Establishment of Passive Hemagglutination Assay (PHA) System for Anti-HBc in Plasma

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Uemura, Y., Fukuyama, K., Nishida, M., Suyama, T. and Ohori, H. Establishment of Passive Hemagglutination Assay (PHA) System for Anti-HBc in Plasma. Tohoku J. exp. Med., 1986, 149 (1), 11-20 —— A sensitive and specific assay system for anti-HBc, the passive hemagglutination (PHA) method, has been established. The reactivity of PHA cells prepared by conjugating purified recombinant HBcAg-particles with fixed sheep blood cells (SRBC) was highly specific to monoclonal- and polyclonal anti-HBc IgGs. The sensitivity of PHA method was higher than that of radioimmunoassay (RIA). However, uncertainty for the positivity of anti-HBc still remained in plasma with the PHA titers lower than 2³. A relatively high ratio (19%, 37 of 196) of anti-HBc-positive plasma, which had been confirmed to be HBsAg negative, was demonstrated in blood donors whose bloods have been considered suitable for transfusion. The hazards of anti-HBc-positive bloods and the importance of anti-HBc detection in plasma are discussed in this paper. ——— PHA; anti-HBc; HBV; plasma

The testing of transfusion bloods for HBsAg has reduced the incidence of post-transfusion hepatitis (PTH), especially of type B hepatitis. However, even when the third generation methods for HBsAg such as radioimmunoassay (RIA), enzyme linked immunoassay (EIA) and reversed passive hemagglutination assay (RPHA) are available, PTH has not been completely extirpated.

Krugman et al. (1974) and Hoofnagle et al. (1974) succeeded in detecting antibody to HB core antigen (anti-HBc) by the complement fixation (CF) method and reported its usefulness for the prevention of hepatitis B virus (HBV) infection. This concept has been supported by later studies (Lander et al. 1978; Ohori et al. 1980a; Rakela et al. 1980). Furthermore, it is worth pointing out that bloods positive for anti-HBc may induce non-A, non-B (NANB) hepatitis for which the infectious candidate(s) have not been identified, because a significant

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Requests for reprints: Dr. Yahiro Uemura, The Central Research Laboratories, The Green Cross Corporation, 3-5-44 Miyakojimanakadori, Miyakojima-ku, Osaka 534, Japan.
reduction of this hepatitis was found in recipients of bloods negative for anti-HBc as compared with those who received anti-HBc-positive bloods (Stevens et al. 1981; Cossart et al. 1982; Vyas and Perkins 1982).

Another pointer to the significance of anti-HBc is the fact that this antibody may play a role in the pathogenesis of liver tissues of type B hepatitis patients (Realdi et al. 1982; Trevisan et al. 1982).

In our earlier study (Ohori et al. 1980a), it was found that sera with high titers of anti-HBc also have a high activity of HBV specific DNA polymerase and large amounts of HBsAg, suggesting that the activity of this antibody may reflect the active replication of HBV in the liver.

Accumulating findings on anti-HBc in type B hepatitis patients led us to consider that screening for anti-HBc in serum or in plasma qualitatively and quantitatively, would be important for the reduction of PTH and for a clear evaluation of the clinical features of type B hepatitis patients.

However, the assay methods for anti-HBc so far established place considerable obstacles in the way of quantitative determination of anti-HBc activity because of their cost and labor intensiveness. In the present study, we have established a sensitive and specific assay system for anti-HBc, the passive hemagglutination (PHA) method, and discuss its usefulness in serum or in plasma of type B hepatitis patients.

**MATERIALS AND METHODS**

*Specimens*

Serum and plasma specimens from HBsAg carriers, blood donors and HBsAg vaccine (plasma HBsAg from The Green Cross Corp., Osaka) inoculated healthy adults who had been proven to be negative for any HBV markers before the inoculation were included in this study.

*Serological testing*

HBsAg was tested by the RPHA method (Antihebscell, The Green Cross Corp.) and retested by the RIA method (Ausria II, Abbott Laboratories, North Chicago, Ill, USA.). HBeAg was tested by the RPHA method (Anti-e-cell-Neo, The Green Cross Corp.). Anti-HBs and anti-HBe were tested by the PHA methods (Hebsgencell and e-cell, respectively, The Green Cross Corp.). Anti-HBc was tested by two methods using PHA (see below) and RIA (Corab, Abbott Laboratories).

*Preparation of PHA cells (Corecell) for the detection of anti-HBc*

The PHA cells were prepared according to a modification of a method described previously (Ohori et al. 1980a). A brief description is as follows; sheep red blood cells (SRBC) were washed 4 times with sodium phosphate buffered saline (PBS, 0.075 M sodium phosphate buffer, pH 7.2, 0.075 M NaCl, 0.1% NaN₃) and suspended in saline to make 5% of hematocrit. The washed SRBC suspension was mixed with one-fifth volume of 2% (w/v) glutaraldehyde solution and stirred for 60 min at room temperature. Then an equal volume of 0.2 mg/ml tannic acid solution was added to the glutaraldehyde treated SRBC suspension and incubated for 15 min at 37°C. The SRBC were washed 2 times with saline and resuspended in saline to make 5% of hematocrit. Purified HBCaAg particles (purchased...
from Biogen S.A., Switzerland) which had been extracted from E. coli cells were dissolved in saline to make an absorbancy at 280 nm of 0.10, and mixed with an equal volume of the tannated SRBC suspension. The mixture was stirred gently for 60 min at 37°C and centrifuged at 1,000 rpm for 10 min. The resulting precipitate of HBcAg-coated SRBC was washed repeatedly with PBS and lyophilized.

**Monoclonal antibodies to HBsAg, HBcAg and HBeAg**

Purified serum derived HBsAg and recombinant HBcAg (purchased from Biogen S.A.) were used for the immunogens. For the preparation of anti-HBe monoclonal antibody, the recombinant HBcAg-particles which were also used for the preparation of monoclonal anti-HBc antibody were treated with 2% sodium lauryl sulfate (SDS)-0.2% 2-mercaptoethanol (2-ME). The complete antigenic conversion of HBcAg to HBeAg was confirmed as described previously (Ohori et al. 1980b).

Each 20 µg of immunogen (HBsAg, HBcAg and HBeAg) was mixed with an equal volume of Freund’s complete adjuvant and separately immunized i.p. to BALB/c mice followed by weekly boost for a month. Four days after the last boost, the fusion of spleen cells with P3UI myeloma cells was carried out by a modification of the procedure described by Galfre et al. (1977). Hybrids were screened by the PHA cells (Hebsgencell for anti-HBs and e-cell for anti-HBe, The Green Cross Corp.). For the screening the hybrid cells producing anti-HBc, RIA (Corab, Abbott Laboratories,) and PHA (Corecell, The Green Cross Corp.) were used.

**RESULTS**

**Specificity of PHA cells**

It was important to use monoclonal antibodies to HBV specific antigens for the evaluation of the specificity of PHA cells because polyclonal antibodies from human or animal sera usually display more than two kinds of antibody activity. Especially, human serum positive for anti-HBc is also positive for anti-HBs and anti-HBe. To avoid such confusion, monoclonal anti-HBc (BH11c and BA8, 1:8,000 and 1:51,200 PHA titers, respectively), anti-HBe (CE1, AH1 and BH11e, 1:51,200, 1:12,800 and 1:6,400 PHA titers, respectively) and anti-HBs (1D6, 2E2, 1E6 and 1D4, 1:25,600, 1:51,200, 1:8,000 and 1:25,600 PHA titers, respectively) were used (Table 1). The PHA cells reacted with the monoclonal antibodies of anti-HBc, but did not with those of anti-HBe and anti-HBs. These results strongly suggest that the PHA cells prepared in this study are highly specific to anti-HBc.

**Sensitivity of PHA cells**

The sensitivity of PHA cells was roughly estimated by using monoclonal anti-HBc IgG listed in Table 1 and polyclonal human anti-HBc IgG. Each two-fold dilution of a known concentration (1 µg/ml) of polyclonal- and monoclonal IgGs was assayed with the PHA cells whose sensitivity to these IgGs was calculated by dividing the amount of IgGs by the corresponding PHA titers. Different sensitivities, 6.5 ng/ml/unit and 0.9 ng/ml/unit, were obtained in monoclonal anti-HBc IgGs of BH11c and BA8, respectively (Table 2). These results suggest that epitopes which react with these monoclonal antibodies may be
different in their distribution and number on the surface of HBcAg particles conjugated with PHA cells or that the affinity of these antibodies to the corresponding epitopes may be different.

On the other hand, a relatively constant sensitivity was demonstrated for the polyclonal human anti-HBc IgGs which had been prepared from the sera of HBeAg-positive asymptomatic HBsAg carriers.

In order to see the recovery of anti-HBc activity, polyclonal- and monoclonal (BH11c) IgGs were added to the plasma negative for anti-HBc (Fig. 1), and the activity of anti-HBc was assayed. Both activities of these IgGs were completely different in their distribution and number on the surface of HBcAg particles conjugated with PHA cells or that the affinity of these antibodies to the corresponding epitopes may be different.

On the other hand, a relatively constant sensitivity was demonstrated for the polyclonal human anti-HBc IgGs which had been prepared from the sera of HBeAg-positive asymptomatic HBsAg carriers.

Table 1. Reactivity of PHA cells with the monoclonal antibodies to HBcAg, HBeAg and HBsAg

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Clone</th>
<th>IgG subclass</th>
<th>Anti-HBc titer (unit*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HBc</td>
<td>BH11c</td>
<td>IgG2a</td>
<td>8,000</td>
</tr>
<tr>
<td></td>
<td>BA8</td>
<td>IgG1</td>
<td>51,200</td>
</tr>
<tr>
<td>Anti-HBe</td>
<td>CE1</td>
<td>IgG2b</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>AH1</td>
<td>IgG1</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>BH11e</td>
<td>IgG1</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Anti-HBs</td>
<td>1D6</td>
<td>IgG1</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>2E2</td>
<td>IgG1</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>1E6</td>
<td>IgG1</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>1D4</td>
<td>IgG2a</td>
<td>&lt;2</td>
</tr>
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* Each IgG solution (50 µg/ml) was two-fold diluted, followed by assaying for anti-HBc activity with the PHA cells. The reciprocal of maximum dilution which gave positive hemagglutination was taken as unit PHA titer.

Table 2. Sensitivity of PHA cells to the monoclonal and polyclonal anti-HBc antibodies

<table>
<thead>
<tr>
<th>Antibody (IgG)*</th>
<th>Sensitivity† (ng/ml/unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal</td>
<td></td>
</tr>
<tr>
<td>BH11c</td>
<td>6.5</td>
</tr>
<tr>
<td>BA8</td>
<td>0.9</td>
</tr>
<tr>
<td>Polyclonal</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* IgGs were prepared by the method described previously (Ohori et al. 1984).
† Each two-fold dilution of a known concentration (1 µg/ml) of polyclonal- or monoclonal IgGs was assayed with the PHA cells and calculated the sensitivity for the IgG by dividing the amount of IgGs by the corresponding unit PHA titer.
recovered even at low concentrations of anti-HBc, indicating the usefulness of the PHA cells in plasma.

**Comparison of PHA and RIA tests for the detection of anti-HBc in plasma**

In order to evaluate the usefulness of the PHA method in the determination of anti-HBc activity in plasma, a total of 235 plasma specimens including those serologically negative and positive for HBV markers were assayed for anti-HBc activity by PHA and RIA methods. Fig. 2 shows the relationship between the two methods. Plasma specimens showing more than 2\(^7\) PHA titers were also confirmed to be positive by the RIA method (more than 70% RIA inhibition). Five plasma specimens having 2\(^6\) PHA titers were judged to be positive (2 of 5) and negative (3 of 5) by the latter method. Three plasma specimens that had been judged to be negative by the RIA method were reexamined by the PHA method. However, all of them again showed the same titers as those obtained in the first experiments. Furthermore, the activities of all five plasma specimens were absorbed by HBCAg (data not shown). On the other hand, all plasma specimens having lower titers than 2\(^5\) by the PHA method were judged to be negative or pending for positive by the RIA method. Whether these plasma specimens were really positive for anti-HBc or not remained unresolved in this study.

Next experiments were conducted to compare sensitivities between PHA and RIA methods. As seen in Table 3, higher titers by PHA than RIA were constantly observed in different 10 plasma specimens. It is of interest that the ratios of RIA titers to those of PHA were different in individual plasma, i.e., higher ratios
(64 to 85) were observed in plasma specimens having low and high activity of anti-HBc and low ratios (12 to 20) were observed in those having intermediate activities of anti-HBc. These results indicate that the composition of immunoglobulin types may be different in different plasma specimens.

**Prevalence of anti-HBc in plasmas with and without HBV markers**

In order to determine which of the PHA titers were really positive for anti-HBc, plasma specimens including 153 seronegative for HBV other than anti-HBc, 14 from the subjects who had received HBV vaccine, 8 positive for anti-HBs but negative for anti-HBe, 21 positive for anti-HBs and anti-HBe and 53 positive for HBsAg were examined for the presence of anti-HBc (Fig. 3). Impressive results were obtained in plasma from vaccinated subjects in whom anti-HBc titers were lower than 25 before the vaccination. There were no changes in anti-HBc titers in plasma which obtained 4 weeks after the final vaccination. From the results obtained in this experiments, as well as from those found in Fig. 2, as far as our PHA method was concerned, 25 PHA titers were the critical titer for the practical evaluation of real positive or real negative. From this procedure, it appeared that 5.9% (9 of 153) of seronegative groups had anti-HBc. Significant high titers were constantly observed in plasma specimens positive for HBsAg, whereas plasma specimens positive for anti-HBs show low titers of anti-HBc.

**Discussion**

A more sensitive and simple assay system for the determination of anti-HBc in plasma has been eagerly demanded. To date, the RIA method has been widely used for the determination of this antibody. In spite of the high sensitivity and specificity of this method, the difficulties inhered in it regarding cost and labor
intensiveness have hindered studies on quantitative determination of anti-HBc in sera of HBV carriers.

To overcome these problems, we have tried to establish a more sensitive and facile assay system of the PHA method. Because of the difficulty in obtaining HBcAg-particles for PHA cell preparation, there has been no information about this method. Pasek et al. (1979) succeeded in preparing E. coli produced HBcAg particles by gene technology, which made it easy to obtain large amounts of purified HBcAg particles.

As reported in other papers (Ohori et al. 1980a, 1984; Mackey et al. 1981), HBCAg particles display their antigenic conversion from HBcAg to HBeAg accompanying with their morphological disintegration. It is important, therefore, to discover whether the PHA cells have cross reactivity with anti-HBc and anti-HBe or not. The PHA cells presented here had not cross reactivity with the two monoclonal antibodies of anti-HBs and anti-HBe (Table 1) indicating that the HBcAg-particles did not disintegrate during the preparation process of the PHA cells.

In this experiment, some comparisons of PHA with RIA methods were
undertaken using human plasma specimens of various origins. The reliable positivity of anti-HBc with the PHA method was estimated to be $2^6$ PHA titers. Plasmas with the titers lower than this value were roughly judged to be negative by the RIA method (Fig. 2). This was reasonably accepted when the plasma was obtained from subjects who had received HBV vaccine. The PHA titers of anti-HBc were under $2^5$ in their plasmas irrespective of whether before or after the vaccination (Fig. 3). These results indicate that the PHA cells prepared in this study may be useful for plasma with more than $2^5$ anti-HBc titers. Plasma showing lower than $2^5$ PHA titers must be reexamined in concentrated plasma or serum.

It is noteworthy that 19% (37 of 196) of the plasmas negative for HBsAg had anti-HBc activity. These plasmas are now considered to be suitable for the transfusion. It is well established that bloods proven negative for HBsAg even by the highly sensitive tests are known to entail the risk of post-transfusion hepatitis (PTH) if they are anti-HBc positive. Furthermore, 69% of patients with chronic liver disease and 39% of acute hepatitis patients have been proven to be positive for anti-HBc, even they are negative for HBsAg (Wada et al., personal communication). In connection with these considerations, it must be emphasized that the screening of bloods with respect to the presence of anti-HBc is very important for the exclusion of post-transfusion hepatitis, as has in fact been advocated by many researchers (Hoofnagle et al. 1978; Ohori et al. 1980a; Holland 1982).

The ratios of anti-HBc positive in blood donors are reported to be 2–3% in the USA (Irwin et al. 1977; Nath et al. 1983) and the Netherland (Katchaki et al. 1979), and 11% in Italy (Ascione et al. 1981). As shown in Fig. 3, 5.9% (9 out of 153) of normal adults were positive for anti-HBc by the PHA method. Testing

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Sensitivity (units)</th>
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<tr>
<td></td>
<td>PHA</td>
</tr>
<tr>
<td>1</td>
<td>1: 128</td>
</tr>
<tr>
<td>2</td>
<td>1: 512</td>
</tr>
<tr>
<td>3</td>
<td>1: 800</td>
</tr>
<tr>
<td>4</td>
<td>1: 3,200</td>
</tr>
<tr>
<td>5</td>
<td>1: 4,096</td>
</tr>
<tr>
<td>6</td>
<td>1: 6,400</td>
</tr>
<tr>
<td>7</td>
<td>1: 6,400</td>
</tr>
<tr>
<td>8</td>
<td>1: 160,000</td>
</tr>
<tr>
<td>9</td>
<td>1: 320,000</td>
</tr>
<tr>
<td>10</td>
<td>1: 640,000</td>
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</table>
for HBsAg alone, therefore is insufficient for the exclusion of highly dangerous blood, no matter how highly sensitive of assay methods for HBsAg may be available. Even though, uncertainty for the evaluation of the positivity of anti-HBc is still remaining, especially in plasma with the PHA titers lower than $2^6$, the sensitivity of this method is constantly higher than that of RIA method (Table 3). Taking into consideration that assay methods for anti-HBc are not widely used at this time because of the labor intensiveness of the technique, the PHA method presented here seems to be eligible as an assay system for anti-HBc in plasma on account of its sensitivity, specificity and simplicity.

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References


