New Assays for Quantitative Determination of Viral Markers in Management of Chronic Hepatitis B Virus Infection

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We performed a quantitative study of serum hepatitis B virus (HBV) markers, including new parameters such as pre-S1 antigen (Ag), pre-S2 Ag, and anti-HBx, in 88 chronic hepatitis B surface antigen (HBsAg) carriers. New IMx assays for HBsAg and immunoglobulin M (IgM) anti-HBc detection were also used. The population studied was composed of 65 chronic hepatitis cases (40 positive for hepatitis B antigen [HBeAg] and 25 positive for anti-HBe) and 23 anti-HBe-positive, asymptomatic HBsAg carriers. Serum HBsAg levels detected by IMx were higher in HBeAg-positive than in anti-HBe-positive HBsAg carriers (all patient subgroups included) and correlated with the serum HBV DNA level (P = 0.0001). Both pre-S1 and pre-S2 Ags were detected by enzyme immunoasays in almost all HBsAg carriers. Both pre-S1 and pre-S2 Ag titers correlated positively with the serum HBsAg concentration (P = 0.0001), but only the pre-S1 Ag titer correlated with the level of serum HBV DNA (P = 0.02). The detection of low levels of IgM anti-hepatitis B core (anti-HBc) antibodies by IMx was associated with the presence of liver disease (P = 0.05) but not with the level of viral replication. The prevalence of anti-HBx antibodies detected by the enzyme immunoassay was slightly, although not significantly, higher in patients with high levels of HBV DNA (>100 pg/ml) than in patients without detectable HBV DNA (P = 0.16). In anti-HBe-positive chronic HBsAg carriers, the quantitative detection of serum HBV DNA, pre-S Ag titers, and IgM anti-HBc allowed us to predict which patients suffered from chronic liver disease and/or supported viral replication (P < 0.05). In a follow-up study of eight patients undergoing antiviral therapy, the clearance of both pre-S1 Ag and HBV DNA was associated with a subsequent clearance of HBV. Therefore, the quantitative determination of HBV DNA, pre-S Ags, and IgM anti-HBc may prove useful for the decision to use and the monitoring of antiviral therapy, especially in anti-HBe-positive HBsAg carriers.

Hepatitis B virus (HBV) infection causes a wide spectrum of clinical conditions. Schematically, patients with chronic HBV infection can be divided into two groups: (i) the healthy asymptomatic, chronic hepatitis B surface antigen (HBsAg) carriers with low-grade HBV replication and normal liver function tests and (ii) the chronic hepatitis (CH) B patients with active viral replication and progressive liver disease that may lead to cirrhosis and hepatocellular carcinoma (these patients may or may not be symptomatic) (1, 17, 20). The natural history of chronic HBV infection is characterized by a slow transition from a phase of active HBV replication to a phase of low viral replication (1, 17, 20). The early replicative phase is associated with the production of hepatitis B envelope antigen (HBeAg) in serum and hepatitis B core antigen (HBeAg) in liver and the presence of CH (17, 33). The later, low-replication phase is accompanied by seroconversion from HBeAg to anti-HBe, which is usually followed by the remission of liver disease (16, 17, 33). The detection of serum HBV DNA has been shown as the most reliable marker of HBV replication, while the HBe versus the anti-HBe status has not always permitted the differentiation of the two phases (the active-replication phase versus the low-replication phase) of chronic HBV infection (5, 14, 29). Indeed, in a significant proportion of cases, the detection of significant levels of serum HBV DNA indicates active HBV replication in anti-HBe-positive chronic HBV infection (3, 14). These atypical forms of CH B have been recently related to mutations in the precore region of the HBV genome leading to a stop codon (TAG) that prevents the synthesis of HBe Ag (7, 32). In some of these cases, the course of CH may be more severe and/or rapidly progressive than with wild-type virus infection (3, 10). The assessment of HBV replication in these anti-HBe-positive CH infections may be difficult since circulating HBV DNA is often transient and fluctuating at low levels (3, 10). The polymerase chain reaction (PCR) is the most sensitive method for the detection of HBV DNA and may be useful especially in anti-HBe-positive CH for the diagnosis of low-grade HBV replication (1, 12, 20). PCR amplification is also necessary for the detection of the mutations in the precore region of the HBV genome (25). However, the PCR is not yet widely available for clinical purposes.

Another postulated serological marker for the presence of active HBV replication and accompanying active liver disease is immunoglobulin M anti-hepatitis B core (IgM anti-HBc) antibodies, but its clinical relevance depends on the sensitivity of the assay and remains undetermined in chronic HBV infection (2, 23). The clinical significance of pre-S1 and pre-S2 protein detection in serum is still a matter of controversy: several studies concerning pre-S detection in relation to markers of HBV replication have led to opposite results (6, 12, 13, 18, 19, 28, 30, 35). Conflicting findings regarding the correlation of anti-HBx antibodies with the intensity of HBV replication or the clinical status of HBV-infected patients have been reported (11, 24, 31, 34). Recently, the development of a quantitative assay for the detection of
serum HBV DNA (22) and fully automated immunoassays for the detection of HBsAg and IgM anti-HBs (9) has provided new tools for the diagnosis of HBV infections. Prompted by the results of a qualitative evaluation of HBV markers (unpublished data), we initiated a study to assess the clinical relevance of the quantitative determination of serum HBV markers (including HBsAg, the new pre-S1 and pre-S2 Ags, anti-HBx antibodies, IgM anti-HBc, and HBV DNA) in a large group of chronically HBV-infected patients. We focused our attention on the anti-HBc-positive chronic HBsAg carriers to determine which marker(s) may be useful to distinguish patients with chronic liver disease and active HBV replication from those who are in the low-replication phase and without liver disease.

MATERIALS AND METHODS

Patients. Serum samples were obtained from 88 chronic HBsAg carriers known to be HBsAg positive for more than 12 months. They included a group of 65 patients with CH B diagnosed by clinical, biological, and histological findings (40 patients were HBsAg positive, and 25 patients were positive for anti-HBc, IgM anti-HBc, and IgM anti-HBs). The histological analysis of 65 CH B patients showed the features of chronic, persistent hepatitis in 1 case, chronic, active hepatitis in 53 cases, and cirrhosis in 11 cases. Another group consisted of 23 asymptomatic chronic HBsAg carriers (ASC) positive for anti-HBc with normal alanine aminotransferase values (10 patients were referred to our liver unit for serum HBsAg positivity and 13 were healthy volunteer blood donors positive for HBsAg on serum screening). Liver biopsies were not performed for this latter group, referred to as healthy HBsAg carriers. Thirty-one serial serum samples were available from eight HBsAg-positive CH patients undergoing antiviral therapy (interferon and/or ara-AMP). All patients studied were negative for anti-hepatitis D virus, anti-hepatitis C virus, and anti-human immunodeficiency virus. The control group consisted of 4 healthy blood donors with normal liver function tests and no HBV markers and 10 patients suffering from CH C (negative for HBsAg and positive for anti-hepatitis C virus. All serum samples were stored at 20°C.

Routine detection of serum HBV markers. HBsAg, anti-HBe, anti-HBc, anti-HBs, and anti-HBx were tested routinely by a radioimmunoassay by using commercially available tests (Abbott Laboratories, North Chicago, Ill.).

HBsAg detection by IMx HBsAg. Each serum sample was tested for HBsAg by IMx HBsAg (Abbott), a fully automated microparticle enzyme immunoassay described in detail elsewhere (9). In the IMx HBsAg assay, the solid phase consists of microparticles coated with anti-HBs. An aliquot of the specimen is incubated with coated microparticles and biotinylated anti-HBs solution in the reaction well, the mixture is transferred to a matrix which captures the microparticles. The excess specimen is washed away, and the microparticles are bathed in anti-biotin-alkaline phosphatase conjugate, which binds to the biotinylated anti-HBs–Ag microparticle complex. The excess conjugate is washed away, and enzyme substrate, 4-methylumbelliferyl phosphate (MUP), is added. The rate of formation of fluorescent product on the matrix is calculated and compared with that obtained with a calibrator to determine the presence of HBsAg. The rate of fluorescent product formation is proportional to the HBsAg concentration. An index value is determined as a sample/positive (S/N) value. Any specimen having an index value greater than or equal to 2.00 was considered reactive for HBsAg. The concentration of HBsAg in serum was assayed according to the 10-fold-dilution positive specimens by using IMx HBsAg and extrapolating the IMx index obtained to a standard curve derived by testing the Abbott HBsAg sensitivity panel with low concentrations of HBsAg (subtypes ad and ay). Results were expressed as micrograms of HBsAg per milliliter of serum. The IMx HBsAg assay was able to detect HBsAg concentrations less than 0.5 ng/ml. The reproducibility and specificity of this assay have been described (9).

Pre-S antigen detection. Pre-S antigen assays were previously evaluated by testing undiluted serum samples from 200 HBsAg-negative hospital patients and 288 HBV carrier mothers (26). One-hundred percent (229 of 229) of HBsAg-positive carriers and 96.6% (57 of 59) of anti-HBe-positive carriers were reactive for pre-S1 Ag, but none of the HBsAg-negative specimens were reactive. Similar findings were observed for the pre-S2 Ag assay, in which 99% (102 of 103) of HBsAg-positive carriers and 95% (81 of 85) of anti-HBe-positive carriers were reactive. These data indicated that the simple presence or absence of pre-S1 Ag or pre-S2 Ag in undiluted specimens was not a useful marker for active viral replication or infectivity. However, the levels of pre-S1 Ag and pre-S2 Ag reactive titers were higher in HBsAg-positive carriers than in anti-HBe-positive carriers (26). Therefore, in the present study, pre-S Ag testing was performed with diluted serum specimens and at further 10-fold dilutions to determine the clinical relevance of this quantitative assay.

Serum samples were diluted 1:10 in 5% bovine serum albumin and then incubated overnight at room temperature for 18 h with Auszyme II beads (Abbott). After being washed with water, the beads were incubated with 200 µl of peroxidase-conjugated monoclonal antibodies for 2 h at 4°C. The beads were washed with water and incubated with 300 µl of a solution containing O-phenylenediamine and H2O2. After 30 min of incubation at room temperature, the reaction was stopped by adding 1 ml of 1 N H2SO4, and the resulting optical density at 492 nm was determined by using a photoenzyme-linked immunosorbent assay colorimeter (Abbott).

For pre-S1 Ag detection, peroxidase-conjugated monoclonal antibodies 116-80 and 116-86, which recognize epitopes of the pre-S1 region, were used as the detection system. The fine specificity of these monoclonal antibodies is described in detail elsewhere (27). The cutoff value was calculated as the mean optical density of negative controls plus 0.05. For a quantitative analysis of pre-S1 Ag, the test sera positive for pre-S1 Ag were diluted 10-fold with 5% bovine serum albumin. The pre-S1 Ag titer was expressed by the highest positive 10-fold dilution of the test serum.

For pre-S2 Ag detection, peroxidase-conjugated monoclonal antibodies 50-80, 25-19, and 116-34, which are described elsewhere (27), were used as the detection system. The cutoff value was calculated as described for the pre-S1 Ag assay. The pre-S2 Ag titer was expressed by the highest positive 10-fold dilution of the test serum.

Detection of IgM anti-HBe by IMx core M. The presence of IgM anti-HBe antibodies was tested in serum by IMx core M (Abbott) as previously described (9). Briefly, each specimen was diluted by the IMx instrument. Microparticles coated with anti-human IgM antibody (µ chain) were used to capture IgM in the diluted specimen. A recombinant-derived HBeAg is added, and if IgM anti-HBe is present in the sample, the HBeAg binds to the microparticle-antibody complex. Human anti-HBe-alkaline phosphatase conjugate is dispensed and binds to the microparticle-antibody-antigen complex. The microparticle complex captured on the matrix
is washed to remove unbound material, and the substrate MUP is added to the matrix. The rate of production of fluorescent product is proportional to the amount of IgM anti-HBc captured and is normalized to the Mode 1 calibrator rate to give an index value. Specimens giving index values between 0.800 and 1.200 were considered “gray zone” reactive, and specimens giving index values greater than 1.200 were considered reactive in the assay. The sensitivity, reproducibility, and specificity of the assay were previously determined (9).

**Detection of anti-HBx antibodies by ELISA.** Serum samples from 84 of the 88 chronic HBsAg carriers were available for anti-HBx testing. Detection of anti-HBx antibodies by an enzyme-linked immunosorbent assay (ELISA) was carried out as described elsewhere (34). Briefly, a purified recombinant HBx preparation was coated on Microwell III assay plates at a concentration of 1 to 1.5 mg/ml in phosphate-buffered saline (PBS), pH 7.2, by incubation overnight at 4°C. After washing with PBS–5% Tween 20, the plates were overcoated with PBS–5% casein for 90 min at 37°C. The microwells were then reacted with human sera at both 1:100 and 1:200 dilutions in PBS-casein for 90 min at 37°C. After washing with PBS–Tween 20, goat anti-human immunoglobulin conjugated to alkaline phosphatase in PBS–5% casein for 1 h at 37°C, the microwells were washed with PBS–Tween 20, and phosphatase substrate in diethanolamine (1 mol/liter, pH 9.8) was added. After 1 h at 37°C, the results were read spectrophotometrically. Sera with absorbance values three times higher than that of normal serum were considered positive for anti-HBx antibodies. To quantify the results, an index value was calculated as the serum sample optical density/cutoff value.

**Solution hybridization assay for HBV DNA.** The solution hybridization assay for HBV DNA in serum was performed as previously described (22), and results were quantified according to the procedures and the formulas described in the Abbott commercial test kit (Genostic). One-hundred microliters of serum was denatured and then hybridized in solution for 16 to 18 h at 65°C. The hybridization mixture was then applied to a gel matrix to separate hybrids from free probe. 125I-DNA probe hybridized to HBV DNA sequences eluted from the matrix, while free probe remained on the gel column. After the radioactivity was determined with a γ counter for 10 min, HBV DNA-positive samples were quantitated by using the positive control standard and results were expressed in picograms of HBV DNA per milliliter. The sensitivity of the assay was 0.15 pg of HBV DNA.

**PCR assay for HBV DNA.** The PCR assay for serum HBV DNA was performed as described previously in detail (8) for a separate additional group of 31 anti-HBe-positive chronic HBsAg carriers (11 CH cases and 20 ASC cases); pre-S1 Ag testing was also performed in the same undiluted serum specimens for comparison.

(i) **Preparation of DNA samples from serum.** Five-hundred microliters of serum was incubated at 55°C for 1 h in Tris-HCl (pH 8)–1 mM EDTA–100 mM NaCl–proteinase K (500 μg/ml). After three phenol-chloroform extractions, the DNA was precipitated overnight with ethanol in the presence of 3 M sodium acetate, pH 5.2, at −20°C. The precipitate was redissolved in 100 μl of H2O.

(ii) **PCR assay.** Oligonucleotides were synthesized by using a DNA synthesizer apparatus (Applied Biosystems). Oligonucleotides synthesized are specific for the most-conserved C and S gene sequences of HBV genome were selected according to the sequence kindly provided by J. Sninsky from the Cetus Corporation, as described in detail (8). Primers MD03 (position 736 to 759) and MD06 (position 637 to 651) and the probe MD09 are located in the S gene sequence. Primers MD25 (position 1959 to 1980) and MD27 (position 1856 to 1881) and the probe MD28 are located in the C gene region.

DNA extracted from 500 μl of serum was amplified as described previously (8). Target sequences were amplified in a 50-μl reaction mixture containing 1 μg of the serum DNA sample, 1 U of Taq polymerase (Perkin-Elmer Cetus), 187 mM each deoxynucleoside triphosphate, 50 pmol each primer, 40 mM KCl, 50 mM Tris-HCl, and 6 mM MgCl2. Mixtures were overlaid with 100 μl of mineral oil to prevent evaporation. The reaction was performed for 30 cycles in a programmable DNA thermal cycler (Perkin-Elmer Cetus). During each cycle, samples were heated to 95°C for 25 s, cooled to 55°C for 25 s, and then incubated for 1 min at 72°C.

To eliminate sources of contamination, reagents were aliquoted and stored in new disposable containers. All experiments were performed in parallel with serum samples from positive and negative controls and with the reaction mixture without DNA. Each sample was tested in at least two different series for each set of primers.

(iii) **Analysis of PCR amplification products.** For each sample, a 10-μl aliquot of the amplified DNA reaction mixture was analyzed by ethidium bromide coloration after gel electrophoresis and Southern blot hybridization (SBH) analysis after electrophoresis as described previously (8). Probes designated MD09 for the S gene and MD28 for the C gene were labeled by terminal transferase with a 3'-end-labeling kit (Boehringer GmbH, Mannheim, Germany) by using [32P]dCTP (3,000 Ci/mmol; Amersham) to a specific activity of 10⁶ cpm/ml in hybridization medium. The hybridization solution for the nucleotide probes contained 3× SSPE (1× SSPE is 0.18 M NaCl, 10 mM sodium phosphate [pH 7.4], and 1 mM EDTA), 5× Denhardt’s solution, 30% (vol/vol) formamide, and 0.5% sodium dodecyl sulfate (SDS). The membrane (Hybond N; Amersham) was hybridized with standard labeled specific probe at 42°C overnight. Excess probe was removed by two washes with 2× SSPE–0.1% SDS and an additional wash with 0.2× SSPE–0.1% SDS for 30 min at 42°C. The membrane was exposed for 1 h and overnight at −70°C with two intensifying screens on X-ray film (Hyperfilm; Amersham) for autoradiography. The sensitivity of the PCR-SBH assay was 10⁻⁵ pg of HBV DNA (8).

**Statistical analysis.** Differences in the mean value of each group were evaluated by an analysis of variance or Kruskall Wallis test for noncontinuous variables. The correlations between the variables were calculated by using simple regression analysis. The prevalences of the different viral markers were compared by using the chi-square test.

**RESULTS**

Assays for HBV markers in patients with chronic HBV infection. (i) **HBsAg detection by IMx.** HBsAg was detected by IMx HBsAg in the serum samples of all CH HBsAg carriers but in none of the control group (Table 1). A quantitative determination of HBsAg showed that the mean serum HBsAg concentration was higher in HBeAg-positive CH than in anti-HBe-positive chronic HBsAg carriers whether they were ASC or patients with chronic liver disease (P < 0.05) (Table 2).

(ii) **Pre-S Ag detection in serum.** By using specimens diluted 1:10, the prevalence of pre-S1 Ag in anti-HBe-positive ASC sera (61%) was significantly lower than that in
TABLE 1. Prevalence of serum HBV markers in the different clinical categories of chronic HBV infection

<table>
<thead>
<tr>
<th>Markers</th>
<th>Anti-HBe+ ASC (n = 23)</th>
<th>Anti-HBe+ CH (n = 25)</th>
<th>HBeAg+ CH (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>23/23 (100)</td>
<td>25/25 (100)</td>
<td>40/40 (100)</td>
</tr>
<tr>
<td>Pre-S1 Ag</td>
<td>14/23 (61)</td>
<td>25/25 (100)</td>
<td>40/40 (100)</td>
</tr>
<tr>
<td>Pre-S2 Ag</td>
<td>23/23 (100)</td>
<td>25/25 (100)</td>
<td>39/40 (98)</td>
</tr>
<tr>
<td>IgM anti-HBc</td>
<td>4/23 (17)</td>
<td>6/25 (24)</td>
<td>7/40 (18)</td>
</tr>
<tr>
<td>Anti-HBs</td>
<td>10/22 (45)</td>
<td>7/24 (29)</td>
<td>18/38 (47)</td>
</tr>
<tr>
<td>HBV DNA+</td>
<td>2/23 (9)</td>
<td>11/25 (44)</td>
<td>32/40 (80)</td>
</tr>
</tbody>
</table>

a Of 14 control subjects, none had any of the markers.
b The prevalence was statistically different in the three clinical categories (chi-square test, \( P = 0.0001 \)).

table: Pre-S1 Ag was detected in the serum samples of all but one chronic HBsAg carriers (Table 1). Pre-S2 titers were higher in both CH patient groups (positive for HBcAG or anti-HBe) than in anti-HBe-positive ASC (\( P < 0.05 \)) (Table 2).

Pre-S2 Ag was detected in the serum samples of all but one chronic HBsAg carriers (Table 1). Pre-S2 titers were higher in both CH groups (positive for HBcAG or anti-HBe) than in anti-HBe-positive ASC (\( P < 0.05 \)) (Table 2).

(iii) IgM anti-HBc antibody detection by IMx. Patient serum samples tested reactive for IgM anti-HBc antibodies by IMX core M in 17% of ASC cases, 24% of anti-HBe-positive CH cases, and 18% of HBcAG-positive CH cases (Table 1). The prevalence of IgM anti-HBc in the different groups of chronic HBsAg carriers was not statistically different (\( P > 0.05 \)). Many serum samples tested fell into the gray zone of reactivity: 5 of 25 (20%) anti-HBe-positive CH cases and 10 of 40 (25%) HBeAG-positive CH cases. Altogether, 28 of 65 (43%) CH cases (HBeAG positive or anti-HBe positive) were positive or weakly reactive for IgM anti-HBc in contrast to 4 of 23 (17%) ASC cases (\( P = 0.05 \)). The mean index value of IgM anti-HBc was not statistically different in the three groups of chronic HBsAg carriers (Table 2).

(iv) Detection of anti-HBx antibodies by ELISA. Anti-hepatitis B x gene product (anti-HBx) antibodies were found in the serum samples of 45% of anti-HBe-positive ASC cases, 29% of anti-HBe-positive CH cases, and 47% of HBeAG-positive CH cases (\( P > 0.05 \)) (Table 5). The mean anti-HBx index value was higher in HBeAG-positive CH cases than in anti-HBe-positive CH cases (\( P < 0.05 \)) (Table 2).

(v) Detection of serum HBV DNA by solution hybridization assay. HBV DNA was detected in the serum samples of 9% of anti-HBe-positive ASC cases, 44% of anti-HBe-positive CH cases, and 80% of HBe-positive CH cases (\( P < 0.05 \)) (Table 1). The mean serum HBV DNA level was higher in the HBeAG-positive CH group than in both groups of anti-HBe-positive chronic HBsAG carriers with or without liver disease (\( P < 0.05 \)) (Table 2).

Determination of HBV markers in relation to the level of HBV replication. We analyzed the correlation between the different HBV markers and the levels of serum HBV DNA in 88 chronic HBsAg carriers. As shown in Fig. 1, the serum HBcAG concentration correlated positively with the serum HBV DNA level (\( r = 0.55; P = 0.0001 \)). Pre-S1 Ag titers correlated positively with the serum HBV DNA level (\( r = 0.24; P = 0.02 \)), while pre-S2 titers did not (\( r = 0.01; P = 0.86 \)) (Fig. 2). It was noteworthy that both pre-S1 Ag and pre-S2 Ag titers correlated positively with the HBcAG concentration in serum (\( r = 0.44; P = 0.0001 \)). The index value of IgM anti-HBc did not statistically correlate with the serum HBV DNA level (\( P = 0.44 \)). The index value of anti-HBx antibodies did not correlate with the serum HBV DNA level (\( r = 0.03; P = 0.76 \)). However, the prevalence of anti-HBx antibodies was higher (53%) in patients with high viral replication (serum HBV DNA, >100 pg/ml) than in patients without detectable serum HBV DNA (33%), but this difference did not reach statistical significance (\( P = 0.16 \)).

Detection of HBV markers in HBcAG-positive CH patients during antiviral therapy. Thirty-one serum samples were available from 8 HBsAg-positive patients undergoing antiviral therapy (Interferon and/or ara-AMP).

In two cases, interferon therapy induced HBV DNA clearance with a persistence of HBsAg. In these two cases, the HBV DNA disappearance from serum samples was associated with a decrease in the HBcAG concentration, in the pre-S2 Ag and pre-S1 Ag titers, and, in one case, in the IgM anti-HBc index from the gray zone to a negative value. Anti-HBx antibodies were negative in one case and remained positive during the follow-up in the other case.

In three cases, anti-HBe sera were observed. Two patients received interferon, and one received interferon in combination with ara-AMP. The serum HBcAG concentration decreased at the time of anti-HBe seroconversion in all three cases. The pre-S1 Ag titer diminished in two cases, while the pre-S2 Ag titer diminished in only one case at the time of anti-HBe seroconversion. The IgM anti-HBc index value, although initially negative in all three cases, decreased after anti-HBe seroconversion. Anti-HBx antibodies were positive in two cases, and the anti-HBx index value decreased in these two cases after anti-HBe seroconversion but remained positive.

Three patients cleared HBsAg from serum samples. Two patients received interferon, and one received interferon in combination with ara-AMP. In these cases, both HBV DNA and pre-S1 Ag first became negative, followed by the con-

TABLE 2. Quantitative determination of HBV markers according to different clinical groups

<table>
<thead>
<tr>
<th>Clinical group</th>
<th>Mean (±SD) of the following marker:</th>
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<tbody>
<tr>
<td></td>
<td>HBsAg (μg/ml)</td>
</tr>
<tr>
<td>Anti-HBe+ ASC</td>
<td>(n = 23)</td>
</tr>
<tr>
<td>22.9 ± 30.6</td>
<td>1.17 ± 1.11</td>
</tr>
<tr>
<td>Anti-HBe+ CH</td>
<td>(n = 25)</td>
</tr>
<tr>
<td>HBeAg+ CH</td>
<td>(n = 40)</td>
</tr>
</tbody>
</table>

* GMT, geometric mean titer.
\( * \) Result is significantly different from those of the other two groups (analysis of variance, \( P < 0.05 \)).
\( * \) Result is significantly different from that of the Anti-HBe+ ASC group (Kruskal-Wallis test, \( P < 0.05 \)).
\( * \) Result is significantly different from that of the Anti-HBe+ CH group (analysis of variance, \( P < 0.05 \)).
secutive clearance of pre-S2 Ag and HBsAg. The IgM anti-HBc index value decreased from a gray zone to a negative value in two cases, while the third case remained negative for IgM anti-HBc antibodies. Anti-HBx antibodies were negative in this third case; in the two other cases, the anti-HBx index value decreased and became negative in one case. The sequence of the disappearance of HBV markers was pre-S1 Ag and HBV DNA and then pre-S2 Ag and HBsAg, as shown in Fig. 3.

Clinical significance of quantitative determination of HBV markers in anti-HBe-positive chronic HBsAg carriers with or without liver disease. In the group of anti-HBe-positive chronic HBsAg carriers, we examined which HBV marker(s) might be useful to distinguish patients with active HBV replication and CH from those who are in the low-replication phase without liver disease. In these anti-HBe-positive HBsAg carriers, the prevalence of serum HBV DNA detected by the solution hybridization assay was significantly higher in CH (44%) than in ASC (9%) cases (Table 1). However, in 56% of anti-HBe-positive CH cases, HBV replication could
FIG. 3. Follow-up study of HBV markers in a CH B patient treated with both ara-AMP and interferon and from whose serum HBsAg was cleared. (A) Titers of pre-S1 Ag and pre-S2 Ag as detected by an enzyme immunoassay. (B) Evolution of serum HBsAg and that of serum HBV DNA. The serum HBsAg concentration as determined by IMx HBsAg is expressed in micrograms per milliliter. The serum HBV DNA was tested with a solution hybridization assay, and the concentration is expressed in picograms per milliliter. (C) Anti-HBx antibodies were detected by an enzyme immunoassay. A serum sample is considered positive for anti-HBx when the index value is higher than 1.000. IgM anti-HBc antibodies were detected by IMx Core M. Specimens giving index values between 0.800 and 1.200 were considered gray zone reactive, and specimens giving index values greater than 1.200 were considered reactive in the assay.

not be demonstrated by serum HBV DNA detection. Since both pre-S1 Ag and pre-S2 Ag titers were higher in anti-HBe-positive CH cases than in ASC cases (Table 2), we looked for a cutoff titer which might be useful to discriminate between CH and ASC cases. We found that 68% of anti-HBe-positive CH cases had pre-S1 Ag titers higher than 1:100 versus 13% of ASC cases (P < 0.001). Furthermore, 84% of anti-HBe-positive CH cases had pre-S2 Ag titers higher than 1:1,000 versus 30% of ASC cases (P < 0.001). The HBsAg concentration was higher in anti-HBe-positive CH cases than in ASC cases, but the difference was not statistically significant (Table 2). Both the prevalence of IgM anti-HBc antibodies and the mean index value were slightly higher in anti-HBe-positive CH cases than in ASC cases, but the differences did not reach statistical significance (Tables 1 and 2). However, when samples gray zone reactive for IgM anti-HBc (index value, >0.800) were taken into account, the prevalence of IgM anti-HBc antibodies became significantly
higher in CH (44%) than in ASC (18%) cases (P = 0.05). Both the prevalence and index values of anti-HBx antibodies were higher in ASC cases than in anti-HBe-positive CH cases, but the difference was not statistically significant (Tables 1 and 2). Thus, HBV DNA detection, pre-S1 Ag titers of >1:100, pre-S2 Ag titers of >1:1,100, and IgM anti-HBc index values of >0.800 were significantly associated with the presence of chronic liver disease in anti-HBe-positive HBsAg carriers (Table 3).

In a separate additional group of 31 anti-HBe-positive chronic HBsAg carriers, pre-S1 Ag was tested with undiluted serum samples and serum HBV DNA by the PCR assay. Pre-S1 Ag was detected in 11 of 11 (100%) serum samples of anti-HBe-positive CH cases and in 18 of 20 (90%) serum samples of anti-HBe-positive ASC cases. Serum HBV DNA was positive by PCR-SBH in 11 of 11 (100%) serum samples of anti-HBe-positive CH cases and 17 of 20 (85%) serum samples of anti-HBe-positive ASC cases. Altogether, in this group of anti-HBe-positive chronic HBsAg carriers, the concordance between pre-S1 Ag detection and that of HBV DNA by PCR-SBH was 28 of 31 (90%).

**DISCUSSION**

We have studied the clinical significance of the quantitative determination of HBV markers in a series of 88 chronic HBsAg carriers carefully selected to represent the different clinical groups and the relevant stages of the natural history of chronic HBV infection.

Serum HBsAg was detected by a very sensitive and fully automated enzyme immunoassay (IMX HBsAg) which allowed the determination of the HBsAg concentration. No discordant result with the widely used radioimmunoassay (AUSRIA) was observed. In agreement with previous studies (16, 33), the serum HBsAg concentration was significantly higher in HBeAg-positive cases than in anti-HBe-positive HBsAg carriers. A positive correlation was also observed between the level of serum HBV DNA and that of HBsAg. This correlation between the HBsAg level and HBV replication was confirmed in our longitudinal study of CH B patients undergoing antiviral therapy. It is noteworthy that all the subsequent HBV marker assays that have become available have served to refine this correlation with HBV replication. HBV replication was assessed by quantitative determination of serum HBV DNA by using a solution hybridization test (22). In our population of chronic HBsAg carriers, almost all (80%) HBeAg-positive CH cases had serum HBV DNA, while 44% of anti-HBe CH cases and less than 10% of anti-HBe positive ASC were positive for serum HBV DNA, in agreement with other reports using the spot hybridization method (3, 5, 10, 14, 29). The serum HBV DNA level was also higher in HBeAg-positive CH cases than in anti-HBe CH cases, as previously described (3, 10). These data further confirm the clinical usefulness of this simple, quantitative, and sensitive solution hybridization assay for the monitoring of HBV replication.

IgM anti-HBc antibodies were detected by IMX Core M in the serum samples of approximately 20% of the chronic HBsAg carriers, a prevalence similar to that observed by Lai et al. (23). As reported by others (2, 23), we found no correlation between IgM anti-HBc detection and the presence of HBV replication. When samples gray zone reactive for IgM anti-HBe were taken into account, a correlation between the detection of IgM anti-HBe and the presence of chronic liver disease was demonstrated. This observation suggests that the detection of IgM anti-HBe even at low levels may indicate an underlying chronic liver disease. Therefore, further studies are warranted to assess the clinical relevance of the rapid and sensitive detection of IgM anti-HBc by IMX Core M in chronic HBV infection, especially in patients classified as ASC.

Both pre-S1 and pre-S2 antigens were determined quantitatively by an enzyme immunoassay. In the pre-S assay system, a collection of labeled monoclonal antibodies directed against different epitopes of the pre-S region (27) was used in order to obtain a very sensitive assay that would not be significantly influenced by HBV subtype, pre-S variant, or epitope masking by endogenous anti-pre-S antibodies. Both pre-S1 and pre-S2 antigens were detected in almost all of the undiluted serum samples of chronic HBsAg carriers, as previously reported by other groups utilizing sensitive enzyme immunoassays or radioimmunoassays for pre-S Ag detection (6, 18, 19, 35). By using serum specimens diluted 1:10, the prevalence of pre-S1 Ag in anti-HBe-positive ASC (61%) was significantly lower than in HBeAg- or anti-HBe-positive CH cases (100%). These changes underscore the point that pre-S1 Ag and pre-S2 Ag can be detected in virtually all chronic HBsAg carriers but that a 1:10 dilution allows a clinically relevant statement to be made. Pre-S Ag titration showed that both pre-S1 and pre-S2 titers were higher in CH cases (whether they were HBeAg positive or anti-HBe positive) than in anti-HBe positive cases (20). These changes confirm that these ASC may support a low level of viral replication (1, 8, 12, 20, 28, 35). The quantitative determination of pre-S antibodies in relation to the level of HBV replication showed that pre-S1 Ag titers correlated positively with the HBV DNA level while pre-S2 Ag titers did not. These data are in agreement with the preferential localization of pre-S1 epitopes on the envelope of Dane particles as detected with monoclonal anti-pre-S antibodies (27). Both pre-S1 and pre-S2 Ag titers correlated positively with the HBsAg level as shown by others (18, 35). Altogether, these findings suggest that the quantitative determination of pre-S1 Ag may prove useful as a marker for the level of HBV replication (13, 19, 28, 30) and that pre-S2 Ag detection is more closely related to that of HBsAg than to the level of viral replication (28).

Anti-HBx antibody detection was performed by using a sensitive enzyme immunoassay (34). The anti-HBx prevalence was approximately 40% in our population of chronic HBsAg carriers. The prevalence of anti-HBx was not associated with the severity of liver disease (31, 34). The high prevalence of anti-HBx in anti-HBe ASC (45%) compared with the results from our first study (16%) may be explained by the lower number of healthy blood donors devoid of HBV replication in our present study (34). There was a trend towards a correlation between the presence of anti-HBx and a high viral DNA level in serum, suggesting that anti-HBx...
may represent a marker of HBV replication (24, 34). We are currently studying the clinical significance of the differential recognition of the N-terminal, C-terminal, and interior portions of HBx by sera from HBV-infected patients in relation to their viral and clinical statuses (21).

The follow-up study of HBcAg-positive CH cases treated with antiviral agents showed a rather complex evolution of serum HBV markers. It is therefore difficult to determine an early predictive marker for seroconversion from HBc to anti-HBc in this small series. More interesting was the fact that HBV DNA clearance associated with that of pre-S1 Ag may be an early marker of HBV clearance in CH B patients treated by antiviral therapies. The sequence of HBV marker disappearance was identical to that observed by Hess et al. (15). Brahman et al. have reported that in CH B infections treated by interferon, pre-S2 Ag clearance is a marker of successful therapy (4); our observation suggests that the clearance of pre-S1 Ag from serum is an earlier marker of successful antiviral therapy than that of pre-S2 Ag.

For anti-HBe-positive chronic HBsAg carriers, we examined which HBV marker(s) may be useful to distinguish patients with active HBV replication and CH from those who are in the low-replication phase and without liver disease. For anti-HBe positive CH cases, HBV replication could be demonstrated in only 44% of cases by serum HBV DNA detection, as previously described in Italy (10). Furthermore, we found that high pre-S1 and pre-S2 Ag titers as well as detectable but low levels of IgM anti-HBc were significantly associated with the presence of chronic liver disease in anti-HBe-positive HBsAg carriers. Therefore, in anti-HBe-positive CH cases negative for serum HBV DNA which may support low levels of viral replication only detectable by PCR (1, 8, 12, 20), the quantitative determination of pre-S antigens and IgM anti-HBc with sensitive enzyme immunoassays may be needed for the decision of whether to use antiviral therapy. Accordingly, for a group of 31 anti-HBe-positive chronic HBsAg carriers, we observed that pre-S1 Ag detection was strongly associated with the presence of serum HBV DNA as detected by PCR-SBH.

In conclusion, by using quantitative and sensitive new assays, the results of this study on the use of HBV markers to diagnose chronic HBV infection suggest that (i) IMx assays are valuable for both HBsAg and IgM anti-HBc testing in clinical practice, (ii) determination of the pre-S1 Ag level may be a useful tool for the monitoring of low-level HBV replication especially during antiviral therapy, and (iii) in anti-HBe-positive chronic HBsAg carriers negative for serum HBV DNA, the quantitative determination of pre-S antigens and IgM anti-HBc may be necessary to determine which patients may benefit from antiviral therapy.

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REFERENCES


