do not reflect merely the production of virions but rather the presence of defective HBsAg particles (spheres and filaments) that exceed competent virions by a factor of 10^2 to 10^5.[25–27] Accordingly and in agreement with previous reports,[28] we found only a weak correlation between HBsAg and HBV-DNA serum levels, particularly in AC ($\rho = 0.321$). However, HBV-DNA/HBsAg ratios differed significantly between active and inactive carriers (1.6 vs 0.25, respectively, $P < .001$) and were similar in both groups of carriers without liver disease, namely in IC (0.25) and AC1 (0.49) (P = NS). Interestingly, the AC1 subgroup of active carriers (31/153 patients, 20%), in whom viremia remained persistently below 20,000 IU/mL throughout the follow-up and any biochemical, elastographic, or histologic evidence of significant liver disease was present, had HBsAg serum levels significantly lower than the remaining 122 active carriers (AC2) with higher levels (>20,000 IU/mL) of viral replication and overt liver disease (883 IU/mL, range, 0.5–7838 vs 4233 IU/mL; range, 164–82,480, $P = .002$). The lower HBV-DNA/HBsAg ratios observed in both IC and AC1 suggest that HBV infection is less productive in carriers without significant liver disease than in CHB patients. Consistently, the HBV-DNA/HBsAg ratios were higher and indicative of even more productive infection in cirrhotic patients as compared with CHB patients without cirrhosis (2.34 vs 1.97, respectively, $P = .023$). Our data agree with original studies characterizing serum HBsAg particles, which suggested that the proportion of defective HBsAg particles increases during the low replicative as compared with the highly replicative phase of chronic HBV infection.[27] If these data will be confirmed in future studies, HBV-DNA/HBsAg ratio in addition to quantitative HBsAg might have relevant clinical implications, contributing to a more accurate characterization of HBV carriers and the identification of those who need treatment.

Serum HBsAg levels are thought to depend mainly on translation of specific messenger RNAs for "s" gene generated from the cccDNA and also from integrated HBV-DNA within the host genome. Our data provide consistent evidence that HBsAg declines during transition from the active to the inactive phase of HBV infection and suggest that HBsAg levels may reflect the "transcriptionally" active cccDNA rather than its absolute amount or HBV-DNA integrated sequences, which are thought to increase over time during chronic infection.[29] Accordingly, preliminary reports showed correlation between intrahepatic cccDNA and HBsAg serum levels[19,20] and between the decline of HBsAg levels and sustained virologic response to pegylated interferon, but not to lamivudine, despite greater suppression of HBV-DNA levels with the latter.[21] All together, these findings suggest that low HBsAg levels could represent the hallmark of the control of HBV infection prompted by the host's immune system that is required both to achieve and sustain the inactive HBV infection profile. Accordingly, in our cohort, HBsAg declined significantly only in inactive carriers, who showed 0.055 per person-year rate of HBsAg clearance. In contrast, in CHB patients with active disease, HBsAg serum levels were stable overtime, with only minimal variations (-0.011 $\log_{10}$ IU/mL). The median decline of HBsAg was less than 0.5 $\log_{10}$ IU/mL, even during hepatitis B exacerbations that characterize the disease profile of a large proportion of HBeAg-negative patients. HBsAg declined by 1.5 $\log_{10}$ IU/mL at the time of the ALT peak in only 1 patient (Figure 4*A*), but, 1 week later, the values had increased again by 1 log while the HBV-DNA value reached its nadir (-3 $\log_{10}$ IU/mL decline in HBV-DNA from baseline). Fluctuations in HBsAg were minimal, even during longer follow-up, in all the remaining CHB patients.

In conclusion, our study shows that HBsAg serum levels vary during the different phases of chronic HBeAg-negative HBV genotype D infection and are below 1000 IU/mL in over 90% of IC but only in 5.7% of patients with CHB. The HBV-DNA/HBsAg ratios declined following the sustained immune control of HBV infection.

Our findings confirm that serum HBV-DNA and HBsAg levels provide complementary information on the status of HBV infection. Accordingly, we showed for the first time that the single point combined quantification of HBV-DNA (<2000 IU/mL) and HBsAg (<1000 IU/mL) allows the identification of IC with a very high diagnostic

accuracy (94.3%) that is comparable with that of 1-year monthly monitoring. This approach represents a new means for an effective management of chronic HBV infection and disease. For a generalized use of quantitative HBsAg in clinical practice, it is mandatory to proceed further with clinical validation testing reference panels of sera containing medium and high levels of HBsAg from patients infected with different HBV genotypes and in different phases of infection (HBeAg positive and HBeAg negative).

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