PRESENCE OF LOWER TEMPERATURE-DEPENDENT ANTIBODY WITH LOW AVIDITY TO C100-3 (HCV) ANTIGEN IN VOLUNTARY BLOOD DONORS

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SUMMARY: Serum samples with lower temperature-dependent antibody with low avidity to C100-3 (HCV) antigen were found in 0.19% of 23,197 voluntary blood donors at this blood center. They showed positive C100-3 antibody activity at 24 C but not at 37 C. The antibody activity bound to C100-3 antigen at lower temperature disappeared after incubation for 60 min at 37 C or treatment with 8 M urea. Other markers of hepatitis C virus infection, especially the presence of HCV-RNA were demonstrated in some of these serum samples and the importance of this phenomenon is discussed with regard to virus screening of blood donors for hepatitis C.

INTRODUCTION

To reduce the post-transfusion nonA nonB hepatitis (PTNANBH) common throughout Japan at that time, the nationwide screening of donated blood for positive hepatitis C antibody (HCVAb) was started in November, 1989 at all Japanese Red Cross Blood Centers. Since then, the incidence of PTNANBH has decreased significantly (1) but still remains above zero and further improvements of screening procedures are certainly required for more effective control of
PTNANBH. We have been increasingly required to supply donated blood to hospitals as rapidly as possible and for that reason, we tried to shorten the time required for testing blood for HCVAb.

We reduced the first incubation time of C100-3 antigen of HCV and test samples from 60 min in an incubator at 37 °C (the manufacturer's standard method) to 30 min. As we carried out this shortened method, we met with an unexpected result. Although our sample number was small, some of the tested samples reacted positively by a 30-min incubation but negatively by the standard method. This phenomenon was due to the presence of a lower temperature-dependent antibody with a low avidity to C100-3 antigen derived from HCV. We thought that this poses an important problem in screening blood for the CV antibody.

**MATERIALS AND METHODS**

**Serum samples:** Serum samples were collected from donated blood in this blood center and tested within 18 hr or stored at −20 °C or below until testing.

**Measurement of C100-3 antibody:** Microwells coated with recombinant C100-3 antigen, HCV ELISA test (Ortho Diagnostic System Inc.) were used. To each well containing 200 μl of a specimen diluent, 20 μl of each undiluted serum sample was added and they were allowed to react in an incubator at 37 °C or at 24 °C for 60 min. After five thorough washings, the captured antibody was measured by addition of anti-human IgG monoclonal antibody labeled with peroxidase followed by the addition of the enzyme substrate as described by the manufacturer. After stopping the enzyme reaction by the addition of 4 N sulfuric acid, the yellow products were read with a microwell reader at 490 ± 2 nm and the cut-off value was set up at the mean absorbance of negative control + 0.400. The antibody activity under various conditions was simply expressed as the absorbance at 490 ± 2 nm.

Ethylenediaminetetraacetate was added to a concentration of 0.04 M, pH 7.4 to the specimen diluent when necessary.

**Urea treatment:** After the first reaction of serum samples with C100-3 antigen at 24 °C for 60 min and five washes, 8 M/liter urea in the wash fluid was added and 10 min later, an additional wash was done with the manufacturer's recommended wash solution to remove the urea.

**Measurement of GOR antibody:** Antibody against GOR antigen was measured by ELISA with spGOR2 peptide (2).
Detection of HCV-RNA: HCV-RNA in test samples was detected by two-stage polymerase chain reaction (PCR) with nested primers derived from 5' non-coding regions of the HCV genome (3).

Immunoblot assays for four bands of HCV antigen epitope (4-RIBA): Antibodies against four antigens, 5-1-1, C100, C33c and C22, were tested by recombinant immunoblot assay, 4-RIBA (4).

RESULTS

Presence of Antibodies Reacting with C100-3 Antigen at a Temperature Lower than 37 C

Among 23,197 donated blood samples, 216 (0.93%) were positive by the standard method described by the manufacturer, that is incubation of test samples and C100-3 antigen at 37 C for 60 min. Among them, additional 45 samples (0.19%) were positive when the incubation time was reduced to 30 min (shortened method) in the 37 C incubator but negative by the standard method.

After setting the microplate in the incubator for 30 min, the real inside temperature remained lower than 37 C. Therefore, we took several samples from the group which were positive by only the shortened method and compared these with the samples positive by the standard method. The results of reaction of C100-3 and serum samples at 37 C for 60 min and those at 24 C for 60 min were compared with incidence of other markers of HCV infection.

Comparison of C100-3Ab Test Results at 37 C and at 24 C with Other Markers of HCV Infection

The results of C100-3 antibody ELISA test under various conditions were compared with those of the 4-RIBA test, GOR antibody test and PCR test for detection of HCV-RNA as shown in Table I.

In group I, all sera were strongly positive at 37 C and at 24 C, and the C100-3Ab activity remained after urea treatment. In group II, C100-3Ab was strongly positive both at 37 C and 24 C but after urea treatment, C100-3Ab became negative. In groups I and II, 4-RIBA, anti GOR and HCV RNA were all positive.

In group III, the serum samples were all negative by 37 C incubation but positive by 24 C incubation. By the 4-RIBA test, all reacted to some antigen
Table I. Comparison of C100-3 antibody test results at 37 C, 24 C and after urea treatment with other markers of HCV infection

<table>
<thead>
<tr>
<th>Group</th>
<th>Lot No.</th>
<th>C100-3 Antibody ELISA Test</th>
<th>4-RIBA epitopes</th>
<th>GOR Antibody</th>
<th>HCV-RNA</th>
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<tr>
<td></td>
<td></td>
<td>37C (1)</td>
<td>24C (2)</td>
<td>UREA (3)</td>
<td>5-1-1</td>
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<tr>
<td>I</td>
<td>042-5428</td>
<td>&gt;3.000</td>
<td>&gt;3.000</td>
<td>&gt;3.000</td>
<td>4</td>
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<tr>
<td></td>
<td>048-6888</td>
<td>&gt;3.000</td>
<td>&gt;3.000</td>
<td>1.621</td>
<td>4</td>
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<td>&gt;3.000</td>
<td>&gt;3.000</td>
<td>4</td>
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<td></td>
<td>70-1918</td>
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<td>&gt;3.000</td>
<td>&gt;3.000</td>
<td>4</td>
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<td></td>
<td>094-9654</td>
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<td>2.654</td>
<td>2.929</td>
<td>± 1</td>
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<tr>
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<td>070-5882</td>
<td>&gt;3.000</td>
<td>2.473</td>
<td>2.175</td>
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<tr>
<td>II</td>
<td>497-5454</td>
<td>&gt;3.000</td>
<td>&gt;3.000</td>
<td>0.201</td>
<td>± 2</td>
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<td>± 2</td>
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<td>1.034</td>
<td>0.210</td>
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<td>0.164</td>
<td>0.410</td>
<td>0.034</td>
<td>± 2</td>
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<td>C.O.V (4)</td>
<td>0.514</td>
<td>0.442</td>
<td>0.418</td>
<td>± 2</td>
</tr>
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</table>

(1) C100-3Ag + Serum 37 C 60 min.
(2) C100-3Ag + Serum 24 C 60 min.
(3) C100-3Ag + Serum 24 C 60 min, wash, 8 M urea 10 min at 24 C, wash.
(4) Cut off value: Mean abosorbance with negative control + 0.400.

epitopes and two and three were positive both for GOR antibody and for HCV-RNA.

In group IV, all were positive at 24 C but all were negative at 37 C. 4-RIBA immunoblot, anti GOR test and HCV-RNA were all negative. Group V is a representing negative samples, gave negative results in all the reactions tested.
Fig. 1. Disassociation of antibody bound to C100-3 antigen at 37 C and at 24 C
First reaction: C100-3 + Samples, at 24 C, 60 min, wash.
Second reaction: Add specimen diluent, incubated at 37 C or 24 C for 60 min, wash.
COV: Cut off value.

Effect of EDTA on the C100-3Ab Assay
In the presence of 0.04 M EDTA which chelates Ca++ and Mg++ in the reaction mixture, samples in group III and IV were all negative by incubation at 37 C for 60 min but positive at 24 C incubation for 60 min. Samples in groups I and II were all positive in the presence of EDTA at 37 C and at 24 C.

Dissociation of Antibody Bound to C100-3 Antigen at 37 C
Serum samples from group I and group III were added to C100-3 antigen-coated microwells and allowed to react at 24 C for 60 min (first reaction). After extensive washing at room temperature, the specimen diluent was added and the mixtures were incubated exactly at 24 C or 37 C for 60 min. After washing,
antibody bound to C100-3 antigen was measured by ELISA. As shown in Fig. 1, serum samples from group I did not dissociate after incubation at 37°C for 60 min. By contrast, serum from group III was first bound at 24°C but was dissociated from C100-3 antigen by incubation at 37°C for 60 min.

DISCUSSION

Although the sample size is small, we did encounter serum samples which gave positive reaction to C100-3 antigen by shorter incubation (30 min) in an incubator presumably held at 37°C but negative by the standard incubation time (60 min) in the same incubator. Actually the real temperature inside the incubator was not high enough in the shortened reaction time. When we carried out the reaction exactly at 24°C or at 37°C, serum samples of this group reacted positively to C100-3 antigen at 24°C for 60 min but not at 37°C for 60 min. These are such antibodies whose reaction with C100-3 antigen is dependent upon a lower temperature and which become negative because of dissociation at 37°C.

The serum complement system has been known to inhibit antigen-antibody reaction (5) or solubilize the antigen-antibody complex (6). However, in the presence of 0.04 M EDTA, which chelates out Ca++ and Mg++ in the reaction mixture, the serum samples in groups III and IV showed lower temperature dependency as did in the specimen diluent without EDTA. Therefore, this phenomenon seems to be dependent not on the complement reaction but rather on the low avidity antibody itself.

There is evidence showing that the low avidity antibody can be dissociated with a mild protein-denaturing agent (8) and in the case of the C100-3 antigen-antibody system, 8 M urea disassociated the low avidity antibody bound to antigen (8). Our observation of the antibody with lower temperature dependency is in line with this. However, it should be noted that in apparently healthy blood donors as described in group II and group III, even though the antibody was dissociated by treatment with 8 M urea, other markers of HCV infection such as 4-RIBA test and anti-GOR test were positive. Moreover, HCV-RNA was detected by nested double PCR with 5' non-coding region of the HCV genome. From these results, it would be improper to conclude that the reactivity of HCVAb disappeared after urea treatment because of a non-specific false-positive reaction. It is more difficult to say that 8 M urea treatment can distinguish between
specific HCV antibody and a non-specific reaction (9) or can correct false-positive anti-HCV results (10). So, even though antibody activity disappeared after 8 M urea treatment in groups II and III, these serum samples were really specific for HCV infection.

Furthermore, the serum samples in group III were all negative by the standard method (37 C 60 min) but positive by shorter incubation or incubation at 24 C for 60 min. HCV-RNA was detected in two or three samples of this group as well as anti-GOR antibody. So, they should not be called false positive or non-specific for CV infection.

In group IV, almost all samples were positive (absorbance higher than the cut-off value) at 24 C, but negative at 37 C and other markers of HCV infection could not be detected. They may be truly non-specific or they might be due to cross reaction to other antigens or antibody produced in a very early stage of HCV infection or by past HCV infection. To confirm the immunological specificity, the specific inhibition test with purified antigen is necessary.

These results raised a problem of the avidity of anti C100-3 antibody in relation to HCV infection. The real nature of the antibody of this type is now under investigation to clarify the immunochemical and clinical significance in HCV infection. Moreover, our finding, particularly with respect to group III serum samples, raised an important problem in screening for blood infected with HCV.

To improve the preventive effect of current HCV screening, adding antigen-epitopes other than C100-3 to the test reagents may improve the sensitivity. On the other hand, the method should be improved to capture most effectively both low temperature-dependent antibody of this type and the ordinary antibody present in active HCV infection.

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