Improvement in the Specificity of Assays for Detection of Antibody to Hepatitis B Core Antigen

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Reducing agents dramatically alter the specificity of competitive assays for antibody to hepatitis B core antigen (anti-HBc). A specificity improvement was demonstrated with a new assay which utilizes microparticle membrane capture and chemiluminescence detection as well as a current radioimmunoassay procedure (Corab; Abbott Laboratories, Abbott Park, Ill.). The effect was most noticeable with elevated negative and weakly reactive samples. In both systems, reductants increased separation of a negative population (n = 160) from assay cutoffs. With a selected population (n = 307), inclusion of reductant eliminated apparent anti-HBc activity in 54 of 81 samples in the 30 to 70% inhibition range. Reductant-stable anti-HBc samples were strongly associated with the presence of antibody to hepatitis B surface antigen (21 of 27). The association persisted below the detection limits of current assays to 0.3 to 0.4 Paul Ehrlich Institute units per ml. Only 1 of 54 reduction-sensitive borderline samples was confirmed to be positive for antibody to hepatitis B surface antigen. The modified procedures had unchanged or slightly improved sensitivity for immunoglobulin G (IgG)-associated anti-HBc activity. Although IgM anti-HBc detection was reduced from four- to eightfold in the presence of reductants, sensitivities remained at least twofold greater than that of an enzyme immunoassay (Corzyme M; Abbott) designed to detect acute-phase levels of IgM anti-HBc. The use of reducing agents should significantly improve the reliability of anti-HBc testing, especially near assay cutoffs.

The core particle of the hepatitis B virus (HBV) is an extremely potent antigen that elicits strong B- and T-cell responses in individuals exposed to the virus (6, 9). After resolution of transient HBV infection, antibody to the core antigen (anti-HBc) usually persists longer than other HBV markers. Anti-HBc is therefore the most universal marker for diagnostic and epidemiologic purposes. In addition, anti-HBc is associated with an elevated risk of transmission of non-A, non-B hepatitis (7, 13) and has been used as a surrogate test to screen blood donors in the United States and France. Despite the recent discovery and development of specific tests for hepatitis C (8), it is anticipated that anti-HBc screening will continue.

Unfortunately, numerous reports have concluded that virtually all commercially available anti-HBc procedures have poor specificity for HBV (1, 4, 5, 10, 12). The problem is most noticeable with low-level anti-HBc-reactive samples which are often from individuals with no history and no other markers of HBV exposure. Detection of anti-HBc reactivity in this range is poorly reproducible and often varies with different testing procedures. For example, a number of investigators (5, 12) have noted discrepancies between the Corzyme (an enzyme immunoassay [EIA]) and Corab (a radioimmunoassay [RIA]) assays, in which the latter procedure is considered more specific.

We have found that mild reducing agents can selectively eliminate false-positive reactivity and greatly improve the specificity of competitive anti-HBc assays (14). In this paper, we present studies with a new assay which uses a latex microparticle solid phase and chemiluminescent conjugate (CLIA) and compare results with those of the Corab (RIA) in the presence and absence of reductant. The ability of the modified procedures to detect both immunoglobulin G (IgG) and IgM anti-HBc activity is assessed.

MATERIALS AND METHODS

Specimens. Reference serum reactive for anti-HBc was obtained from the Paul Ehrlich Institute (PEI; Frankfurt, Federal Republic of Germany) and was used to prepare an IgG anti-HBc panel with activity from 0.28 to 4.68 PEI units per ml. A population of 307 EDTA plasma samples from volunteer donors was obtained from the Oklahoma Blood Institute (Oklahoma City, Okla.). These samples originally tested positive for anti-HBc by using the Corzyme 2-h procedure, but upon retest about 20% were negative. This population (subsequently referred to as the selected population) was very useful in our analysis because it contained a number of specimens with borderline anti-HBc reactivity. Specimens were kept at −20°C for prolonged storage and were kept at 2 to 8°C for the duration of this study. A negative plasma population from 160 volunteer blood donors (Lifesource, Chicago, Ill.) was assayed within 3 days of collection.

Assay methods. (i) Chemiluminescence assay for anti-HBc (CLIA). A detailed description of this procedure and accessory equipment appears elsewhere (16). Briefly, DEAE-purified polyclonal human anti-HBc was coupled to carboxylated polystyrene latex microparticles (Seradyn, Indianapolis, Ind.) by using 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (Sigma Chemical Co., St. Louis, Mo.). Recombinant hepatitis B core antigen was then immunologically adsorbed to the undercoated particles. The same polyclonal human anti-HBc was used to prepare a chemiluminescent conjugate by reaction with an acridinium sulfonamide coupled through a carbodiimide condensation reaction with an activated N-hydroxysuccinimide (Sigma) intermediate (15).

(ii) One-step CLIA. In the absence of reductant, the CLIA assay for anti-HBc was a one-step competitive procedure. The following were added to a microtiter tray: 100 µl of plasma (or serum) sample or control, 50 µl of acridinium anti-HBc conjugate (150 ng/ml), and 50 µl of recombinant hepatitis B core antigen-coated microparticles (typically

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0.02% solids). The tray was covered, and after a 40-min incubation at 40°C, the particles were separated from the reaction mixture by capture on a glass-fiber matrix. The capture membrane vessels were pretreated with 50 μl of 0.01 M sodium phosphate, pH 7.2, containing 0.15 M NaCl and 0.1% peroxide-free Tween 20 (Pierce, Rockford, Ill.) (wash solution). A total of 180 μl of the reaction mixture was transferred to the capture membrane, which was then washed three times with 100 μl (each wash) of wash solution. Bound conjugate was quantitated on a semiautomated chemiluminescence reader (16). Results were expressed as percent inhibition: ([negative control counts – sample counts]/[negative control counts – positive control counts]) × 100. A sample with greater than 50% inhibition was considered reactive.

(iii) Two-step CLIA. For assay of anti-HBc activity in the presence of reductant, the CLIA assay for anti-HBc was performed in two steps utilizing the above reagents. Reductant was most effective when added at the beginning of the assay. The following were added to a microtiter tray: 100 μl of plasma (or serum) sample or control, 50 μl of 0.2 M cysteine (Sigma; prepared fresh in distilled water), and 50 μl of microparticles. The tray was covered and incubated for 20 min at 40°C. The reaction mixture was transferred to a capture membrane pretreated with 50 μl of wash solution, the membrane was washed with 50 μl of the same solution, and 50 μl of acridinium anti-HBc conjugate was added directly to the membrane surface. Capture vessels were covered and incubated for an additional 20 min at 40°C before being washed three times with 100 μl of wash solution. Bound conjugate was determined as described previously (16). A nominal 50% inhibition cutoff was used for this procedure except as noted below.

(iv) Other assay methods. Anti-HBc reactivity was determined by RIA (Corab; Abbott Laboratories, North Chicago, Ill.) according to the manufacturer's instructions. The assay was modified to include reductant by pipetting 10 μl of 0.5 M sodium metabisulfite (Sigma; freshly prepared) into assay wells and then following the standard RIA procedure. Anti-HBc was also determined by ELISA (Corzyme, procedure A; Abbott) and by a reductant-modified ELISA which also utilizes sodium metabisulfite, as described elsewhere (2). IgM anti-HBc and antibody to hepatitis B surface antigen (anti-HBs) were assayed by following kit instructions (Corzyme M and Ausab [RIA], respectively; Abbott).

IgM anti-HBc panel. A strongly positive IgM anti-HBc specimen was used to prepare an IgM-specific panel. Serial dilutions of this specimen in negative control were tested in the one-step CLIA assay and compared with IgG anti-HBc standards. At 50% inhibition, the anti-HBc activity was calculated to be 7,000 PEI units per ml. An aliquot of this plasma (0.25 ml, 1,750 PEI units) was applied to a column of Sephacryl S-300 (2.5 by 45 cm) (Sigma) equilibrated with 0.1 M sodium phosphate, pH 7.2, containing 0.15 M NaCl and 0.1% sodium azide. Fractions (2.5 ml) were collected at a flow rate of 30 ml/h, while absorbance was monitored continuously at 280 nm. Fractions in the IgM region were diluted 1/10 and tested for activity in the one-step CLIA assay. Peak activity fractions (32 through 39) were pooled and passed through a 1-ml column of Protein G-Sepharose 4B (Pharmacia Inc., Piscataway, N.J.) equilibrated in S-300 column buffer. This procedure removed residual IgG anti-HBc which was bound by the column. IgM anti-HBc was recovered in the flowthrough (volume, 21 ml). A 50-ml stirred flow cell (Amicon Corp., Danvers, Mass.) utilizing a YM membrane (molecular weight cutoff, 30,000) under N2 at 50 lb/in² was used to concentrate the protein G pool to a final volume of 3.3 ml. A total of 1,220 PEI units was recovered, representing 70% of the starting activity. An aliquot of the concentrated pool was dilute into recalcified plasma negative for anti-HBc to produce a nine-member IgM-specific panel consisting of twofold serial dilutions having anti-HBc activity from 64 to 0.25 PEI units per ml.

RESULTS

Effect of reductants on anti-HBc population distributions. Figure 1 shows the frequency distribution of a volunteer donor population in assays with and without reductant. All specimens were negative except for a single positive result in the Corab RIA that was negative upon repeat testing. As shown, the presence of reductant resulted in tightened negative population distributions, especially in the two-step CLIA assay, with less tendency to overlap the 50% inhibition cutoff.

The distribution of anti-HBc activities within the selected plasma population (n = 307) is shown in Fig. 2. Qualitatively, the impact of reductant in both assays was to decrease the number of samples within a broad borderline region from approximately 30 to 70% inhibition. There was also a tightened distribution of samples having reactivities near that of the negative control. The change in the CLIA assay distribution was due to the addition of reductant, since changing from a one- to a two-step format had no significant effect on reactivities (data not shown).

When we restricted our analysis to the effect of reductant on 81 samples from the selected population, defined as having reactivities reflecting from 30 to 70% inhibition in either the CLIA one-step or Corab procedures, we found that the addition of reducing agents resolved this single
borderline group into two populations (Fig. 3). There was general agreement between the two assay systems in identifying reductant-sensitive and reductant-stable specimens. Twenty-seven specimens retained anti-HBc reactivities reflecting greater than 30% inhibition in both assay systems in the presence of reductants. Fifty-four samples displayed sensitivity to reducing agents: 51 had reactivities reflecting less than 30% inhibition in both assays, and 3 samples had reactivities reflecting between 30 and 45% inhibition in the CLIA two-step procedure but less than 30% inhibition in the modified RIA.

Association of anti-HBc reactivity with anti-HBs. Serological testing following HBV infection has revealed that antibodies to the core and surface antigens usually occur together (4). Within the selected population analyzed here, anti-HBc reactivity is closely associated with the presence of anti-HBs (Table 1). An association of about 95% was seen with samples having greater than 70% inhibition in the CLIA and RIA anti-HBc assays and was not affected by the presence of reducing agent. Negative anti-HBc samples with reactivities reflecting less than 30% inhibition showed only a 5% association with anti-HBs, again independent of reductant. However, the presence of reductant significantly increased the association of anti-HBs with borderline reactive anti-HBc samples in both the CLIA and RIA systems. When we examined the total of 81 unique samples having reactivity in this range, we found that 21 of 27 reductant-stable anti-HBc samples had detectable anti-HBs, while only 1 of 54 reduction-sensitive samples was anti-HBs positive (Fig. 3).

Detection of IgG and IgM anti-HBc in the presence of reductants. Since reducing agents can alter immunoglobulin structure, it was necessary to determine the effect of reductants on the ability of modified assays to detect true anti-HBc activity. Table 2 summarizes the results of testing a five-member anti-HBc panel constructed from the PEI stan-

dard. Gel filtration and DEAE analysis have confirmed that this standard is exclusively IgG-associated anti-HBc (data not shown). There was no decrease in IgG detection in the presence of reductants. In fact, a slight increase was seen from the CLIA one-step procedure to the two-step procedure. The CLIA two-step and RIA procedures had similar sensitivities of approximately 0.5 PEI units per ml at a nominal 50% inhibition cutoff. This was about twofold greater than that of the Corzyme EIA (with no reductant), shown for comparison.

IgM is well known to be much more sensitive to reduction than IgG. Therefore, a specific panel was constructed to assess IgM anti-HBc detection in the modified assays (Table 3). In addition to the CLIA and RIA systems, this panel was also tested in the Corzyme EIA and the reductant-modified EIA. We found that there was a four- to eightfold loss of detection of IgM-associated anti-HBc activity, depending on the assay system. However, modified-assay sensitivities all remained at least one twofold dilution greater than that of an IgM anti-HBc EIA (Corzyme M).

**DISCUSSION**

Numerous reports have appeared over the last several years assessing the significance of low positive anti-HBc results (1, 4, 5, 10, 12). Evidence presented has been based on clinical and epidemiological arguments (4, 5), observed EIA and RIA discrepancies (1, 5, 12), and the immunological response to hepatitis B core antigen (10). A consensus has been reached that most if not all of the current tests are nonspecific and poorly reproducible, especially near assay cutoffs. When we first observed the dramatic effect of reductants on borderline anti-HBc reactive samples, it ap-
TABLE 1. Association of anti-HBc with anti-HBs in a selected population

<table>
<thead>
<tr>
<th>% Inhibition of anti-HBc</th>
<th>No. positive for anti-HBs/total no. tested (%) detected by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CLIA&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>−RA</td>
</tr>
<tr>
<td>&lt;30</td>
<td>2/62 (3.2)</td>
</tr>
<tr>
<td>&gt;30, &lt;70</td>
<td>23/76 (30.3)</td>
</tr>
<tr>
<td>&gt;70</td>
<td>159/169 (94.1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The CLIA was performed as a one-step assay without reducing agent (−RA) and as a two-step assay with reducing agent (+RA), as described in Materials and Methods.

<sup>b</sup> The RIA was performed with (+RA) and without (−RA) the reducing agent, metabisulfite.

peared very likely that this could be a breakthrough in anti-HBc testing. To substantiate these observations, we used some of the criteria mentioned above to evaluate reduction-sensitive and reduction-stable low-level reactive anti-HBc specimens.

We have shown here that mild reducing agents selectively eliminate apparent low-level anti-HBc reactivity from samples generally having no other markers of HBV infection (Fig. 3). On the other hand, 21 of 27 reductant-stable borderline anti-HBc samples were associated with anti-HBs, presumably reflecting prior exposure to hepatitis B. We have also observed that elimination of very low levels of anti-HBc inhibitory activity in the negative population (Fig. 1) yields a tighter distribution in the CLIA two-step procedure and to a lesser extent in the modified RIA, reducing trailing into the assay cutoffs. Thus, reduction-sensitive anti-HBc activity seems to be quite common at low concentrations, only occasionally occurring at levels high enough to give false-positive results in competitive assays.

The effects of reductant were similar in the competitive assays studied, which have different solid phases and detection systems. Within the selected population, there was excellent agreement in identification of reductant-sensitive and reductant-stable samples (Fig. 3). Although cysteine (final concentration, 50 mM) was optimum for the CLIA system because it has a minimal effect on generation of the chemiluminescent signal (about a 10% signal loss is observed), it was, in fact, virtually interchangeable with sodium metabisulfite (final concentration, 25 mM) in the elimination of reduction-sensitive activity in the CLIA system (data not shown). Other reducing agents, such as 2-mercaptoethanol and dithiothreitol, were also effective (data not shown). Reducant-modified EIA and RIA also have an improved correlation in the borderline reactive region, as discussed elsewhere (2). Thus, in the presence of reducing agents, these competitive anti-HBc systems become more equivalent.

Sällberg and Magnius (10) have demonstrated that the immunoglobulin class can discriminate positive anti-HBc results not caused by HBV exposure. Their data suggest that only IgG-associated anti-HBc activity in discrepant samples reflects a T-cell-specific activation of B lymphocytes that is due to previous exposure to HBV (9). We have shown here that detection of IgG anti-HBc activity is unaffected by the levels of reducing agents employed in the modified assays (Table 2).

From the selected population indicate that samples with very low levels of reductant-stable activity are associated with anti-HBs and presumably are IgG anti-HBc (4, 10). This suggests that the sensitivity of anti-HBc assays may need to be increased (0.3 to 0.4 PEI units per ml) in order to detect true low-level reactive samples. On the basis of the limited data presented here, the required sensitivity could be achieved in the CLIA two-step assay by lowering the cutoff to about 30% inhibition. This appears to be feasible given the tightened negative population distribution of the modified assay: the 30% cutoff is 4.8 standard deviations from the negative mean in the CLIA two-step assay compared with 4.0 standard deviations from a 50% cutoff in the one-step assay. By the same argument, the data suggest that it would be possible to lower the modified RIA cutoff to 40% inhibition to achieve the required sensitivity without sacrificing the specificity improvement; however, more studies are needed to establish optimum cutoffs.

Detection of IgM anti-HBc is useful in differentiating recent or current HBV infections from remote infections (3). Since reducing agents are known to affect IgM, the loss of IgM sensitivity in the modified assays was one potential disadvantage of this approach. Since samples containing exclusively IgM occur only rarely, if at all (11), an artificial panel was created in order to test specific IgM detection. Although there was a significant decrease in the detection of IgM anti-HBc (Table 3), modified assays were at least one twofold dilution more sensitive than Corzyme M (3). By matching or slightly exceeding Corzyme M sensitivity, we have shown that the modified assays still detect a total immunoglobulin response to HBV, except that IgM anti-HBc sensitivity has been reset, in essence, to a more appropriate level.

In conclusion, we have shown that the specificity of competitive assays for anti-HBc is significantly improved by addition of mild reducing agents. The data strongly suggest that modified anti-HBc procedures can correctly identify individuals with prior exposure to HBV, even near assay cutoffs.
TABLE 3. Sensitivity of anti-HBc assays as evaluated with a specific IgM panel

<table>
<thead>
<tr>
<th>Panel member (PEI units/ml)</th>
<th>Result by Corzyme M&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Result and % inhibition by:</th>
<th>CLIA</th>
<th>RIA</th>
<th>EIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−RA</td>
<td>+RA</td>
<td>−RA</td>
<td>+RA</td>
</tr>
<tr>
<td>1 (64)</td>
<td>+ (1.56)</td>
<td>+ (90.6)</td>
<td>+ (76.3)</td>
<td>(75.2)</td>
<td>+ (64.1)</td>
</tr>
<tr>
<td>2 (32)</td>
<td>− (0.93)</td>
<td>+ (86.6)</td>
<td>+ (73.0)</td>
<td>(72.9)</td>
<td>+ (55.4)</td>
</tr>
<tr>
<td>3 (16)</td>
<td>− (0.61)</td>
<td>+ (84.0)</td>
<td>+ (59.1)</td>
<td>(67.0)</td>
<td>− (46.8)</td>
</tr>
<tr>
<td>4 (8)</td>
<td>− (0.41)</td>
<td>+ (75.3)</td>
<td>− (47.2)</td>
<td>(54.5)</td>
<td>− (42.1)</td>
</tr>
<tr>
<td>5 (4)</td>
<td>− (0.32)</td>
<td>+ (70.3)</td>
<td>− (40.8)</td>
<td>(45.5)</td>
<td>− (29.2)</td>
</tr>
<tr>
<td>6 (2)</td>
<td>NT</td>
<td>+ (60.3)</td>
<td>− (37.2)</td>
<td>(34.3)</td>
<td>− (20.0)</td>
</tr>
<tr>
<td>7 (1)</td>
<td>NT</td>
<td>− (48.8)</td>
<td>− (21.5)</td>
<td>(17.2)</td>
<td>− (12.9)</td>
</tr>
<tr>
<td>8 (0.5)</td>
<td>NT</td>
<td>− (39.7)</td>
<td>− (12.2)</td>
<td>(6.3)</td>
<td>− (4.7)</td>
</tr>
<tr>
<td>9 (0.25)</td>
<td>NT</td>
<td>− (26.2)</td>
<td>− (2.8)</td>
<td>(4.4)</td>
<td>− (0.4)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results with each procedure listed as + or − on the basis of the cutoff for each assay as described in Materials and Methods. RA, Reducing agent.

<sup>b</sup> Numbers in parentheses are the sample-to-cutoff ratios. NT, Not tested.

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REFERENCES


