Characterization of a Reduction-Sensitive Factor from Human Plasma Responsible for Apparent False Activity in Competitive Assays for Antibody to Hepatitis B Core Antigen

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Addition of reducing agents to competitive assays for antibody to hepatitis B core antigen (anti-HBc) eliminates apparent false reactivity of specimens obtained from individuals with no prior history of hepatitis B virus (HBV) infection and without other serological markers of HBV infection. We have purified and characterized a reduction-sensitive factor (RSF) isolated from the plasma of several volunteer blood donors. Column fractions were assayed for anti-HBc by using a highly sensitive chemiluminescence assay with a detection of 0.15 Paul Ehrlich Institut units per ml at 50% inhibition. Gel filtration on Sephacryl S-300 indicated that reductant-sensitive samples possessed anti-HBc activity that was associated with immunoglobulin M (IgM), whereas reductant-stable activity was associated with IgG. Gel filtration followed by metal chelate affinity chromatography resulted in a 55-fold purification and demonstrated that RSF activity copurifies with IgM. RSF was recovered from a recombinant hepatitis B core antigen matrix and shown to be an IgM species by immunoblot. In addition, RSF activity coeluted with IgM protein from anti-μ-chain Sepharose. Discrepancies between enzyme immunoassay and radioimmunoassay procedures for anti-HBc (Corzyme and Corab, respectively; Abbott Laboratories, North Chicago, Ill.) appear to be due to the relative sensitivity of the enzyme immunoassay for IgM anti-HBc (sevenfold greater than the radioimmunoassay using a specific panel). The biological basis for the occurrence of low levels of nonspecific IgM anti-HBc reactivity in individuals not previously exposed to HBV remains to be elucidated.

The core particle of hepatitis B virus (HBV) is a potent immunogen that elicits a strong antibody response in individuals exposed to HBV. Detection of antibodies directed against the core antigen (anti-HBc) is useful in the diagnosis of recent or past HBV infection (7). The anti-HBc assay has also been used as a surrogate test to identify blood donors likely to have been exposed to non-A, non-B hepatitis (hepatitis C) (9, 16). When large-scale anti-HBc testing in blood banks was initiated in 1986, it was found that some otherwise acceptable blood donors who tested positive for anti-HBc had no history of exposure to HBV. Since then, evidence based on clinical and epidemiological findings (4, 5), enzyme immunoassay (EIA) and radioimmunoassay (RIA) discrepancies (1, 14), and immunological analysis (13) has suggested that most, if not all, current assays for anti-HBc have a significant proportion of results which are nonspecific. The problem is especially apparent in populations at low risk for HBV, such as volunteer blood donors.

Recently, it has been shown that treatment of plasma samples with mild reducing agents dramatically improves the specificity of competitive anti-HBc assays (2, 18). Here we report the isolation and partial characterization of a reduction-sensitive factor (RSF) responsible for nonspecific anti-HBc assay results. In light of our findings, the basis of discrepancies between the EIA and RIA is discussed.

MATERIALS AND METHODS

Specimens. Studies were performed by using 80 weakly reactive anti-HBc specimens obtained from volunteer donors at the Sacramento Medical Foundation Center for Blood Research, Sacramento, Calif., drawn from November 1987 to November 1988. All individuals satisfied the criteria of the American Association of Blood Banks and Food and Drug Administration qualified blood donors and provided 450 ml of whole blood. Specifically, none had a clinical history of viral hepatitis or potential exposure within the prior 6 months to individuals with viral hepatitis, including HBV. Donors with a repeatedly reactive anti-HBc test were deferred. Samples for this study were selected on the basis of initial sample-to-cutoff ratios from 0.45 to 1.2 (corresponding to 50 to 85% inhibition) by using the Corzyme 2-h procedure. The majority of these specimens were only reactive in the anti-HBc EIA, i.e., nonreactive for hepatitis B surface antigen (HBsAg) and antibody to HBsAg (anti-HBs). Some samples with low-level anti-HBc reactivity were also positive for anti-HBs. Plasma units separated from erythrocytes were stored at −20°C for up to 3 months before being thawed and refrozen in aliquots (10 ml). Once thawed, aliquots were kept at 2 to 8°C for subsequent analyses. A second plasma unit was obtained from one blood donor, a 17-year-old female with no subsequent evidence of HBV infection, for additional characterization studies.

A reference serum from the Paul Ehrlich Institute (PEI; Frankfurt, Federal Republic of Germany) was used to construct an eight-member immunoglobulin G (IgG) anti-HBc sensitivity panel with activity from 0.035 to 4.68 PEI units per ml. IgM-associated anti-HBc was purified from a strongly positive specimen (7,000 PEI units per ml) by gel

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filtration and protein G chromatography as described previously (18) and used to construct an IgM-specific panel.

**Assay methods.** (i) **High-sensitivity anti-HBc CLIA.** A high-sensitivity chemiluminescence microparticle immunoassay (HS-CLIA) was developed to allow detection of anti-HBc in diluted column fractions. Reagents and instrumentation for this procedure were essentially as described in detail elsewhere (20), except that a monoclonal anti-HBc conjugate was employed.

Mouse monoclonal anti-HBc, produced in an airlift fermentor, was purified by ammonium sulfate precipitation and hydroxyapatite chromatography. Purified IgG was reacted with a 15-fold molar excess of β-alanine acridinium sulfonamide [10-methyl-N-tosyl-N-(2-carboxyethyl)-9-acridinium carboxamide] activated with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide and N-hydroxysuccinimide (Sigma Chemical Co., St. Louis, Mo.) (19). Conjugate was purified by gel filtration on a TSK 250 column (BioRad Laboratories, Richmond, Calif.), and fractions containing the IgG peak were pooled and stored at 2 to 8°C in the dark, even when prepared at the relatively high specific activity (6 mol of acridinium per mol of IgG) required for the HS-CLIA procedure. Conjugate was diluted into 0.01 M sodium phosphate (pH 6.3) containing 1.5% EDTA, 0.1% sodium azide, 0.5% Tween 20, 50% normal calf serum, and 2% normal human serum before each assay. For the HS-CLIA procedure, column fractions (100 µl) were added to 100 µl of negative control plasma and incubated in a covered microfuge tray with 50 µl of latex microparticles (0.02% solids; Seradyn, Indianapolis, Ind.) coated with recombinant hepatitis B core antigen (HBcAg) in the presence or absence of 20 µl of 0.675 M cysteine (prepared fresh). Corzyme (Abbott) controls and panel members, diluted in the appropriate column buffer, were run at least duplicate. After 2 h at 40°C, the reaction mixture was transferred to a glass fiber matrix prewet with 50 µl of 0.01 M sodium phosphate, pH 6.3, containing 0.15 M NaCl, 0.1% Tween 20, and 0.1% azide. Membranes were washed with 50 µl of the same solution, and 20 µl of monoclonal anti-HBc acidium conjugate (100 ng/ml) was added directly to the surface. The capture vessels were covered and incubated for 10 min at room temperature before being washed three times with 100 µl of the same solution. Bound conjugate was quantified on a semiautomated chemiluminescence reader (20).

(ii) **Other assay methods.** A standard chemiluminescence assay (CLIA) for anti-HBc in plasma samples was performed as described previously in the absence (one-step assay) (20) or presence (two-step assay) (18) of a reducing agent (cysteine; final concentration, 50 mM). Anti-HBc reactivity was also determined by RIA (Corab; Abbott) with and without reducing agent (sodium metabisulfite; final concentration, 25 mM) (18) and by EIA (no reductant) (Corzyme; Abbott). IgM anti-HBc, HBsAg, and anti-HBs were assayed according to kit instructions (Corzyme M, Auszyme, and Ausab [RIA], respectively; Abbott).

**Analytical methods.** (i) **Gel filtration.** Gel filtration was performed on columns of Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with phosphate-buffered saline (PBS) (pH 7.2) containing 0.1% sodium azide. Plasma samples were filtered through 0.2-µm membranes, applied to the top of the columns, and eluted with the equilibration buffer.

(ii) **Metal chelate affinity chromatography.** Metal chelate affinity chromatography was performed on a column (2.5 by 10 cm) of iminodiacetic acid-coupled, epoxycarbonyl activated Sepharose 6B (Pharmacia) (10). The column was activated with ZnCl₂, washed with 0.05 M sodium acetate (pH 5.0) containing 0.5 M NaCl (buffer A), and finally equilibrated in 0.02 M sodium phosphate (pH 6.0) containing 0.15 M NaCl (buffer B) prior to sample application. Sequential elution was with buffer B, buffer A, and 0.05 M EDTA (pH 7.0) containing 0.15 M NaCl.

(iii) **Anti-μ affinity chromatography.** Affinity-isolated goat IgG anti-human IgM-μ chain (Sigma) was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to manufacturer's specifications at a ratio of 5 mg of IgG per ml of swollen gel. Filtered plasma was diluted in an equal volume of 0.1 M Tris-HCl (pH 7.5) containing 0.15 M NaCl (TBS) and mixed with the affinity gel overnight at 2 to 8°C. After washing with TBS and TBS containing 2 M NaCl, bound proteins were eluted with 4 M urea in TBS. After a TBS wash, a second elution was performed with 0.5 M glycine hydrochloride, pH 2.3. Fractions were neutralized with tris-buffered sodium phosphate.

(iv) **Affinity chromatography.** Recombinant HBcAg (1.5 mg) was immobilized onto a Nalgene affinity chromatography membrane (cat. no. 750-2012; Nalco Co., Rochester, N.Y.) according to manufacturer's instructions and equilibrated with PBS (pH 7.2) containing 0.1% sodium azide. Plasma samples were recirculated for 3 h through the membrane to promote maximum binding. The cartridge was washed with equilibration buffer, and bound proteins were eluted with 1.0 M glycerine hydrochloride, pH 2.3. Fractions were neutralized with tris-buffered sodium phosphate.

(v) **Assay of column fractions.** Column fractions were assayed for anti-HBc activity by using the HS-CLIA, as described above. Results were expressed as percent inhibition or as PEI units per milliliter. For quantitation, fractions were assayed at serial dilutions and compared with the linear portion of the IgG panel standard curve between 20 and 80% inhibition (Fig. 1). Dilutions of fractions giving a nonparallel dose-response were quantitated at 50% inhibition. Undiluted fractions having less than 20% inhibition in the HS-CLIA assay were considered to have zero anti-HBc activity.
TABLE 1. Anti-HBc testing on selected, borderline-reactive blood donor samples

<table>
<thead>
<tr>
<th>Assay*</th>
<th>Mean % inhibition (SD)*</th>
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<tbody>
<tr>
<td>Corzyme</td>
<td></td>
</tr>
<tr>
<td>Initial</td>
<td>68.40 (7.70)</td>
</tr>
<tr>
<td>Repeat</td>
<td>55.92 (10.28)</td>
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<tr>
<td>Corab</td>
<td></td>
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<tr>
<td></td>
<td>40.68 (14.88)</td>
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<tr>
<td>Corab + RA</td>
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<tr>
<td></td>
<td>9.85 (22.95)</td>
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<tr>
<td>CLIA</td>
<td></td>
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<tr>
<td></td>
<td>47.24 (16.36)</td>
</tr>
<tr>
<td>CLIA + RA</td>
<td></td>
</tr>
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<td>18.79 (21.83)</td>
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</tbody>
</table>

* RA, Reducing agent. n = 80 in all assays.  
* Percent inhibition = [(negative control - sample)/(negative control - positive control)] x 100.

Protein was assayed by A280 (where 1 absorbance unit = 1 mg/ml).

(vi) Detection of IgM protein. Column fractions were analyzed for protein purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the procedure of Laemmli (11). The presence of IgM was confirmed by Western immunoblot, by the general procedure of Towbin et al. (17), utilizing affinity-purified goat anti-human \( \mu \) chain followed by alkaline phosphatase-labeled rabbit anti-goat IgG.

**RESULTS**

**Anti-HBc HS-CLIA.** By using a reference panel, we found that the HS-CLIA detected approximately 0.15 PEI units per ml at 50% inhibition, compared with 0.55 PEI units per ml for the standard CLIA two-step procedure. At 20% inhibition, the limit for quantitative analysis, the sensitivity was less than 0.05 PEI units per ml. The increase in sensitivity was due both to an extended first incubation (120 versus 20 min) and to a decreased conjugate concentration (2.0 versus 7.5 ng per ml). The increased specific activity of the acridinium monoclonal anti-HBc conjugate resulted in acceptable assay performance (negative-to-positive ratio > 5) at the lower conjugate concentration.

**Anti-HBc results on plasma samples from volunteer blood donors.** Eighty plasma units obtained from volunteer blood donors were evaluated in this study. Seventy-five were initially reactive by EIA testing (Corzyme 2-h procedure), with sample-to-cutoff ratios from 0.46 to 0.99, while five others were close to the cutoff but considered negative for anti-HBC (sample-to-cutoff ratios from 1.12 to 1.20). After a maximum of two freeze-thaw cycles, aliquots were retested in several anti-HBc assays. The results, summarized in Table 1, are expressed in terms of percent inhibition, which facilitates comparisons between the assays. Results from Corzyme repeat testing indicated that most samples had lower anti-HBc activity than seen initially, an average of more than 10% inhibition. This apparent loss of some anti-HBc reactivity from individual samples varied from no loss in a few cases up to a 30% inhibition loss in others. Samples with lower initial activity tended to be less stable. Testing by RIA yielded on average significantly lower percent inhibition, while the CLIA one-step procedure gave results intermediate between the RIA and EIA.

In the presence of reductants, there was a sharp drop in the anti-HBc reactivity of most, but not all, of the samples in both the RIA and CLIA systems. The shift in the population distributions is illustrated in Fig. 1. Samples that retained at least 30% inhibition in the presence of reducing agents in either assay system were frequently found to be positive for anti-HBs (solid portion of each bar). For the CLIA two-step assay, 12 of 24 (50%) reductant-stable samples were anti-HBs positive. In the reductant-modified RIA procedure an even higher percentage (11 of 15; 73%) was anti-HBs positive. With one exception, in the case of the modified RIA, reductant-sensitive samples displaying less than 30% inhibition were anti-HBs negative.

**Gel filtration of reductant-sensitive and reductant-stable anti-HBc specimens.** Plasma samples displaying reductant-sensitive and reductant-stable anti-HBc activities were subjected to gel filtration on Sephacryl S-300. Fractions were tested for protein by absorbance and for anti-HBc activity in the HS-CLIA. Typical results are shown in Fig. 2. The reductant-sensitive sample displayed a single activity peak that was eluted near the void volume of the column. As expected, no anti-HBc activity was detectable in the presence of 50 mM cysteine. Note that this plasma sample was...
more reactive by the EIA than by either the CLIA or the RIA without reductant. In contrast, a low-level reductant-stable sample showed an activity peak centered on the IgG protein fractions that was especially prominent in the presence of reductant. Detection of this sample, which was also anti-HBs positive, was similarly increased in plasma assays in the presence of reductants. Fluctuations of up to 20% inhibition in the IgM region were seen in the HS-CLIA in the absence of reductant (Fig. 2B). Fractions of an anti-HBc-negative plasma pool also showed similar baseline variation in the IgM region (data not shown).

Two-step purification of the RSF. In order to further characterize the nature of the RSF, an additional plasma unit was obtained from one of the blood donors (donor 2) 8 months after her original donation. The second plasma remained reactive in anti-HBc assays without reductants, but at a somewhat decreased level: 56.1 versus 64.6% inhibition in the CLIA one-step procedure.

A 10-ml aliquot of the second plasma unit was applied to a column (5 by 50 cm) of Sephacryl S-300. As shown previously with another reduction-sensitive sample (Fig. 2A), the anti-HBc activity emerged near the void volume. SDS-PAGE and Western immunoblot analyses showed that the RSF activity overlapped with two major plasma proteins: IgM and α2-macroglobulin (data not shown). In order to resolve these two proteins, pooled S-300 fractions were concentrated and applied to a column of iminodiacetic acid charged with ZnCl2 and sequentially eluted (Fig. 3). RSF activity was unbound and coeluted with IgM protein. α2-macroglobulin remained bound and was subsequently eluted at pH 5.0. IgM purity was greater than 90%, as judged by SDS-PAGE. Overall, the procedure achieved a 55-fold purification with 43% recovery of RSF activity (Table 2).

Efforts to isolate a subspecies of IgM with RSF activity from the metal chelate pool were not successful because of the instability of purified material.

Anti-μ affinity chromatography. Results of anti-μ affinity chromatography are shown in Fig. 4. A 2-ml aliquot of plasma from donor 2 (first donation) containing 5.8 PEI units was applied to the column. No anti-HBc activity or IgM protein was detected in the passthrough fractions. After washes, activity was eluted in two steps. Because of pooling of fractions prior to assay, activity was determined for the total yield from each elution. IgM protein was confirmed by SDS-PAGE and Western immunoblotting. Recovery from the combined peaks was 18% of the starting activity, with a specific activity of 0.75 PEI units per mg. The low specific activity may have been due to the harsh elution conditions utilized.

Affinity chromatography. A 3-ml aliquot of plasma from donor 2 (5.3 PEI units), filtered through a 0.2-μm membrane, was recirculated, washed, and eluted as described in Materials and Methods (Fig. 5). RSF activity was quantitatively bound by the membrane, whereas the majority of IgM protein, as judged by Western blot, was present in the passthrough fraction. Recovery of reduction-sensitive anti-HBc activity was only 13%, again probably because of elution conditions, but the purified material had the highest specific activity seen with any procedure utilized here, 2.9 PEI units per mg of protein, a 108-fold increase.

Relative sensitivity of Corzyme and Corab for IgM anti-HBc. A specific IgM anti-HBc panel was prepared from a strong positive sample, as described in Materials and Methods. The nine panel members were twofold serial dilutions of the purified IgM, ranging from 64 to 0.25 PEI units per ml. The panel was tested by using the EIA (Corzyme) and RIA (Corab) procedures, and results are displayed in Fig. 6. This analysis indicates that at the assay cutoffs the EIA is about
It has previously been observed that reducing agents dramatically improve the specificity of anti-HBc assays (2, 18). By using a selected volunteer blood donor population (n = 307), Weare et al. (18) found that only reductant-stable activity was associated with anti-HBs. Low-level reactivity persisted below the detection limit of current assays to 0.3 to 0.4 PEI units per ml. Our results with the 80 blood donor samples in this study are completely consistent with the earlier report: anti-HBs was again closely associated with reductant-stable activity down to 30% inhibition in either the RIA or CLIA system (Fig. 1). Very similar results on two different populations were thus obtained with assays using different solid phases, conjugates, and reductants.

Given the known lability of IgM in the presence of reductants, we anticipated the nature of RSF from the earliest observations. Characterization of reduction-sensitive activity by several methods has demonstrated that it is indeed an IgM anti-HBc-reactive species. This conclusion is supported by the observation (data not shown) that these low-level anti-HBc-reactive species were generally neutralizable by HBCAg (15). In addition to the methods shown here, we have also found that RSF activity and IgM protein coelute in several additional chromatographic techniques, including DEAE ion-exchange, proteamine agarose, lectin affinity chromatography, and hydrophobic interaction chromatography. Although the most detailed characterization of RSF was performed with plasma from only one healthy blood donor, many samples have been partially characterized and displayed similar characteristics. We have subjected an additional 12 samples to S-300 chromatography, and more than 50 samples have been analyzed in the same manner by Ed Chun (3). It appears that low-level-IgM anti-HBc is responsible for the vast majority of false-positive anti-HBc assay results. Since RSF activity is found in individuals who have not been exposed to HBV, it is by definition nonspecific, even though RSF has characteristics of IgM anti-HBc.

Gel filtration on Sephacryl S-300 has shown that reductant-stable anti-HBc is associated with the IgG fraction. We have also used DEAE chromatography (2) to confirm that the stable specimens contain IgG anti-HBc (data not shown). On the basis of its association with anti-HBs, even low-level IgG-positive anti-HBc should be indicative of prior HBV exposure. More specific anti-HBc assays will need increased sensitivity to detect all true reactivates. This is illustrated by the reductant-stable borderline sample shown in Fig. 2B. In this case, a true-positive sample apparently of mixed immunoglobulin type falls below the cutoff of either the RIA or CLIA. Its detection by the EIA presumably is because of greater sensitivity for concomitant IgM anti-HBc (see below).

Efforts to obtain a preparation of the nonspecific IgM anti-HBc in high yield with maximum specific activity were unsuccessful because of the extreme lability of even partially purified RSF. For example, gel filtration of sample 9 (Fig. 2A) on Sepharose 6B resulted in complete inactivation of RSF activity, even in the absence of reductant (data not shown). Since this was not generally true of other reduction-sensitive samples, RSF may be heterogeneous. The basis for RSF inactivation in the absence of reductant is not known. However, the general instability of RSF probably accounts for the long-standing observation (4, 5, 14) that low-level anti-HBc reactivity in the Corzyme EIA is poorly reproducible. We have found that IgG-associated, reductant-stable activity is much more reproducible in the modified anti-HBc assays even at low levels.

FIG. 5. Purification of RSF by affinity chromatography. A 3-ml aliquot of plasma from blood donor 2 was recirculated, washed, and eluted as described in Materials and Methods. Total protein (■), IgM protein, and anti-HBc activity (no reductant) (+) were determined as described in the text.

FIG. 6. Relative sensitivity of EIA (Corzyme) and RIA (Corab) procedures for IgM anti-HBc as determined with a specific panel. Panel members were twofold serial dilutions of purified IgM from 64 to 0.25 PEI units per ml. Detection limits at assay cutoffs of 50% (RIA) and 60% (EIA) inhibition are indicated. See text for details.

FIG. 2B. A factor responsible for anti-HBc false-positives...
Previous studies have generally concluded that the anti-HBc RIA is more specific than the EIA (1, 5, 14). In order to understand the basis for these observations in light of our characterization studies, we compared the relative sensitivity of the EIA with that of the RIA by using a panel constructed from purified IgM anti-HBc. Surprisingly, we found that the EIA was about seven times more sensitive for IgM anti-HBc at its cutoff than was the RIA (Fig. 6), whereas the latter procedure is about twice as sensitive for IgG anti-HBc (data not shown). This result, together with the IgM nature of nonspecific anti-HBc activity shown here, strongly suggests that the primary basis for discrepancies between EIA and RIA is the relative sensitivity of the EIA for detection of IgM anti-HBc. Binding studies (data not shown) have suggested that because of the size of the EIA conjugate, it may be sterically restricted to the outer portion of the solid phase surface, resulting in more direct competition with the large IgM molecule.

Hoefnagle et al. (6) have pointed out that when very sensitive assays are used, naturally occurring IgM antibodies directed against HBsAg can be detected in many animal species. In the present case, detection of nonspecific anti-HBc activity, especially by the EIA, is apparently the result of this same type of immune response rather than a technical failure of the assay system. Indeed, there is evidence that this nonspecific phenomenon may extend to the assay of other immunoglobulins, e.g., anti-HBs (8), imposing a fundamental limitation on this type of serological test.

In conclusion, we have determined that the RSF responsible for apparent false anti-HBc activity is a nonspecific IgM anti-HBc species. Isolated low-level reagent-sensitive reactivity appears unrelated to past or present HBV infection. Anti-HBc assays modified by inclusion of a reducing agent increase their relative specificity for IgG anti-HBc, which is indicative of previous exposure to HBV.

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