Micromethod for Phosphonoformate Inhibition Assay of Hepatitis B Viral DNA Polymerase

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A micromethod for the specific measurement of hepatitis B viral DNA polymerase in serum is presented, based on the phosphonoformate inhibition assay (J Med Virol 12: 61–70, 1983). In the micromethod, sample volume is reduced to 120 µL and the ultracentrifugation step is eliminated. The method allows good discrimination between serum infected with hepatitis B virus and uninfected serum. The cutoff value for rate of nucleotide incorporation, based on assays of 41 serum specimens negative for hepatitis B serological markers, was about 15 nU/L (90th percentile). Serum containing hepatitis B surface and e antigens exhibited rates of phosphonoformate-inhibitive nucleotide incorporation of 150 (SD 150) nU/L, with an upper 90th percentile range of 17 to 667 nU/L (n = 41). The micromethod makes use of commercially available [32P]dCTP (specific activity about 7000 cCi/mol). [32P]-labeled dCTP was found to be unsuitable for this assay. Human DNA polymerases in serum are detected by this method but are excluded from the phosphonoformate-inhibitive fraction.

Hepatitis B infection is endemic in many parts of the world (1). Measurement of hepatitis B viral DNA polymerase (DNA nucleotidyltransferase, EC 2.7.7.7) in serum is important in assessing the stage of infection: because the enzyme is found only in the virus (2), its detection indicates the presence of the infectious particle in blood. The usual method of measuring hepatitis B viral DNA polymerase requires separation of viral particles from serum by ultracentrifugation, followed by serological tests to ensure specificity (3, 4). Recently, an assay method was reported (5) in which the inhibition by phosphonoformic acid (PFA) of nucleotide incorporation is a measure of hepatitis B viral DNA polymerase activity.4 The PFA inhibition assay eliminated the need for serological checks, but entailed a 6-h ultracentrifugation step of 6-mL serum samples. Here we report a micromethod version of the PFA inhibition assay. Requiring 120 µL of serum and no ultracentrifugation, the micromethod has the advantages of greater convenience and speed. Analysis time has been reduced from three days to 1.5 days. The micromethod enables one person to carry out 60 tests per week, and makes the PFA inhibition assay suitable for routine use.

The principle of the micromethod is the same as that in the macro procedure (5). PFA is used at a concentration that inhibits the viral enzyme but not human DNA polymerases. Samples are incubated with and without PFA, with high specific activity [32P]dCTP. After incubation the [32P]DNA and serum proteins are coprecipitated and free [32P]dCTP is removed by washing the precipitate. The difference in the amount of [32P] incorporated in the absence of PFA and that in its presence is a measure of hepatitis B viral DNA polymerase activity. PFA also inhibits DNA polymerases from other viruses, including cytomegalovirus, herpes simplex virus, and Epstein–Barr virus (6, 7) but these viruses are not found in blood in numbers that permit their detection by means of their DNA polymerase activity. Thus the PFA inhibition assay of serum is considered to be specific for hepatitis B viral DNA polymerase.

Materials and Methods

Reagents

Except where specified, chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

PFA, 7.5 mmol/L. Dissolve 22.5 mg of the trisodium salt of PFA hexahydrate in 10 mL of water.

Detergent and antioxidant. Per liter, 50 mL of "Nonident P-40," and 5 mL of 2-mercaptoethanol. Nonident P-40 is octylphenoxy polyethoxylated alcohol.

Buffer-salts solution, pH 7.4. Per liter, 0.4 mol of Tris, 1.6 mol of KC1, and 0.1 mol of magnesium acetate.

dATP, dGTP, and dTTP (about 65 mmol/L each). To 10-mg vials of each chemical add 270 µL of water.


Bacterial DNA polymerase. To 10 U of DNA polymerase (from Micrococcus lysodeikticus) dissolved in 50 µL of a solution containing, per liter, 0.1 mol of Tris and 1 mL of 2-mercaptoethanol (pH 7.4), add 500 µL of serum and 50 µL of activated DNA. The latter is prepared by heating to 80°C (5 min) DNA dissolved in a solution containing, per liter, 50 mmol of Tris and 6 mmol of magnesium acetate (pH 7.4) at a concentration of 250 mg/L, then cooling the solution in a 4°C bath.

Trichloroacetic acid (TCA)–pyrophosphate. Dissolve 100 g of TCA and 53.2 g of anhydrous Na2P2O7 in water; dilute to 2 L.

Scintillation fluid. Mix 1 L of "Lipotron" with 100 mL of "Solotron" and 20 mL of water (both products of Kontron AG, Zurich, Switzerland).

Store the PFA and TCA solutions at 4°C, the scintillation fluid at room temperature, and all other reagents at −20°C.

Other materials. 5-125I-labeled 2'-deoxyctydine 5'-triphosphate (specific activity 2000 1Ci/mol) and deoxy-[5-3H]-cytidine 5'-triphosphate (specific activity 19 1Ci/mol) were obtained from Amersham International.

Specimens

We used serum from volunteers who were undergoing serological screening in a hepatitis B vaccination program. Plasma from blood-bank specimens that had been rejected because of the presence of HBsAg was also used. Blood-bank plasma was recalified before use in order to complex the

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4 Nonstandard abbreviations: PFA, phosphonoformic acid; TCA, trichloroacetic acid; HBsAg, HBeAg, surface antigen and e antigen, respectively, of hepatitis B virus; HBsAb, HBeAb, antibodies to hepatitis B surface antigen and core antigen, respectively.

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citrate that would otherwise have bound Mg$^{2+}$. Serum was stored at -20 °C.

Serological markers for hepatitis B virus infection were tested with commercial enzyme-linked immuno-absorbent assay kits (Abbott Laboratories, N. Chicago, IL): AUS-)

Zyme II, AUSAB-EIA, CORZYM, and HBe-EIA for HBsAg, anti-HBs, anti-HBc, and HBeAg, respectively.

Procedure

1. Use four disposable 12 × 75 mm round-bottom polypropylene tubes (Elkay Products, Inc., Shrewsbury, MA) for each specimen. Add 10 μL of water or PFA to duplicate tubes, then add 15 μL of Nonidet P-40 reagent and 30 μL of serum to each tube. Vortex-mix and let the tubes stand at room temperature for 30 min. (Use the bacterial DNA polymerase preparation when the assay mixture needs checking or for quality control. Its polymerase activity after five days of storage at -20 °C is about 90% of that on the first day.)

2. Prepare a volume of radioactive mixture equal to (n + 1), where n is the number of tubes set up in step 1. The 20-μL volumes per tube consist of 18.75 μL of the Tris-K-Mg buffer; 0.35 μL each of dATP, dGTP, and dTTP solutions; and 0.20 μL (2 μCi) of $^{32}$PdCTP. Keep the mixture at 4 °C.

3. Add 20 μL of the radioactive mixture that was prepared in Step 2 to the tubes prepared in step 1. Vortex-mix and place the tubes in a bath at 37 °C for 2 h. Then transfer the tubes to an ice bath (4 °C) and add 1 mL of ice-cold TCA-pyrophosphate. Vortex-mix the tubes' contents and let them stand in an ice bath for 1 h.

4. Centrifuge the contents of the tubes at 1000 × g for 10 min at room temperature. After placing the tubes in an ice bath, aspirate the supernates with a Pasteur pipet that is connected to a suction flask for collecting radioactive waste fluids.

5. To each tube add 2 mL of ice-cold TCA-pyrophosphate. Vortex-mix until the disc of precipitated serum is lifted off the bottom of the tube. The efficiency of washing is not improved by breaking up the disc. Centrifuge the tubes and their contents at 1000 × g for 5 min. Again place the tubes in an ice bath and aspirate the supernates. Repeat step 5 three more times.

6. Add to each tube 1.5 mL of scintillation fluid and vortex-mix. Transfer the entire contents of each tube to a 5-mL scintillation vial. Wash each tube with two more 1.5-mL portions of scintillation fluid, and add the washes to the vial. Let the vials stand overnight, and shake them the next day to disperse the precipitate.

7. Measure the radioactivity with a liquid scintillation counter, counting for 5 min per vial (counting efficiency, 100%; estimated SD of PFA-inhibitory radioactive activity per specimen at 8000 and 2000 cpm is 1.4% and 2.8%, respectively). The counts per minute are corrected for radioactive decay to the reference date, by use of standard tables. After calculating the difference in radioactivity incorporated in the absence and presence of PFA, multiply by the factor F to obtain hepatitis B viral DNA polymerase activity, in nUL.

\[
F = \frac{(4 \times 10^8 \text{ μL/L})(1 \text{ dpm/cpm})(1000 \text{ nUL} \cdot \text{min/μmol})}{(30 \text{ μL})(120 \text{ min})(2220 \text{ dpm/μCi})(\text{specific activity})}
\]

For the $^{32}$PdCTP we used, the specific activity was 7000 nCi/μmol, so that F = 0.0715 nUL · cpm. Where the difference in $^{32}$P incorporation with and without PFA was a small negative value, as was sometimes the case, we used a result of zero.

Other Procedures

Assays with $^{125}$I-labeled dCTP were performed as above, through step 5. Radioactivity of the precipitates was counted in a gamma counter. Assays with $^{3}H$dCTP were performed as described elsewhere (5). In both experiments, we used 2 μCi of radioisotope per tube, as above.

Results

Figure 1 shows the time course of $^{32}$PdCTP incorporation into TCA-insoluble constituents of serum. PFA had no effect on $^{32}$P incorporation in uninfected serum, but hepatitis B-infected serum typically showed pronounced effects. The dosage of added PFA (nominal final concentration, 1 mmol/L) inhibited more than 90% of the viral enzyme but none of the human DNA polymerases in serum (5). Relatively high amounts of $^{32}$P were incorporated, both in uninfected serum and in infected serum incubated in the presence of PFA.

The time course of PFA-inhibitable nucleotide incorporation was curvilinear (Figure 2). Use of a shorter incubation period might have produced a more nearly linear curve, but radioactivity counting rates (cpm) for samples would be lower and the assay would be less sensitive. The amount of PFA-inhibitable nucleotide incorporation was consistently greater after 2 h than after 1 h, but changes observed during the third hour were more variable, as shown by the disproportionately larger SD. Because of the nonlinear kinetics, we express hepatitis B DNA polymerase activity as nUL to denote the average rate of incorporation in a 2-h incubation period.

![Graph](image-url)
Figure 3 illustrates the clinical application of the method for two groups of sera (n = 41 each): those negative for all three markers of exposure to hepatitis B virus (HBsAg, HBsAb, and HBeAb) and those showing markers for infection (HBsAg, HBeAg). The cutoff value, calculated as the 90th percentile of values in the uninfected group, was about 210 cpm or 15 nU/L (8). We have adopted 200 cpm as a convenient cutoff value, consequently excluding 10% of the results on HBsAg-negative specimens from the DNA polymerase-negative group. Results in infected cases ranged from 0 to 665 nU/L (0 to 9300 cpm), with a median value of 100 nU/L (1400 cpm). Four of the infected serum specimens repeatedly fell below the cutoff value. These points could represent false negatives or they could reflect the absence of virus (see Discussion).

We evaluated the precision of the micromethod with sera having different amounts of viral DNA polymerase activity (Table 1). As expected, the CV increased at lower enzymic activity. Specimens of HBsAg-positive serum that had less than 500 cpm of activity (about one in eight) were usually retested. The mean difference between two successive tests on the same serum was about 140 cpm or 10 nU/L.

We compared results with those by our previous method (5), using [3H]dCTP as the radioactive precursor, for 16 specimens of recalculated HBsAg-positive plasma. As Table 2 shows, the correlation between the two methods was excellent, and the radioactivity counting rates in the macro-

![Graph of hepatitis B viral DNA polymerase activity in serum](image)

**Fig. 2.** Kinetics of hepatitis B viral DNA polymerase activity in serum. Summary of experiments on three specimens that showed nucleotide incorporation of 83 to 150 nU/L at 1 h. Vertical axis represents amounts of incorporation relative to that incorporated at 1 h; bars indicate 1 SD.

**Table 1. Precision of the Micromethod for Hepatitis B Viral DNA Polymerase**

<table>
<thead>
<tr>
<th>n</th>
<th>PFA-inhibitable nucleotide incorporation</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean (and SD), nU/L</td>
<td></td>
</tr>
<tr>
<td>Within run</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>272 (22)</td>
<td>8.1</td>
</tr>
<tr>
<td>4</td>
<td>150 (18)</td>
<td>12.0</td>
</tr>
<tr>
<td>4</td>
<td>60 (13)</td>
<td>21.7</td>
</tr>
<tr>
<td>Between run</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>215 (18)</td>
<td>8.4</td>
</tr>
<tr>
<td>5</td>
<td>165 (22)</td>
<td>13.3</td>
</tr>
<tr>
<td>4</td>
<td>120 (20)</td>
<td>16.7</td>
</tr>
<tr>
<td>4</td>
<td>80 (15)</td>
<td>18.8</td>
</tr>
<tr>
<td>4</td>
<td>15 (6.7)</td>
<td>44.7</td>
</tr>
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</table>

method (5) and micromethod were comparable. Furthermore, we detected HBeAg only in those specimens that exhibited PFA-inhibitive nucleotide incorporation rates exceeding the cutoff limit for the micromethod.

In the course of developing a micromethod for the viral DNA polymerase, we tried using [125I]-labeled dCTP, whose longer half-life than [32P]dCTP appeared to offer a clear advantage. Table 2 summarizes the results for 23 serum specimens that were analyzed with the two radiolabeled nucleotides. The results obtained with [125I]-labeled dCTP were disappointing, generally yielding lower sample counting rates than would be expected from their specific activities. The correlation of results with those obtained by the [32P]-labeled nucleotide was only $r = 0.75$. We attribute the poor performance of the [125I]-labeled dCTP to several factors: its lower radiolabel purity, its greater chemical instability, and the demonstrable inhibitive effect of the iodide ion (or one of its decomposition products) on DNA polymerase activity. Despite its usefulness in similar applications (9),
we concluded that $^{125}$I-labeled dCTP was unsuitable for the assay of hepatitis B viral DNA polymerase.

Discussion

The key change in converting the PFA inhibition assay to a micromethod was the use of radiolabel of high specific activity to offset the decrease of changing the size of the serum sample. A commercially produced [32P]dCTP filled this requirement, and had the added advantage of greater counting efficiency than was obtained with [3H]dCTP. However, the rate of nucleotide incorporation was decreased as the result of the low concentration of dCTP (4 nmol/L), so that we had to establish a new reference interval for the micro-method. Eliminating the ultracentrifugation step probably increased the fraction of viral particles assayed, given the incomplete sedimentation of the virus under specified conditions (5). The overall effect of these positive and negative factors was that PFA-inhibitable radioactivity incorporated in the samples in the macro- and micromethods was comparable, and there was no loss of sensitivity in adopting the micromethod.

The sensitivity of the proposed assay is about the same as that of another test for the presence of the virus, molecular hybridization to hepatitis B DNA. Independent estimates of the proportion of HBeAg-positive blood specimens that contain hepatitis B DNA or viral DNA polymerase agree closely and suggest that virus may be absent in about 10–15% of this group. Molecular hybridization tests carried out in two laboratories showed 84 to 88% of HBeAg-positive sera to be positive also for hepatitis B DNA (10, 11). The remainder showed equivocal or negative results. This compares with our previous report that about 85% of HBeAg-positive blood specimens contain PFA-inhibitive DNA polymerase activity (5). The micromethod provides similar results, detecting about 90% (37/41) of HBeAg-positive serum tested in the present study as having hepatitis B DNA polymerase activities above the cutoff value.

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References