A New Solid Phase Passive Hemagglutination Test for IgM Antibody to Hepatitis B Core Antigen

BENCHA PETCHCLAI, M.D., KALAYANEE KHUPULSUP, M.Sc., AND SUCHA KURATHONG, M.D.

There is a need for a simple, sensitive, specific, and inexpensive test for immunoglobulin M antibody to hepatitis B core antigen (anti-HBc IgM). A solid phase passive hemagglutination test (SP-PHA) was developed for this purpose and compared with the enzyme-linked immunosorbent assay (ELISA) test. Hepatitis B core antigen (HBcAg) used in PHA and SP-PHA was synthesized in Escherichia coli. Human IgM was captured to a microtiter plate coated with anti-human IgM, and the presence of anti-HBc IgM was demonstrated by the adherence of HBcAg-sensitized erythrocytes to the bottom of a U-shaped microtiter plate. ELISA and SP-PHA were made at 1:100 and 1:1,000 serum dilution, respectively. Both were positive in 100% of 36 cases of acute hepatitis B, 68.18% of 22 cases of chronic hepatitis B, and 20% of 75 healthy carriers of hepatitis B surface antigen (HBsAg) but none in 65 anti-HBc-positive blood donors that had negative results for HBsAg. Results of both tests were identical but were false positive because rheumatoid factor was found only in ELISA. End-point titration by SP-PHA and PHA was also found useful for the differentiation of acute hepatitis B from chronic hepatitis B and HBsAg carriers. (Key words: Hepatitis B; Anti-HBc IgM; Passive hemagglutination) Am J Clin Pathol 1987; 87: 267-270

IMMUNOGLOBULIN M antibody to hepatitis B core antigen (anti-HBc IgM) is present in almost all patients with acute hepatitis B and considered the most sensitive and specific marker for the serodiagnosis of acute hepatitis B. However, this diagnostic potential is hampered by the presence of anti-HBc IgM, at lower titers and lower percentages, in chronic hepatitis B, as well as carriers of hepatitis B surface antigen (HBsAg). Differentiation of acute hepatitis B from both chronic hepatitis B and HBsAg carriers can be improved by end-point titration of anti-HBc IgM\(^5,8\) and can establish a cut-off titer differentiating acute hepatitis B from the others. The end-point titration has been made by the two methods: enzyme-linked immunosorbent assay (ELISA)\(^4-6\) and radioimmunoassay (RIA),\(^2,9,10\) but both are technically complicated, interfered with by rheumatoid factor,\(^2,4,5,8\) and available only at the reference laboratories. A recently introduced ELISA kit claimed excellent results,\(^6\) but it needs additional evaluation in areas hyperendemic for hepatitis B virus (HBV) infection. We developed and evaluated, in the hyperendemic area of HBV, a simple test for anti-HBc IgM that is highly suitable for end-point titration.

Materials and Methods

Sera

Sera used in the evaluation were obtained from 75 HBsAg-positive blood donors, 65 HBsAg-negative (anti-HBc-positive) blood donors, 36 patients with acute hepatitis B, 22 HBsAg-positive chronic hepatitis B patients, and 10 sera positive for rheumatoid factor by Rose-Waaler test) that were negative for HBsAg. HBsAg was made by a reversed passive hemagglutination test.

Sheep Erythrocytes (SRBCs) Sensitized with HBcAg

SRBCs sensitized with HBcAg used in the following passive hemagglutination (PHA) and solid phase PHA (SP-PHA) were prepared in our laboratory according to the described technic.\(^7\) Corecell\(^5\) (Green Cross Corporation, Inc., Osaka, Japan), a commercially available HBcAg-sensitized SRBC, was used in the initial development of SP-PHA. Both Corecell and our sensitized SRBC's used HBcAg synthesized in Escherichia coli by recombinant DNA technic and supplied by Biogen S.A. of Switzerland. Biogen S.A. donated HBcAg for the present study. Corecell and our sensitized SRBCs were similar in sensitivity and specificity in the test for anti-HBc.\(^7\)

Preparation of Control SRBCs

SRBCs were collected in Alsever's solution, washed three times in normal saline, and packed. To 0.1 mL of packed SRBCs, 1.2 mL of 0.15 mol/L phosphate-buffered saline, pH 7.2 (0.15 mol/L PBS, pH 7.2), and 0.25 mL of 2.5% glutaraldehyde in distilled water were added. The mixture was rotated at room temperature for two hours; washed three times in 0.15 mol/L PBS, pH 7.2; and suspended as a 0.5% SRBC suspension in 0.15 mol/L PBS, pH 7.2, containing 0.5% BSA and 0.1% sodium azide.

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Table 1. Comparison of SP-PHA and ELISA Tests for Anti-HBc IgM in Various Hepatitis B and Control Groups

<table>
<thead>
<tr>
<th>Study Group</th>
<th>PHA No. Positive/ Total No.</th>
<th>ELISA No. Positive/ Total No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg healthy carriers</td>
<td>15/75 (20%)</td>
<td>15/75 (20%)</td>
</tr>
<tr>
<td>HBsAg-negative anti-HBc-positive persons</td>
<td>0/65 (0%)</td>
<td>0/65 (0%)</td>
</tr>
<tr>
<td>HBsAg-positive acute hepatitis B</td>
<td>36/36 (100%)</td>
<td>36/36 (100%)</td>
</tr>
<tr>
<td>HBsAg-positive chronic hepatitis</td>
<td>15/22 (68.18%)</td>
<td>15/22 (68.18%)</td>
</tr>
<tr>
<td>Rheumatoid factor-positive sera</td>
<td>0/10 (0%)</td>
<td>10/10 (100%)</td>
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</table>

PHA Test for Anti-HBc

Anti-HBc (IgG and IgM) was tested at 1:32 and 1:64 serum dilution by a PHA technic according to the method already described and a two-plus hemagglutination of HBcAg-sensitized SRBCs at a minimum titer of 1:32 without hemagglutination of the control SRBCs at 1:16 serum dilution was considered positive for anti-HBc.

ELISA Test for Anti-HBc IgM

The technic followed the methods described. The following four incubations, in microtiter plates (Immulon II, Dynatech, Alexandria, VA), each at 37 °C for two hours, were in this order: 0.1 mL of rabbit antihuman IgM (Dako, Copenhagen, Denmark) diluted 1:800 in 0.05 mol/L carbonate buffer, pH 9.6; 0.1 mL of test serum diluted at 1:100 in diluent; 0.05 mL of crude HBcAg in diluent; 0.05 mL of human anti-HBc-peroxidase conjugate in diluent containing 20% normal human serum (negative for all HBV markers). Each incubation was followed by three washings with diluent, which was 0.15 mol/L phosphate-buffered saline, pH 7.4, with 0.05% Tween 20 and 0.5% bovine serum albumin.

After the last washing, peroxidase substrate, ortho-phenylenediamine (Sigma Chemical Company, St. Louis, MO) (1 mg/mL) and H₂O₂ (1 μL/mL) in 0.1 mol/L citrate phosphate buffer, pH 5, was added, 0.1 mL per well, and incubated in the dark at room temperature for 30 minutes, and the reaction was stopped with 0.025 mL of 4 mol/L H₂SO₄. The optical density at 490 nm was measured by an ELISA reader (Micro ELISA Reader, Dynatech). All tests were done in duplicate, with positive and negative controls included in each run. An OD 2.1 times over the negative controls was considered positive.

HBCAg was prepared from a liver of a gibbon with chronic hepatitis B. Liver was homogenized and the tissue fragments removed by low-speed centrifugation; then HBcAg-containing supernatant was used in the ELISA test.

IgG fraction of a human serum with an anti-HBc titer of 1:20,000 by ELISA (Corzyme, Abbott Laboratories, North Chicago, IL) was coupled to peroxidase Type VI (Sigma Chemical Company, St. Louis, MO) by one-step glutaraldehyde technic, and the anti-HBc peroxidase conjugate was used in the ELISA test.

Anti-HBc IgM by SP-PHA

The principle of this test is similar to that of ELISA. Human IgM was absorbed to the antihuman IgM–coated U-shaped microtiter plate, and the presence of anti-HBc IgM was demonstrated by the adherence to the surface of the well by HBcAg-sensitized erythrocytes. The technic followed those of the ELISA as described above up to adding test serum to an antihuman IgM–coated ELISA plate (Immulon II). Sera were tested at 1:1,000, 0.1 mL per well, in duplicate: one for test and another for control. After the incubation of test serum with the antihuman IgM–coated microtiter plate for two hours at 37 °C and three washings with diluent, 0.025 mL of HBcAg-sensitized erythrocytes and control SRBCs were added to the test and control wells, respectively. The microtiter plate was left undisturbed at room temperature for two hours and the results read. A mat of erythrocytes covering the bottom of the test well with a button of control erythrocytes in the control well was considered positive for anti-HBc IgM. In contrast, a negative result was shown by a button of erythrocytes on the bottom of the test well.

Anti-HBc and Anti-HBc IgM Titers in Acute Hepatitis B and HBsAg Carriers

Sera from 26 cases of acute hepatitis B, 22 cases of HBsAg-positive chronic hepatitis B, and 18 HBsAg carriers with positive anti-HBc IgM were tested for anti-HBc and anti-HBc IgM by PHA and SP-PHA in decimal dilutions starting from 1:10³ to determine if acute hepatitis B can be differentiated from HBAG carriers by the two tests.

Results

Comparative results of SP-PHA and ELISA tests for anti-HBc IgM in various groups of sera are shown in Table 1. Both tests were positive in all 36 sera from acute hepatitis B and in 68.18% of 22 HBsAg-positive chronic hepatitis B patients. Among 75 HBsAg-positive healthy carriers, SP-PHA and ELISA tests for anti-HBc IgM were also positive in 20%. All 65 sera negative for HBsAg but positive for anti-HBc by PHA were found negative by both SP-PHA and ELISA tests for anti-HBc IgM. However, rheumatoid factor caused false positive reactions in
the ELISA, while SP-PHA was unaffected (Table 1). Differentiation between positive and negative reactions in SP-PHA was very clear cut and the results obtained within three to four hours after addition of serum. In addition, Corecell can substitute our HBCAg-sensitized SRBCs because both were equivalent in sensitivity and specificity in tests for anti-HBc.\(^7\) When Corecell was used in SP-PHA in the comparison with ELISA in 15 patients with acute hepatitis, 22 patients with chronic hepatitis, 25 HBsAg carriers, and 35 anti-HBc-positive but HBsAg-negative blood donors, both tests were positive in 100%, 68.18%, 20%, and 0%, respectively.

Anti-HBc and anti-HBc IgM titers by PHA and SP-PHA in acute hepatitis B, chronic hepatitis B, and anti-HBc IgM-positive HBsAg carriers were compared and are shown in Table 2. There were overlapping anti-HBc IgM titers in all groups. Out of 18 HBsAg carriers that were positive for anti-HBc IgM, 17 had total anti-HBc titers (by PHA) of 1:10\(^5\) or more while all acute hepatitis B had total anti-HBc titers of 1:10\(^4\) or lower. It was impossible to differentiate chronic hepatitis B from anti-HBc IgM-positive HBsAg carriers by the combination of PHA and SP-PHA.

**Discussion**

The SP-PHA test for anti-HBc IgM was found to be as sensitive as the ELISA counterpart. It is also simple and inexpensive. In addition, SP-PHA was not interfered with by rheumatoid factor and did not need absorption with either aggregated IgG or IgG-coated latex particles required in some ELISA\(^4,5,8\) and RIA\(^2\) tests for anti-HBc IgM. The results of anti-HBc IgM in control and study groups were similar to those of other studies.\(^3-6,8,9\) However, SP-PHA is a much simpler test, based on the well-accepted IgM capture technic. It required fewer reagents and steps and had only two variables: dilutions of anti-human IgM and test serum. HBCAg-sensitized erythrocytes were already prepared at optimal conditions. Results were easy to read and reproducible. The high quality of synthetic HBCAg used in the PHA test must contribute to the success of SP-PHA shown in this study. HBCAg-sensitized SRBCs can be prepared as described\(^1\) or obtained from a test kit for anti-HBc (Corecell).

The continuing virus replication following acute hepatitis B is considered responsible for the presence of anti-HBc IgM in chronic hepatitis B and some HBsAg carriers. Titers of anti-HBc IgM were found to be associated with disease activities.\(^5,8\) It is higher in acute hepatitis B and lower in chronic hepatitis B\(^5,8\) and healthy HBsAg carriers.\(^2,5\) In contrast, total anti-HBc appears to increase with chronicity.\(^2\) The proportion between anti-HBc IgM and total anti-HBc was found useful for monitoring disease activities.\(^9\)

**Table 2**. Reciprocal of Titers of Total Anti-HBc and Anti-HBc IgM by PHA and SP-PHA in Acute Hepatitis B, Chronic Hepatitis B, and HBsAg Carriers

<table>
<thead>
<tr>
<th>Study Group</th>
<th>SP-PHA</th>
<th>PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute hepatitis B</td>
<td>&gt;10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>Chronic hepatitis B*</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>HBsAg carriers with anti-HBc IgM†</td>
<td>18</td>
<td></td>
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</tbody>
</table>

* Anti-HBc IgM was negative in seven, including the two with reciprocal PHA titers less than or equal to 10<sup>6</sup>.† A titer of 1:10<sup>6</sup> was used as the cut off titer.

Our study has shown that titration of both total anti-HBc and anti-HBc IgM was needed for differentiating acute hepatitis B from HBsAg carriers positive for anti-HBc IgM. Positive anti-HBc IgM at 1:1,000 by SP-PHA together with an anti-HBc titer less than or equal to 1:10<sup>5</sup> by PHA were found only in acute hepatitis B. SP-PHA used in combination with the PHA test for total anti-HBc is indeed an excellent tool for diagnosis of acute hepatitis B.

**Acknowledgments.** Dr. Rapin Snitbhan, M.D., Department of Virology, Armed Forces Research Institute of Medical Sciences, Bangkok, kindly provided HBCAg for the ELISA test and sera from 15 patients with acute hepatitis B. Dr. P. Wingfield of Biogen S.A., Switzerland and Professor K. Murray donated HBCAg for this study. Professor F. Deinhardt's correspondence lead to the development of this test in response to our request to have a test for anti-HBc IgM readily available in Thailand.

**References**

Comparison of Clostridium difficile Detection by Monolayer and by Inhibition of Nucleoside Uptake

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Detection and identification of Clostridium difficile toxin by traditional monolayer assay were compared with results obtained by a new procedure based on toxin-dependent inhibition of target cell uptake of a radioactive nucleoside. A high degree of correlation was noted between the two determinations. Although the new procedure was quantitative and objective, its value is seen at present as a rapid screen that may support results obtained in monolayers and as a potential assay for other, currently unidentified, toxins. (Key words: Clostridium difficile toxin; Nucleoside uptake) Am J Clin Pathol 1987; 87: 270–272

ANTIBIOTIC-ASSOCIATED colitis and the more severe pseudomembranous colitis have been shown to be caused by a toxin from Clostridium difficile. At least two major toxins, in fact, are produced by that organism. The first, toxin A, has been associated with the in vivo development of colitis. The second, toxin B, has been shown to produce characteristic cytopathic changes in cells grown in monolayers. Because the two toxins are believed generally to be associated, diagnosis of C. difficile–dependent colitis has, for several years, relied on identification of toxin B by its in vitro effects on tissue culture cells.

Although identification of the toxin is believed to be relatively efficient, sensitive, and specific, the degree of clinical severity of the colitis has not always corresponded to the concentration of toxin detected. In addition, detection of toxin, in some cases, may be obscured by the simultaneous presence of other unidentified toxins that may interfere with interpretation of in vitro changes. For this reason, we evaluated the possibility of detecting C. difficile toxin by means of metabolic changes induced in tissue culture cells grown in suspension culture. Previous reports have indicated toxins from C. difficile inhibit incorporation and membrane transport of nucleic acid precursors. It was hoped that this approach would lead to an assay that would not only be sensitive and specific, as the current monolayer system, but would also be objective, quantitative, and more rapid.

Methods

Monolayer assays for C. difficile toxin were performed in the routine manner on stool specimens that were initially extracted with 3 volumes of phosphate-buffered saline (PBS), pH 7.4. The proposed assay involved suspension of L5178Y murine lymphoma cells. Cells were cultured in Fischer's media containing 10% horse serum and added sodium bicarbonate. Flasks were maintained sealed during cell growth at 37 °C. Routinely, cells at the plateau stage of growth were removed from seed tubes, expanded with 4 volumes of fresh media, and cultured for two days. This working culture was thereafter fed each day with an equal volume of fresh media. Aliquots were removed before feeding for each day's toxin assay. For the assay of C. difficile toxin, 150-μL aliquots of cell suspensions (7–9 × 10⁵ cells/mL) were incubated in microtiter wells. Iodo-deoxyuridine-125 (125IUrD) (0.3 μCi) was added to all cultures, and incorporation into cells was measured after three hours incubation of sealed plates at 37 °C. Cells were collected semiautomatically by means of the Skatron® cell harvester, whereby cells were trapped on fiber mats and washed repetitively. Radioactivity within the cells was determined by removing individual fiber circles (one per well) from the mat and placing them in glass test tubes for counting. Toxin effect was determined by monitoring changes in 125IUrD incorporation in control and treated cells. All assays were performed in triplicate.

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