Improvements in Detection of Antibody to Hepatitis B Core Antigen by Treating Specimens with Reducing Agent in an Automated Microparticle Enzyme Immunoassay

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A fully automated microparticle enzyme immunoassay (EIA), IMx Core, was developed for the detection of antibody against hepatitis B core antigen (anti-HBc). IMx Core sensitivity was less than 0.5 Paul Ehrlich Institut units per ml and was greater than that of the commercial radioimmunoassay (RIA) or EIA, Corab and Corzyme, respectively. Specimens from blood donors and diagnostic and hospital patients, which included individuals with a variety of infectious and immune diseases, were tested in parallel by the IMx Core and EIA. Overall agreement of 99.1% (4,797 of 4,841) was obtained. Prevalence of anti-HBc tested by IMx Core ranged from 1.2% in volunteer blood donors to 9.1% in hospital laboratories. Discordant specimens reactive by IMx Core but negative by Corzyme or Corab resulted from the increased sensitivity of the IMx Core assay, since other hepatitis B markers were usually present. However, most discordant specimens were positive by the EIA or RIA but negative by IMx Core. No other hepatitis B markers could be detected in these discordants, and after addition of reducing agent, these specimens also became negative by EIA or RIA. In clinical trials, 30% (14 of 47) of volunteer blood donors and 8% (9 of 119) of hospital patients testing repeatedly reactive by the EIA had reduction-sensitive (unspecific) anti-HBc reactivity. The reducing agent, dithiothreitol, was added to each specimen automatically in the IMx assay to eliminate these unspecific reactions without significantly affecting anti-HBc reactivity resulting from hepatitis B virus infection as judged by the correlation with other hepatitis B markers.

Antibody to hepatitis B core antigen (anti-HBc) is one of the earliest serological markers during hepatitis B virus (HBV) infection and is generally present during acute, convalescent, and chronic phases of the disease (4, 17). Anti-HBc usually persists for many years after infection and may be the only marker of previous exposure, since antibody to hepatitis B surface antigen (anti-HBs) may wane with time (14).

Since 1986, tests for anti-HBc have been widely used in the United States for screening blood donors, since epidemiological studies have shown that anti-HBc could serve as a surrogate marker for non-A, non-B hepatitis (6, 16). Several investigators have observed low-level anti-HBc test results near the assay cutoff in healthy blood donors testing negative for hepatitis B surface antigen (HBsAg) and have suggested that most of these results were due to poor specificity of the current, commercial immunoassays (1, 2, 13).

Several lines of evidence indicate that many low-level anti-HBc-reactive specimens are reproducible and have a factor which specifically binds hepatitis B core antigen (HBcAg). Staller et al. (15) showed that 92% of low-level reactive specimens were confirmed by neutralization with HBcAg derived from recombinant DNA in Escherichia coli (rHBcAg) or purified from Dane particles. Similar results were obtained in other neutralization studies (8).

Alternative anti-HBc assay formats have been devised, including a direct sandwich in which anti-HBc is captured by binding to rHBcAg on a solid phase and then detected by using HBcAg labeled with $^{125}$I or horseradish peroxidase (9). These low-level anti-HBc reactivities were also positive in this sandwich assay, indicating that an HBcAg binding substance may be detected regardless of assay format.

Chau et al. (2) and Weare et al. (18) have demonstrated that many of these specimens become nonreactive for anti-HBc after treatment with reducing agent (sodium metabisulfite, 2-mercaptoethanol, or cysteine). They have purified this reductant-sensitive factor and identified it as an immunoglobulin M (IgM) or IgM-like molecule (11).

Sallberg and Magnus (12), using an antibody capture format for detection of subclass-specific anti-HBc, found low levels of IgM or IgA anti-HBc in sera and suggested that the presence of these subclasses in sera may result from the unspecific activation of immature B lymphocytes and may not be the result of previous exposure to HBV.

In this study, numerous specimens from blood banks and hospital and reference laboratories were tested for anti-HBc reactivity by using a microparticle enzyme immunoassay (EIA) (IMx Core) which was fully automated on the Abbott IMx instrument. The addition of a reducing agent, dithiothreitol (DTT), to the specimen significantly decreased the number of discrepant specimens reactive by anti-HBc assays but negative for other hepatitis B markers. DTT addition destroyed the reactivity of unspecific IgM anti-HBc in sera from individuals not previously exposed to HBV without significantly affecting the ability of the immunoassay to detect anti-HBc resulting from HBV infection. The IMx Core also showed improved within-run and between-run reproducibility and had sensitivity of better than 0.5 Paul Ehrlich Institut (PEI) units per ml.

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MATERIALS AND METHODS

Test kits. Serum and plasma specimens were tested with commercial radioimmunoassay (RIA) and EIA reagents supplied by Abbott Laboratories: Auszyme Monoclonal for HBsAg, Corzyme and Corab for total anti-HBc, Corzyme-M for IgM anti-HBc, and Ausab for anti-HBs. For some testing, specimens were incubated with a reducing agent, 25 mM sodium metabisulfite, prior to assay in Corab or Corzyme (2).

Samples. In-house and clinical testing was performed on surplus, unlinked specimens obtained from a public health laboratory (San Bernardino, Calif.), reference laboratories and hospitals (New England Deaconess Hospital, Boston, Mass.; Howard Brown Clinic, Chicago, Ill.; Cedars Medical Center, Miami, Fla.), and volunteer and commercial blood banks (Ochsner Foundation Hospital Blood Bank, New Orleans, La.; Community Blood Center, Kansas City, Mo.; Central Indiana Regional Blood Center, Indianapolis). Specimens from patients with diseases other than hepatitis B included specimens from infections with hepatitis A virus, Epstein-Barr virus, cytomegalovirus, hepatitis C virus, human immunodeficiency virus type 1, rubella virus, and herpes simplex virus. Serial bleed specimens were collected at a plasmapheresis center from regular donors who became positive for HBsAg.

IMx Core. The IMx assay for anti-HBc is based on microparticle EIA technology used in the IMx instrument system (3). Briefly, the microparticle EIA technology uses microparticles approximately 0.2 μm in diameter as the solid phase. Separation of bound from unbound material is accomplished by capture of the microparticles on a glass fiber matrix. Microparticles allow increased kinetics of reaction (total time of assay is less than 45 min for 24 specimens) compared with those of other solid phases and may be easily manipulated by the instrument robotics for automation of the assay steps. No operator intervention is required other than for pipetting of the samples into the specimen wells, placing the reagent into the analyzer, and initiating the assay.

Carboxylated latex particles (0.1 to 0.3 μm in diameter) (Seradyn, Indianapolis, Ind.) were coupled to DEAE-purified human anti-HBc (Abbott Laboratories) by using 1-ethyl-3,3-(dimethylaminopropyl)carbodiimide chemistry (10). After the unbound anti-HBc was washed away, the microparticles were overcoated with purified rHBcAg. DEAE-purified human anti-HBc (Abbott Laboratories) was coupled to alkaline phosphatase by using a heterobifunctional cross-linking reagent (N-hydroxy succinimide ester and maleimide) (19).

In the first step of the IMx assay, a specimen diluent containing DTT is added. Microparticles coated with rHBcAg are then added to the reaction mixture. This mixture is transferred to the glass fiber matrix and anti-HBc-alkaline phosphatase conjugate is added. Anti-HBc, if present in the specimen, blocks the binding of anti-HBc-alkaline phosphatase conjugate. Finally, the substrate, methylumbelliferyl phosphate, is added, and a fluorescent signal is generated. This signal is inversely proportional to the amount of anti-HBc captured by the microparticles from the specimen.

IMx Core assay cutoff. Results are given as fluorescence rate values (counts per second per second) and as specimen rate divided by cutoff rate (S/CO). The assay cutoff is calculated as the calibrator rate divided by 2. The calibrator is a pool of recalified human plasma testing negative for anti-HBc. A specimen is considered reactive for anti-HBc when S/CO is less than or equal to 1.000.

Statistical methods. Intraassay (within-run), interassay (between-run), and total variations were calculated as described by Krouwer and Rabinowitz (7).

Sensitivity panel. Recalified pooled human plasma testing strongly reactive for anti-HBc was diluted into pools of plasma which tested negative for anti-HBc to produce a sensitivity panel containing various anti-HBc concentrations. Concentrations were determined by comparing each panel member to a standard provided by the Paul Ehrlich Institute, Frankfurt, Federal Republic of Germany. Concentration values are reported as PEI units per milliliter. Sensitivity of an assay was determined as the anti-HBc concentration in PEI units per milliliter at the assay cutoff read from a standard curve generated from the sensitivity panel.

RESULTS

Sensitivities. Typical sensitivities for IMx Core, Corab (RIA), and Corzyme (EIA), determined by using a sensitivity panel calibrated against the PEI standard, were 0.4 to 0.5, 0.6 to 0.7, and 1.0 to 1.1 PEI units per ml, respectively.

Sensitivity of IMx Core was further evaluated by testing two-fold serial dilutions of 10 different anti-HBc specimens with widely varying titers of 2 to 8,192. The IMx assay showed sensitivity for each of the 10 specimens two- to fourfold greater than that of the EIA. When these data were plotted as S/CO versus dilution factor (Fig. 1), all 10 specimens gave parallel dilution curves, demonstrating that endpoint titers can give a semiquantitative measure of anti-HBc concentration in a specimen.

Reproducibility. The IMx assay reproducibility was determined by using a panel of five specimens containing various concentrations of anti-HBc. The panel was run in duplicate in 80 separate runs on seven instruments. The within-run, between-run, and total variation ranged from 3.5 to 6.2, 2.3 to 6.2, and 3.7 to 6.8%, respectively.

Specificity. Five clinical sites tested 4,841 specimens in parallel with the IMx Core and IMx 1 (Table 1). The following samples were included in the study: 2,371 serum samples and 541 plasma specimens from volunteer blood donors, 1,201 diagnostic specimens from hospital patients, 102 specimens from obstetrical and gynecological patients, 372 specimens from patients with known diseases other than hepatitis B, 92 HBsAg-positive specimens, and 162 specimens which had previously tested positive for anti-HBc. Overall agreement between the IMx and EIA was 99.1% (4,797 of 4,841).

Histograms comparing the IMx and EIA for 2,371 blood donor serum samples show that the EIA gave a significantly broader population distribution than the IMx (Fig. 2). In this population, the population mean S/CO obtained in the IMx was 5.0 standard deviations from the cutoff, compared with 3.2 standard deviations for the EIA.

This tighter distribution in the IMx Core assay can be attributed to the addition of reducing agent, DTT, to the specimens, since IMx Core run without reducing agent shows a significant broadening of the population around the negative population mean (data not shown). Furthermore, pre-treatment of the specimen with the reducing agent sodium metabisulfite prior to testing by the EIA also significantly shifts the negative population distribution away from the cutoff for these assays (2).

Forty-four specimens gave discordant results between the IMx and EIA, 40 of which were resolved by further testing. Thirty-three of the 44 discordant were classified as reduc-
These 32 reactives were all reduction-sensitive with resolved or unresolved. The positive test was described for other hepatitis A virus (HAV) and herpes simplex virus (HSV) infections. A volunteer blood donor (n = 32) with a positive HBsAg test was run in the presence of reducing agent (Fig. 3). Seven discordants were reactive only in the IMx assay because of improved sensitivity over Corzyme. All of these seven specimens were positive for other hepatitis B markers. One specimen was positive in the EIA and RIA but negative by IMx. This specimen was positive for HBsAg and negative for IgM anti-HBc and was unresolved. The three remaining discordants could not be resolved with follow-up testing either because of insufficient volume for further testing or because no other hepatitis B marker was detectable to indicate previous exposure to HBV.

In populations with low prevalence of anti-HBc, i.e., volunteer blood donors, in which the Corzyme reactive rate was 1.61%, 30% of all reactives resulted from the presence of a reduction-sensitive factor in sera (Table 1). Most of these specimens had low-level Corzyme reactivity with an S/CO between 0.7 and 1.0, and these specimens became unreactive upon addition of reducing agent (Fig. 2). In high-risk populations in which anti-HBc prevalence may range from 9.1 to 94%, a significantly lower percentage (7.6 to 3.9%) of Corzyme reactives is sensitive to reductant (Table 1).

**Effect of reductant on IgG and IgM anti-HBc.** To determine

![Graph](image)

**FIG. 1.** Reactivity of 10 anti-HBc-positive specimens in IMx Core as a function of specimen dilution factor. Twofold serial dilutions of each positive specimen were made into human plasma testing negative for anti-HBc.

### TABLE 1. Comparison of IMx Core and Corzyme

<table>
<thead>
<tr>
<th>Specimen source or type (n)</th>
<th>No. (%) reactive by:</th>
<th>% Agreement</th>
<th>No. of:</th>
<th>% Corzyme reagents sensitive to reductant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IMx Core</td>
<td>Corzyme</td>
<td>Discrants</td>
<td>Reduction-sensitive discordants</td>
</tr>
<tr>
<td>Volunteer blood donor (2,912)</td>
<td>34 (1.2)</td>
<td>47 (1.6)</td>
<td>99.5</td>
<td>15</td>
</tr>
<tr>
<td>Diagnostic and obstetric and gynecological (1,303)</td>
<td>113 (8.7)</td>
<td>119 (9.1)</td>
<td>99.1</td>
<td>12</td>
</tr>
<tr>
<td>Anti-HBc reactive (162)</td>
<td>148 (91.4)</td>
<td>153 (94.4)</td>
<td>95.7</td>
<td>7</td>
</tr>
<tr>
<td>HBsAg positive (92)</td>
<td>88 (95.6)</td>
<td>88 (95.6)</td>
<td>100.0</td>
<td>0</td>
</tr>
<tr>
<td>Patients with diseases other than HBV* (372)</td>
<td>90 (24.2)</td>
<td>90 (24.2)</td>
<td>97.3</td>
<td>10</td>
</tr>
</tbody>
</table>

* Includes dialysis patients (n = 120), hemophiliacs (n = 50), patients with autoimmune disorders (n = 20) or with antibodies against rubella virus (n = 7), hepatitis A virus (n = 49), cytomegalovirus (n = 18), Epstein-Barr virus (n = 8), hepatitis C virus (n = 30), human immunodeficiency virus type 1 (n = 10), or herpes simplex virus (n = 21).
the effect of DTT on IgG and IgM anti-HBc reactivity in the IMx, IgG and IgM from four IgM anti-HBc-positive serum samples were fractionated by gel filtration. Peak fractions of IgG and IgM antibody were diluted into pools of plasma which tested negative for anti-HBc and tested by IMx in the presence and absence of DTT. The reactivity of IgG anti-HBc was not decreased by the addition of DTT; however, 93.7 to 99.2% of IgM anti-HBc reactivity was destroyed when reductant was present. The IMx Core assay with DTT addition was able to detect fractionated IgM anti-HBc at twofold-higher to fourfold-lower dilutions than Corzyme-M.

To determine whether this destruction of IgM anti-HBc reactivity had any clinical relevance, specimens were drawn 1 to 12 weeks after onset of symptoms from 42 patients with acute hepatitis B. All specimens that were reactive or borderline reactive for IgM anti-HBc were tested by IMx Core in the presence of DTT and were all strongly reactive.

To compare the abilities of the IMx and EIA to detect anti-HBc early in HBV infection, weekly serial bleeds from three plasmapheresis donors (1, 2, and 3) who became infected with HBV were tested. For patient 1, IMx Core, Corzyme, and Corzyme-M all became reactive in bleeds containing peak levels of HBsAg at 39 days after first detection of HBsAg. In patient 2, anti-HBc was detected by
IMx and EIA 40 days after HBsAg was first detected. Corzyme-M was negative or borderline reactive at day 40 but was considered reactive at the next bleed date 20 days later. Patient 3 was first detected as HBsAg positive 21 days after entering the study. Twenty-seven days later, he became initially reactive by the IMx and EIA, at which time Corzyme-M was borderline reactive. In all three cases, the detectability of anti-HBc early in infection was not affected by the presence or absence of DTT in the specimen diluent.

DISCUSSION

We demonstrate in these studies that a fully automated, microparticle EIA for the detection of anti-HBc, IMx Core, gives better sensitivity and better agreement with other hepatitis B markers than current methods. Serum and plasma specimens from patients with a variety of infectious diseases, random blood donors and hospital patients with acute and chronic hepatitis B, and individuals having serological components which are potential interfering substances were tested by the IMx Core assay along with the commercial EIA, Corzyme. Overall agreement between IMx Core and Corzyme in clinical studies was 99.1% (4,797 of 4,841) (Table 1).

Most discrepant specimens were low-level reactive by EIA or RIA but negative when tested by IMx. These specimens had no other HBV markers and after pretreatment of the specimen with the reducing agent sodium metabisulfite became negative by EIA and RIA. Recently, Chau et al. (2) and Robertson et al. (11) have shown that anti-HBc reactivity in some low-level specimens is destroyed by treatment with a reducing agent. This reductant-sensitive factor was identified by sodium dodecyl sulfate gels and Western immunoblotting as IgM antibody.

Several investigators have questioned the significance of the low-level anti-HBc reactivity observed in healthy blood donors having no other hepatitis B marker and suggested that current, commercial immunoassays are nonspecific (1, 2, 5). Several lines of evidence indicate that some low-level anti-HBc reactivities are reproducible and have an immunoglobulinlike factor, IgM or IgA, which specifically binds HBcAg (2, 8, 11, 13, 15, 18). The presence of this antibody in sera does not serve as a true marker for previous exposure to HBV or HBV infection and may arise unspecifically (12).

The overall prevalence of Corzyme-reactive individuals is strongly dependent upon the population tested; for example, 1.6% of random volunteer blood donors (47 of 2,912) were Corzyme reactive, compared with 9.1% of individuals tested in diagnostic or hospital settings (119 of 1,303). We found that 30% of the blood donors and 8% of the hospital population testing reactive for anti-HBc by Corzyme may not have been previously exposed to HBV (Table 1). The overall prevalence of unspecific anti-HBc (defined as specimens whose reactivity in Corzyme is destroyed by treatment with reducing agent) was similar among these two groups, suggesting that both low- and high-risk populations may have similar rates of unspecific activation of immature B lymphocytes leading to detectable anti-HBc. We find that the reducing agent DTT, when added to the specimen during the IMx Core assay, significantly decreases the number of these unspecific reactive specimens.

Does the DTT treatment which destroys unspecific IgM anti-HBc significantly affect the measurement of specific anti-HBc arising from exposure to or infection with HBV? Although we demonstrate that the reductant has no effect on IgG anti-HBc reactivity in these assays, reductant destroys most of the reactivity of IgM anti-HBc purified from individuals having high titers of both IgM and IgG anti-HBc. We have been unable to find any Corzyme-M-reactive or borderline-reactive specimens which are nonreactive by the IMx
Core. Furthermore, in serial bleeds from infected individuals, anti-HBc was detectable by IMx Core earlier or in the same bleeds that IgM anti-HBc became detectable by Corzyme-M. Therefore, the amount of specific IgM anti-HBc reactivity that may be destroyed by treatment of specimens with DTT in the IMx assay has little clinical relevance. The addition of reducing agent to specimens in anti-HBc assays, e.g., IMx Core, eliminates unspecific reactions without significantly affecting anti-HBc reactivity resulting from HBV infection.

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REFERENCES