Original article

A decline in hepatitis B virus surface antigen (HBsAg) predicts clearance, but does not correlate with quantitative HBeAg or HBV DNA levels

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Background: The elimination of hepatitis B virus surface antigen (HBsAg) is the final goal of hepatitis B treatment, but is rarely achieved. As quantitative assays for HBsAg recently became available, we have investigated whether quantitative HBsAg measurements can substitute for hepatitis B virus (HBV) DNA quantification in treatment monitoring.

Methods: Within this study, 23 liver transplant patients and 18 heart transplant recipients were retrospectively analysed. Patients had been treated with famciclovir and/or lamivudine, in addition some had also received adefovir in cases of lamivudine resistance. Quantitative HBsAg and hepatitis B virus e antigen (HBeAg) levels were determined with the Architect assay. HBV DNA levels were determined with different assays available at given time points.

Results: We did not find a significant correlation between either HBsAg or HBeAg and HBV DNA levels – both in treated and untreated patients. More importantly, there was no significant concordance between an increase or decrease of HBsAg or HBeAg with HBV DNA. However, the curve and decline of quantitative HBsAg enabled prediction of eventual viral clearance. Eight patients showed a 2 log10 drop of HBsAg levels and eight patients demonstrated a reduction of HBsAg levels below 100 IU/ml; five patients fulfilled both criteria. Three of those five cleared HBsAg and became positive for antibodies against HBsAg.

Conclusions: Quantitative HBsAg and HBeAg cannot substitute for HBV DNA quantification during the assessment of antiviral therapy; however, the decline of HBsAg does predict eventual HBsAg clearance. A 2 log10 drop to below 100 IU/ml is associated with a high likelihood of HBsAg clearance.

Introduction

In many viral diseases viral load correlates with viral proteins in the serum; for example, a strong correlation has been observed between cytomegalovirus (CMV) pp65-positive cells and serum CMV DNA viral loads [1]. Likewise, a relationship was reported between levels of p24 and HIV viral load [2]. We previously described a good correlation between levels of hepatitis C virus (HCV) core antigen determined by the Trak-C assay and HCV RNA viral load [3]; the study showed a higher correlation between these assay results and viral load than that observed between two different earlier generation viral load assays [3]. Other studies have also reported a good correlation between levels of HCV RNA and the HCV core antigen, and have highlighted the potential use of the assay in predicting treatment response [4–8].

In hepatitis B virus (HBV) infection the role of quantitative HBV surface antigen (HBsAg) is unknown, although HBV e antigen (HBeAg) detectability is known to correlate with high viral load [9]. In addition, electron microscopy studies revealed an overexpression of HBsAg in relation to complete viruses. Although the complete virus is relatively monomorphic, HBsAg forms pleomorphic...
particles that are predominantly spherical and partly filamentous with a diameter of approximately 20 nm. These particles are estimated to be approximately 1,000 times more prevalent than complete HBV virus [10]. This excess of surface protein and HBeAg, which is not required for the viral life cycle, might have implications for immune evasion [11].

Attempts to quantify HBsAg date back to the mid-seventies [12]. However, methods of quantification have been highly labour-intensive [13], prohibiting their widespread use despite reported usefulness in predicting treatment responses to interferon [14]. Accordingly, few studies have looked into the role of HBsAg and HBeAg quantification in relation to treatment response and its prediction [14,15].

In light of the fact that quantitative assays for HBsAg have recently become available, the aim of this study was to investigate the correlation and concordant increase and decrease of HBsAg and HBeAg in relation to HBV DNA. The study used stored samples from heart and liver transplant patients who underwent antiviral therapy with famciclovir, lamivudine and/or adefovir. We demonstrate here that HBsAg kinetics can predict eventual HBsAg clearance, but also report that HBsAg and HBeAg quantification cannot substitute for HBV DNA testing.

Methods

Within this study, 23 liver transplant patients (22 males, 1 female) and 18 heart transplant recipients (17 males, 1 female) were retrospectively analysed. The liver transplant patients received their grafts between 1991 and 2001, but suffered from HBV reinfection despite prophylaxis with hepatitis B immunoglobulin (HBIG; 10,000 IU administered intraoperatively and 10,000 IU/day until HBsAg became negative) [16]. Thereafter, the aim was to achieve long-term titres >100 IU/ml, but these were not always reached (also documented in an earlier publication reporting the use of combination therapy [17]).

All patients received their transplants before initiation of combination prophylaxis comprising HBIG plus lamivudine. Subsequently, most patients received sequential therapy with famciclovir and lamivudine [18], but those transplanted after 1997 were immediately started on lamivudine after reinfection if not pre-treated with lamivudine before orthotopic liver transplantation (OLT). In the latter case, or in other cases of lamivudine resistance, adefovir was also included.

The heart transplant recipients had developed chronic HBV infection 3 to 40 months after heart transplantation and received famciclovir (as described earlier [19]) or were switched to other nucleos(t)ide regimens enabling improved survival [20]. The antiviral therapies were performed within Institutional Review Board (IRB) approved treatment protocols.

HBV DNA quantification

HBV DNA quantification was performed within the routine clinical care using assays available at the given time. Specifically, two assays were employed. The first was the Genostics Assay (Abbott Laboratories, North Chicago, Illinois, USA), a radioactive hybridization assay with a level of detection of 3 pg/ml (equivalent to ~10^6 copies/ml or 2x10^4 IU/ml). Second, the HBV-Test Hybrid Capture II (Digene/Abbott Laboratories, North Chicago, Illinois, USA) was used. This assay is based on the formation of hybrids between HBV DNA and HBV RNA, which can then be quantified using antibodies in conjunction with chemiluminescence technology. The lower level of detection of this test was 0.5 pg, equivalent to 140,000 copies/ml (28,000 IU/ml).

In some of the most recent samples, a third HBV DNA quantification method was also used: the COBAS Amplicor HBV-Monitor (Roche, Basle, Switzerland) PCR-based quantification assay. This assay has a much lower level of detection at 400 copies/ml (80 IU/ml). All samples of adefovir-treated patients used this PCR-based technology.

HBsAg and HBeAg determination

Qualitative HBsAg and HBeAg measurements were determined with a commercial assay (Abbott Laboratories, Wiesbaden-Delkenheim, Germany). The quantification of HBsAg was performed using the Architect™ HBsAg assay (Abbott Laboratories), a two-step immunoassay based on the use of chemiluminescence microparticles (CIMA). Briefly, samples are mixed with paramagnetic beads coated in polyclonal antibodies against HBsAg (anti-HBs). HBsAg in the sample attaches to the magnetic beads through the presenting antibodies. After a washing step, a conjugate and reactant are added leading to the emission of light, which is proportional to the determined HBsAg concentration. A level >0.5 IU/ml is considered positive. Because this is a polyclonal approach, viral mutants seem to be of little relevance [21].

Likewise, HBeAg was also determined using the Architect™ assay; the main difference here was the use of an anti-HBe antibody instead of the anti-HBs antibody. The concentration was determined using pre-established calibration curves. A level ≤0.28 IU/ml is considered reactive and therefore positive.

The study was carried out retrospectively with samples left over after use for diagnostic work-up during the patients' antiviral therapy. Thus, there are no standardized time points when HBsAg and HBeAg could be quantified.

Statistical analysis

Statistical analysis was performed using the SPSS software 14.0. Correlations between HBsAg, HBeAg
and HBV DNA levels were calculated according to Pearson. Differences in efficacy were not the primary interest of this work, but the non-parametric Kruskal–Wallis test was used here. Difference in concordant results was tested by the $\chi^2$ test.

**Results**

Correlation of HBsAg, HBeAg and HBV DNA levels

HBsAg levels were correlated to 187 HBeAg-positive sera. Surprisingly, there was no correlation between HBsAg and HBeAg levels ($r=-0.014; P=0.85$). Likewise, neither HBeAg nor HBsAg correlated with HBV DNA levels ($n=218$), as determined by hybridization assay ($r=0.022$ and $r=-0.069; P$-values 0.7 and 0.4, respectively).

To exclude that this lack of correlation is a treatment effect, we next studied only those patients who had a sample available before therapy. HBsAg, HBeAg and HBV DNA were correlated in 25 patients prior to initiation of therapy. In these treatment-naive patients there was a weak correlation of HBV DNA with HBsAg ($r=0.547; P=0.005$; Figure 1A). Surprisingly, a correlation of HBV DNA with HBeAg levels was not observed ($r=-0.01; P=0.9$; Figure 1B). Likewise, there was no correlation of HBeAg with the HBsAg ($r=-0.151; P=0.429$; Figure 1C).

Concordance of decrease and increase of HBsAg or HBeAg with HBV DNA during antiviral therapy

We evaluated whether there is a concordance concerning the decrease and increase of HBsAg and HBeAg (Table 1) in relation to HBV DNA changes. This analysis revealed no significant difference for HBV DNA and HBsAg (Table 1) or for HBV DNA and HBeAg. Even if an increase is only considered in cases of at least a 1 log$_{10}$ increase of HBV DNA there is no good concordance, as in some individuals HBsAg decreases despite increasing HBV DNA levels (data not shown).

Level of decrease of HBsAg, HBeAg and HBV DNA during antiviral therapy

The number of patients achieving at least a 0.5 log$_{10}$ or a 1 log$_{10}$ HBsAg reduction was also analysed (Table 2). This analysis indicates increased reductions of HBsAg with increasing known antiviral activity ($P=0.038$). However, this study was not extended to analyse the different strengths of different treatments, as too few patients at scattered time points could be included prohibiting a real analysis.

**HBsAg quantification can predict HBsAg clearance**

In patients responding well to modern antiviral therapy, HBV DNA will become undetectable at some
point; however, it would be useful to be able to predict whether a patient might eventually become HBsAg-negative or whether an alternative treatment approach is required.

Figure 2 indicates the potential strength of HBsAg quantification in this regard: it enables further monitoring in patients who have already become HBV DNA negative. This effect is even more pronounced for patients on adefovir. Within the observation period, three of seven patients (one was excluded because of only two available time points) became HBV DNA negative (Figure 3A). However, discriminating their HBsAg curves indicates that only one of these patients will probably clear HBsAg in the long term (Figure 3B). Indeed, some years after completing this study, one of the three patients who eventually became HBsAg-negative on adefovir became HBsAg-negative and developed an anti-HBs titre of 20 IU/ml (Figure 3B, patient 3; Figure 4, patient 3).

Analysing patients with a drop of HBsAg to <100 IU/ml suggests that there are three patients likely to eventually become HBsAg-negative on the basis of a continuous steep decline of their HBsAg levels. Because of the delay between performing this study and preparation for publication, we were able to prove this hypothesis. None of the five patients with an eventually flat curve of their HBsAg levels was able to clear HBsAg (nor any of the patients showing no drop of HBsAg <100 IU/ml within the study), whereas all three patients with a continuous and steep decline of HBsAg levels eventually became HBsAg-negative (P=0.018).

Performing a multiple regression analysis including baseline HBsAg, baseline HBV DNA, HBeAg levels in the case of HBeAg-positive patients, form of therapy and kind of organ received (liver versus heart) revealed that a reduction of HBsAg to <100 IU/ml was the most relevant predictor of potential HBsAg clearance (P<0.001). A 2 log10 drop of HBsAg was found in eight patients, five of whom also showed a reduction of their HBsAg levels to <100 IU/ml. All three patients who eventually became HBsAg-negative fulfilled both criteria: a 2 log10 drop and HBsAg <100 IU/ml.

Discussion

In this study, we expected to demonstrate a high correlation between HBV DNA and HBsAg and HBeAg. However, only a moderate correlation for HBsAg and HBV DNA and a poor correlation for HBeAg and HBV DNA could be shown. The poor correlation of HBV DNA with HBeAg could result from methodological problems, as the assay might not have been optimized for a quantitative approach. The data illustrating the low correlation for HBsAg and HBV DNA are, however, consistent with a recent

Table 1. Concordance of HBsAg or HBeAg and HBV DNA decrease and increase

<table>
<thead>
<tr>
<th></th>
<th>HBsAg decrease, n</th>
<th>HBsAg increase, n</th>
<th>HBsAg and HBV DNA concordance</th>
<th>HBsAg decrease, n</th>
<th>HBsAg increase, n</th>
<th>HBsAg and HBV DNA concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Famiclovir</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV DNA decrease, n</td>
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<td>8</td>
<td>23</td>
<td>5</td>
<td></td>
<td></td>
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<tr>
<td>HBV DNA increase, n</td>
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<td>9</td>
<td>2</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concordance of total, n/n (%)</td>
<td>34/47 (72.3)</td>
<td></td>
<td>32/39 (82.1)</td>
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<tr>
<td>Lamivudine</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HBV DNA decrease, n</td>
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<td>11</td>
<td>19</td>
<td>9</td>
<td></td>
<td></td>
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<tr>
<td>HBV DNA increase, n</td>
<td>4</td>
<td>28</td>
<td>11</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concordance of total, n/n (%)</td>
<td>51/66 (77.3)</td>
<td></td>
<td>33/53 (62.3)</td>
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<td>Adefovir</td>
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<tr>
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<td>8</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV DNA increase, n</td>
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<td>2</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concordance of total, n/n (%)</td>
<td>29/32 (90.6)</td>
<td></td>
<td>8/11 (72.7)</td>
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</tbody>
</table>

Table 2. Number of patients with ≤0.5 log10 or 1 log10 HBsAg reduction

<table>
<thead>
<tr>
<th>HBsAg reduction</th>
<th>Famiclovir (n=27)</th>
<th>Lamivudine (n=23)</th>
<th>Adefovir (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤0.5 log10</td>
<td>19</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>0.5 log10</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>&gt;1 log10</td>
<td>4</td>
<td>9</td>
<td>5</td>
</tr>
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χ² overall: P=0.038.
This paper did report a correlation but the r-value was only 0.21 [15], which is much lower than the r-value of ≥0.5 usually accepted as correlation. A slightly higher r-value of 0.33 was observed in 200 HBsAg-positive blood donors, where high HBsAg levels were also determined in some donors in the absence of detectable HBV DNA [22]. Much higher correlations with r-values up to 0.8 were reported from Asia [23–25]. When we tested 168 immuno-competent patients with the Architect™ assay, their HBsAg levels correlated well with HBV DNA (r-values of 0.709; P<0.001).

More important than the correlation of HBsAg or HBeAg with HBV DNA levels, is the concordance of decrease and increase of these parameters. In our analysis, no concordance was observed between changes in HBsAg and HBV DNA levels or between changes in HBsAg and HBV DNA levels. Similarly, discordant fluctuation of HBsAg levels versus HBV DNA levels were described in patients continuously responding to lamivudine, patients developing resistance to lamivudine [15] and in treatment-naïve patients [26], respectively. As in some of our patients, others have also observed a delayed increase of HBsAg in cases of resistance [15]. Thus, HBsAg cannot substitute for HBV DNA quantification.

If a parameter cannot substitute for another, it might disclose additional information. Clinical improvement has been associated with HBV DNA control [20,27], yet clearance of HBsAg remains the eventual goal of HBV therapy. It has previously been shown that HBsAg kinetics can predict HBsAg clearance during interferon therapy [15]. A recent abstract further suggested an HBsAg reduction of >2 log_{10} or a reduction to levels <10 IU/ml to be associated with HBsAg elimination in HBsAg decline and clearance on antivirals

Figure 2. Decline in HBV DNA, HBsAg and HBeAg levels in response to famciclovir treatment in two patients over time

Decline in HBV DNA (pg/ml), HBsAg (IU/ml) and HBeAg (IU/ml) in two patients with long-lasting response to famciclovir. HBeAg, hepatitis B virus e antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus.
Figure 3. Decline in HBV DNA, HBsAg and HBeAg levels in response to adefovir treatment

Response to adefovir in relation to (A) HBsAg, (B) HBV DNA and (C) HBeAg in individual patients. Although three patients became HBV DNA negative, only one showed a pronounced constant decline of HBsAg (patient 3). The other two HBV DNA negative patients showed a plateau of HBsAg levels. Patients A, C, 3 and II are also shown in Figure 4. HBsAg, hepatitis B virus surface antigen; HBeAg, hepatitis B virus e antigen; HBV, hepatitis B virus.
HBsAg decline and clearance on antivirals

Figure 4. HBsAg decline in the eight patients with a reduction of HBsAg to levels <100 IU/ml during therapy with adefovir

Patients
1, LAM
2, FCV
3, ADV
I, LAM-R
II, ADV-R
A, ADV
B, FCV
C, ADV

Patients 1, 2 and 3 eventually became HBsAg negative; patients I and II showed a good response, but eventually became resistant to their antiviral therapy; patients A, B and C initially showed some decline in HBsAg levels, but levelled off thereafter. ADV, adefovir; FCV, famciclovir; LAM, lamivudine; R, resistance.

Antiviral therapies can lead to changes of the HBsAg sequence [18]. This can result in diminished immunogenicity [29] and impaired recognition in diagnostic assays. However, these influences seem to be negligible, as a good performance of the Architect™ assay conducted with different HBsAg mutants has been reported [21,30].

Antiviral agents such as lamivudine and adefovir mainly inhibit the generation of new viruses by inhibiting plus strand synthesis and thereby reducing viral load. However, they do not directly modify the amount of covalently closed circular (ccc) DNA, which is stored as minichromosomes in the nucleus of infected cells [31]. The cccDNA is the major replication intermediate of HBV and serves as a matrix for the HBV mRNA to be translated into HBV viral proteins and for the HBV pregenomic transcript; in light of this, one would not expect a direct effect of these antiviral agents on cccDNA. Moreover, one would not expect to see a reduction of HBV viral proteins in the serum, unless the amount of cccDNA is reduced. Thus, a reduction of HBsAg, as seen in some but not all patients (as shown in Figures 3 and 4), most likely reflects cccDNA reduction. In line with this assumption, HBsAg reduction was reported to correlate with cccDNA reduction [32], which is an increasingly targeted aim of HBV therapy [33,34].

As HBV DNA mostly becomes undetectable during treatment with potent antivirals [35–38], quantification of HBsAg will help to further monitor the therapeutic efficacy of nucleos(t)ide analogues once HBV DNA has become negative, especially if the aim is eventual HBsAg elimination. Our study shows for the first time that – even in transplant recipients – the decline of HBsAg during therapy with different antivirals predicts HBsAg clearance.

Acknowledgements

HBsAg and HBeAg test kits were provided by Abbott Diagnostics GmbH, Wiesbaden, Germany.

This work is dedicated to Peter Magerstedt on the occasion of his 65th birthday.

Disclosure statement

HW, MPM and HLT have received honoraria and grant support from Abbott Laboratories.

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