New Enzyme Immunoassay for Detection of Hepatitis B Virus Core Antigen (HBcAg) and Relation between Levels of HBcAg and HBV DNA

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A new enzyme immunoassay specific for hepatitis B virus (HBV) core antigen (HBcAg) was developed. In order to detect HBcAg, specimens were pretreated with detergents to release HBcAg from the HBV virion and disassemble it to dimers, and simultaneously, the treatment inactivated anti-HBc antibodies. HBcAg detected by the assay peaked with HBV DNA in density gradient fractions of HBV-positive sera. The assay showed a wide detection range from 2 to 100,000 pg/ml. We observed no interference from anti-HBc antibody or blood components, but the assay was inhibited by very high concentrations (>1 µg/ml; corresponding to 80 signal/cutoff) of HBcAg. When the cutoff value was tentatively set at 4 pg/ml, all healthy control (HBsAg and HBV DNA negative, n = 160) and anti-hepatitis C virus-positive (n = 55) sera were identified as negative. HBcAg concentrations correlated very closely with HBV DNA (r = 0.946, n = 145) in 216 samples from 72 hepatitis B patients. In seroconversion panels, HBcAg concentrations changed in parallel with HBV DNA levels. The assay, therefore, offers a simple method for monitoring hepatitis B patients. With a series of sera during lamivudine therapy, HBV DNA levels fell sharply and the HBcAg concentration also decreased, but the change in HBcAg was smaller and more gradual. The supposed mechanism of these changes and their clinical significance are discussed.

Diagnosis of chronic hepatitis B virus (HBV) infection has long been based on HBV serology and measurement of liver enzymes. With the development of therapies for chronic HBV infection including interferon and lamivudine (9, 16), quantitative detection of HBV DNA has been used increasingly as the most important marker for monitoring HBV replication activity and disease progression as well as for assessing responses to antiviral treatment of patients with chronic hepatitis B (7, 8). Several assays for the quantitative measurement of HBV DNA have been developed, such as the branched-chain DNA signal amplification assay (5, 7, 28) and transcription-mediated amplification (TMA)-based (10) or PCR-based (6, 11, 13, 17) nucleic acid amplification assays. However, these methods tend to generate highly divergent results (20, 21, 22, 31) and require cumbersome procedures and expensive equipment, in turn requiring considerable skill and high costs.

On the other hand, immunoassays are generally easy and inexpensive. The nucleocapsid of HBV is composed of either 90 or 120 dimers of HBV core antigen (HBcAg) (3), released into circulation after envelopment. Hence, the quantity of HBcAg in serum would demonstrate virus load as well as HBV DNA. Serum HBcAg assays with specimen pretreatment have been reported previously (4, 29), and the concentration of HBcAg in these assays correlated with levels of HBV-associated DNA polymerase (4). Thus, HBcAg could be a marker for virus load.

However, the use of these assays was limited because of relatively low sensitivity and complexity in the procedures.

We have developed an enzyme immunoassay (EIA) for hepatitis B virus core-related antigens (HBcCrAg), which reflects HBV load corresponding to HBV DNA (14, 23). The HBcCrAg is comprised of HBcAg and hepatitis B e antigen (HBeAg); both are products of precore/core gene and share the first 149 amino acids of HBcAg (25). The HBcCrAg assay measures HBcAg and HBeAg simultaneously by using monoclonal antibodies that recognize both denatured HBcAg and HBeAg (14).

In the present study, we developed a new EIA specific for HBcAg. The specimens were pretreated in order to release HBcAg from the virion and to inactivate antibodies before the assay. The correlation between concentrations of HBcAg and HBV DNA was assessed in the sera of hepatitis B patients. With a series of sera from patients undergoing lamivudine therapy, HBcAg concentration decreased less drastically than the HBV DNA level. The supposed mechanism of this difference and its clinical significance are discussed.

MATERIALS AND METHODS

Serum samples and patients. Hepatitis B sera panels were purchased from Boston Biomedica, Inc. (BBI; West Bridgewater, Mass.) or Clinical Science Laboratory, Inc. (CSL; Mansfield, Mass.). Control samples negative for HBV were obtained from blood donors or from chronic hepatitis C patients at the Shinshu University Hospital (Matsumoto, Japan) in 1997. Seventy-two patients with persistent HBV infection (42 males and 30 females [age range, 14 to 82 years]) were examined at least 3 times in 1997, and serum samples were collected 3 times from each patient. Of the 72 patients, 56 showed abnormal levels of serum alanine aminotransferase; the remaining 16 did not and were classified as asymptomatic carriers. None of the 72 patients was treated with antiviral agents such as interferon or lamivudine. Among the 216 sera from the 72 patients, 18
Specimen pretreatment and EIA for HBcAg. The HBcAg assay contains a monoclonal antibody as detector antibodies that react with denatured HBcAg and HBeAg, thus, the assay detects precore and core proteins. HBV markers and HBV DNA measurement. HBsAg, anti-HBe, and anti-HBc were measured by clinically applied radioimmunoassay (Dinabott, Tokyo, Japan). HBsAg was measured by chemiluminescent immunoassay (Dinabott). Samples showing values over the detection range were remeasured after dilution to obtain quantitative results.

HBV DNA was detected by TMA (Chugai Diagnostics Science Co., Ltd., Tokyo, Japan), which has a detection range between 3.7 and 8.7 log genome equivalents (LGE)/ml (corresponding to $5 \times 10^7$ to $5 \times 10^9$ copies/ml), or by PCR (AmpliCor HBV Monitor test; Roche Molecular Systems, Inc., Branchburg, NJ,) with a detection range between $4 \times 10^6$ and $4 \times 10^8$ copies/ml.

In the BBI PHM 935/A/B panels, the results for HBV DNA by the AmpliCor HBV Monitor test were obtained from the supplier’s data sheet.

Sucrose density gradient ultracentrifugation. HBcAg-positive serum (0.1 to 1.0 ml) was layered on a linear 10 to 60% (wt/wt) sucrose gradient, and centrifugation was carried out at 200,000 $\times g$ (33,400 rpm) for 15 h at 4°C with a Beckman Sw40Ti rotor. The centrifuged solution was fractionated (40 fractions of 300 $\mu$l) by micropipette. The density of each fraction was calculated from the weight and volume. Each fraction was diluted 10-fold and tested for HBcAg as well as for HBsAg, HBeAg, and HBV DNA by PCR.

RESULTS

Detection range, linearity, and reproducibility of the HBcAg assay. We constructed a new EIA specific for HBcAg released from the HBV virion by pretreatment with SDS. In the HBcAg assay, the calibration curve was determined by using the HBcAg standard serially diluted in normal human serum (Fig. 1). The upper detection limit of this assay was approximately $1 \times 10^5$ pg/ml (Fig. 1). The analytical lower detection limit was 2 pg/ml (Fig. 1), as the concentration at which the mean $\pm$ 2 standard deviations (SD) of the RLI did not overlap with the analytical blank.

![FIG. 1. Standard curve and detection limit of the HBcAg assay.](image)

![FIG. 2. Dilution linearity of hepatitis B sera. Two HBcAg-positive sera were serially diluted in normal human serum and measured by the HBcAg assay.](image)
mean + 2 SD of the zero calibrator (n = 6) (Fig. 1, inset). This assay displayed a broad dynamic range, from 2 to 10^7 pg/ml.

The linearity of the assay was examined in sera serially diluted in normal human serum (Fig. 2). Two HBcAg-positive sera (CSL P0339/2-02 and -09) were used; the former was anti-HBc positive, and the latter was anti-HBe negative. The quantities of HBcAg decreased linearly with serial dilution on a straight line through the zero point.

Intra-assay reproducibility was assessed from 10 measurements of three specimens. The mean HBcAg values of the specimens were 3.0 × 10^5, 4.1 × 10^5, and 1.3 × 10^5 pg/ml, and the coefficients of variation were 9.3, 16.9, and 5.4%, respectively.

**Distribution of HBcAg in sucrose density fractions.** Seven sera positive for HBV DNA were subjected to ultracentrifugation on a 10 to 60% (wt/wt) sucrose density gradient, and fractions were diluted and tested for HBcAg, HBsAg, HBeAg, and HBV DNA by PCR. The results for a serum sample are shown in Fig. 3. HBcAg appeared in fractions around a density of 1.12 g/ml and peaked at fraction 25, as did HBV DNA. HBsAg was distributed in fractions of lower density (1.09 g/ml) and peaked at fraction 25, as did HBV DNA. HBeAg was dispersed widely in fractions of even lower density. The same results were observed in the other six sera. These data provide support for the hypothesis that the assay indeed detects nucleocapsid protein specifically.

**Recovery of the HBcAg assay.** Aliquots of 10 μl of HBcAg-positive serum (BBI PHJ201-03 and -07) were added to 90 μl of reference serum. A normal human serum and four hepatitis B sera (CSL P0339/2-01, -03, -04, and -08) were utilized as reference sera. Three of these hepatitis B sera (CSL P0339/2-01, -03, and -04) were anti-HBc positive. No HBcAg was detected in any reference sera. The antigen measured in these samples was designated as recovered HBcAg, and recovery rates were calculated as ((recovered HBcAg)/[added HBcAg]) × 100% (Table 1). Recovery rates ranged from 79 to 121%, and there were no significant differences between anti-HBc antibody positivity and negativity among the samples. These data indicate that anti-HBc did not interfere with the HBcAg assay.

**Interference of the HBcAg assay.** The influence of various blood elements was assessed by using an interference check kit (International Reagents Corporation, Kobe, Japan). Five concentrations of each blood element were added to two samples of HBcAg-positive sera, and HBcAg was measured. Unconjugated bilirubin (<19 mg/dl), conjugated bilirubin (<20 mg/dl), hemoglobin (<440 mg/dl), chyle (<2,350 formazin turbidity units), and immunoglobulin M rheumatoid factor (<50 IU/ml) did not interfere with this assay (data not shown).

Because the HBcAg assay uses monoclonal antibodies that react to both HBcAg and HBeAg as the immobilized antibody, HBeAg may interfere with the HBcAg assay. The influence of HBcAg was assessed by using rHBeAg. rHBeAg (0.1, 1, 10, or 100 ng/ml) was mixed with rHBcAg (1, 10, or 100 ng/ml) or 1 or 10 μg/ml and measured by the HBcAg assay. Regardless of the rHBeAg concentration, no significant influence (−10 to +9%) was observed when 1 μg or less of rHBeAg/ml was added. However, considerable (32 to 43%) inhibition was observed when 10 μg of rHBeAg/ml was added (data not shown).

**TABLE 1. Recovery of HBcAg**

<table>
<thead>
<tr>
<th>Reference serum</th>
<th>Anti-HBc</th>
<th>PHJ201-03 (207 pg/ml)</th>
<th>PHJ201-07 (3,339 pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>−</td>
<td>163 (79)</td>
<td>3,007 (90)</td>
</tr>
<tr>
<td>P0339/2-08</td>
<td>−</td>
<td>251 (121)</td>
<td>3,573 (107)</td>
</tr>
<tr>
<td>P0339/2-01</td>
<td>+</td>
<td>222 (108)</td>
<td>3,138 (94)</td>
</tr>
<tr>
<td>P0339/2-03</td>
<td>+</td>
<td>235 (114)</td>
<td>3,443 (103)</td>
</tr>
<tr>
<td>P0339/2-04</td>
<td>+</td>
<td>247 (119)</td>
<td>3,545 (106)</td>
</tr>
</tbody>
</table>

a Aliquots of 10 μl of HBcAg-positive serum were added to 90 μl of reference serum. The antigen concentrations measured in these samples are shown as recovered HBcAg. Recovery = (Recovered HBcAg/Added HBcAg) × 100%. Anti-HBc values (signal/cutoff) of P0339/2-08, -01, -03, and -04 are 1.113, 0.051, 0.024, and 0.018. Ratios of <1.0 are considered positive.
were examined in series sera of chronic hepatitis B patients who were treated by lamivudine (Fig. 5B and C). Changes in the HBcAg concentration nearly paralleled those of HBV DNA before lamivudine treatment had begun. After lamivudine administration had started, HBV DNA levels fell sharply while HBcAg concentrations also decreased, but the change in HBcAg was smaller and more gradual.

**DISCUSSION**

Recently, HBV DNA TMA and PCR have been widely used to monitor virus load (6, 8, 10, 13, 17, 20, 21). However,
conventional EIA has some advantages over nucleic acid amplification assays. EIA is a relatively simple method and provides a low cost and quantitative analysis with high reproducibility.

Measurement of the HCV core antigen after specimen pretreatment has been reported as useful for diagnosing and monitoring hepatitis C (12, 15, 18, 26). A highly sensitive EIA was previously developed for the HCV core antigen (1, 2, 27) in which the HCV core antigen was released from the virion by SDS pretreatment prior to EIA detection. We have applied this detection system to HBV. In a previous study, an EIA for HBcAg was developed (14, 23) that measures HBcAg and HBsAg simultaneously by using monoclonal antibodies that recognize denatured HBcAg and HBsAg commonly. The level of HBcAg reflects the HBV load nearly as well as HBV DNA (14, 23).

In the present study, we developed an EIA specific for HBcAg, which includes a single-step pretreatment with SDS. In density gradient fractions, HBcAg peaked with HBV DNA (Fig. 3), showing the assay detected the HBcAg of the HBV virion in serum. As a result of pretreatment, the HBcAg was released from the virion and dissociated to HBcAg dimers that were confirmed by gel filtration analysis (data not shown). The pretreatment also inactivated anti-HBc antibody in specimens, and therefore, HBcAg could be measured quantitatively even in anti-HBc-positive specimens. This was confirmed by the recovery test with anti-HBc-positive sera (Table 1).

HBcAg shares an identical 149-amino-acid sequence with HBcAg (25). The immobilized monoclonal antibodies of the HBcAg assay capture not only HBcAg but also HBsAg. Thus, theoretically, HBcAg would inhibit the HBcAg assay. However, the inhibition was observed only at very high concentrations (>1 μg/ml, corresponding to 80 s/co) of HBcAg. In order to avoid the inhibition and scale out, a sample dilution was needed for 21 of 217 serum samples from hepatitis B patients. Moreover, the inhibition rate was 32 to 43% at 10 μg of HBcAg/ml (corresponding to 166 s/co). This is lower by only 0.16 to 0.24 log units. The results of the HBcAg assay were not seriously affected by HBsAg.

HBcAg concentrations and HBV DNA levels were very closely correlated in hepatitis B patients (r = 0.946) (Fig. 4). The correlation between HBcAg and HBV DNA levels was better than that of HBcAg versus HBV DNA (14, 23), as might be expected for a capsid protein-specific assay. HBcAg levels also paralleled HBV DNA levels in seroconversion panel sera (Fig. 5A) and in serum from a hepatitis B patient before lamivudine therapy (Fig. 5B and C). Thus, the HBcAg assay could be a virus load marker alternative to HBV DNA assays.

The lower and upper detection limits of the HBcAg assay were 2 and 105 pg/ml, respectively (Fig. 1), which corresponded to approximately 5 and 9 LGE of HBV DNA/ml (105 and 109 copies/ml) (Fig. 4). This broad dynamic range of over 4 orders of magnitude is appropriate for quantitative detection of HBV virus loads that vary over a wide range. The HBcAg assay was as sensitive as HBV DNA branched-chain DNA but less sensitive than HBV DNA TMA or PCR. However, particularly in areas in which TMA or PCR is not widely used, the virus load marker measured by simple EIA would be needed for blood screening of HBV infection as well as for virus load monitoring. The HBcAg assay also could be used to screen for HBsAg-negative HBV infection.

In the series sera, changes in HBcAg concentrations nearly paralleled those of HBV DNA prior to medical treatment. To the contrary, a decrease of HBcAg concentrations was much slower than that of HBV DNA concentrations during lamivudine administration (Fig. 5B and C). Lamivudine, a nucleoside analogue, strongly inhibits HBV reverse transcriptase and thus lowers HBV DNA production rapidly (19, 24). However, lamivudine has little or no effect on covalently closed circular DNA (16) and does not inhibit virus DNA transcription to mRNA or its translation. We hypothesized that during lamivudine treatment, a considerable amount of HBV covalently closed circular DNA remains in the liver, and HBcAg would therefore be translated and released into circulation as empty virus (HBV DNA defective virus-like particle). We verified this to some extent (unpublished data).

If this hypothesis is true, HBcAg levels could reflect HBV levels remaining in the liver. Hence, the measurement of HBcAg in addition to that of HBV DNA may be useful for estimating the efficacy of lamivudine treatment or the risk of the emergence of YMDD variants and for deciding whether to discontinue administration of lamivudine. To confirm these preliminary observations, additional clinical and diagnostic studies of much larger populations are required.

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REFERENCES
