Development of a Fully Automated Chemiluminescent Enzyme Immunoassay for GOR Antibody


Summary We developed an anti-GOR assay using the Luminomaster™ system, a fully automated, random access chemiluminescent enzyme immunoassay system. GOR antibody, which is often detected in the serum of patients infected with Hepatitis C virus (HCV), is closely associated with HCV infection. This new assay provides detection of GOR antibody in 45 min, about half the time of existing EIA methods. The standard curve is 0.1-300 GOR unit/ml (1/10th-300 times cut-off value), giving a detection range about 30 times greater than that of existing assays. This greater detection range allows quantitative determination of low-titer samples without the need for additional detection procedures. This assay can also be used to evaluate the clinical efficacy of interferon (IFN) therapy in patients with HCV.

Keywords: GOR antibody, CLEIA, Luminomaster

Introduction

In the 1970s and 1980s, it became clear that non-A non-B Hepatitis (NANBH) was the most common cause of posttransfusion hepatitis. In the course of their investigation into NANBH, Mishiro et al. reported the isolation of a cDNA clone, designated GOR47-1, from the plasma of a chimpanzee infected with human NANBH agent. The epitope of this clone is not encoded by host cellular sequences. The GOR antibody developed by these workers for the screening and time-course observation of HCV antibody is encoded by a gene found in both humans and chimpanzees. The peptide coded by the GOR gene (GOR47-1) is GOR antigen; the amino sequence of this antigen has five amino acids corresponding with a part of the HCV core protein. GOR antibody, which recognizes the GOR epitope, is often found in the plasma of HCV patients in the infectious stage. Furthermore, the presence of GOR antibody is closely correlated with HCV infection. GOR antibody is therefore used as a marker in serum diagnosis of HCV, as well as in HCV antibody kits for the detection of C100-3 antibody or core antibody.

We have developed an anti-GOR assay using the Luminomaster™ system, a fully automated, random access chemiluminescent enzyme immunoassay system. The automated operation of this assay reduces biohazard risk to health care workers. The assay employs the sp-GOR2 peptide (27 amino acids), which shares some sequences...
Fig. 1 Homology between the GOR epitope and HCV core

with HCV core antigen shown in Fig. 1b. The peptide is immobilized on the tube and the anti-human IgG is labeled with glucose oxidase. After immuno reaction the enzyme activity is detected by chemiluminescence using luminol and microperoxidase.

This report describes the basic elements of automated GOR antibody detection in the Luminomaster™ system3).

Materials and Methods

Reagents

Glucose oxidase (GOD) (EC1.1.3.4, 104 U/mg) was purchased from Toyobo Co., Ltd. (Japan), luminol from Tokyo Chemical Industry Co., Ltd. (Japan), microperoxidase (m-POD) from Sigma Chemical Co., Ltd. (USA), bovine serum albumin (BSA) from Wako Pure Chemical Industries, Ltd. (Japan), and antibody-labeled binding reagent (N-e-maleimidocarproyloxy succinimide, EMCS) and 2-(cyclohexylamino) ethane sulfonic acid (CHES) from Dojindo Laboratories (Japan). All other reagents were of analytical grade.

Reagents (Solution)

A GOR antigen-immobilizing reagent and a washing reagent for the solid phase were prepared at pH 7.0 with 10 mmol/l of sodium hydrogen phosphate-10 mmol/l of sodium dihydrogen phosphate. Immuno reaction buffer and post coating solution were prepared by the same reagent with 0.1% BSA. Substrate was prepared at pH 5.8 with 50 mmol/l acetic acid-50 mmol/l sodium acetate and 18 mg/ml of glucose. Chemiluminescent reagent was prepared at pH 9.5 with 10 mmol/l luminol and 120 mg/l m-POD in 10 mmol/l CHES.

Preparation of the GOR antigen-coated tube

Three hundred microliter of sp-GOR2 peptide solution (2.5 μg/ml) was poured into a polystyrene tube and left to stand overnight at 4°C. The tube was then washed with immuno reaction buffer and 1 ml of post coating solution was added.

Preparation of enzyme-labeled antibody

GOD was labeled by the maleimido method to F(ab’)2 fragment produced by pepsin proteolysis from anti-human IgG0). The labeled antibody was further diluted with a reaction buffer for the labeled enzyme at the time of use.

Apparatus

A Luminomaster™ LEIA-2000 (Sankyo Co., Ltd. (Japan)) was used.

Analysis of the polymerase chain reaction

Determination of the RNA sequence of HCV was entrusted to a Special Reference Laboratory (Japan)5).

Methods

Thirty microliter of sample solution previously diluted 10 times with an automatic diluting system in the Luminomaster™ and 270 μl of reaction buffer were placed in a tube coated with spGOR2 peptide. The first immuno reaction was started under stirring for 15 min at 37°C. After washing with buffer, 300 μl of GOD-labeled anti-human IgG was added, and the second immuno reaction was started under stirring for 15 min at 37°C. After washing again with reaction buffer, 300 μl of substrate solution was added to produce an enzyme reaction under stirring for 15 min at 37°C. One hundred microliters of the reacted solution was then mixed with 100 μl of the luminescent reagent to measure luminescence. The Luminomaster™ is a fully automated apparatus which performs all functions from sample dispensing to data output.

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Results

Standard curve and standardization
A commercial ELISA kit (Institute of Immunology Co., Ltd., Japan) was used as the reference standard method. The absorption rate of 0.3, the cut-off value of this ELISA kit, was set at a GOR antibody concentration of 1 GOR unit/ml. Figure 2 shows that the standard curve for GOR antibody could be calculated from 0.1 to 300 GOR unit/ml. The assay developed here has a wide standard curve range, about 30 times greater than that of existing ELISA methods, and can be used in the Lumino-master™ system.

Precision of the assay
Simultaneous reproducibility (n=10) was between 2.3% and 4.6% when 5 kinds of serum were used, namely a GOR antibody-negative control sample (0.37 GOR unit/ml), a sample near the cut-off value (1.18 GOR unit/ml), and 3 positive samples with low, intermediate, and high GOR antibody concentrations (3.36, 9.72, 74.7 GOR unit/ml, respectively).

Interday reproducibility over five days was between 4.7% and 5.9% on the same samples. This study was conducted using a stored calibration curve.

Dilution curves
Accuracy of this anti-GOR assay was investigated using dilutions of serum samples. Three different GOR antibody serum samples (0.80, 9.72, 74.7 GOR unit/ml) were serially diluted with reaction buffer and the GOR antibody concentration was determined. As shown in Fig. 3, the dilution curves were linear and passed through the origin.

Good linearity was confirmed, even at a range lower than the cut-off value of 1 GOR unit/ml. This showed that quantitative assay from a range lower than cut-off value to high concentrations is available.

Recovery Test
GOR antibody-supplemented serum samples were prepared by adding known concentrations of exogenous GOR antibody to four serum samples. Analytical recovery was assessed by analyzing the samples before and after supplementation. As shown in Table I, recovery ranged from 97.0% to 106.8%.

Qualitative agreement with enzyme immnosorbert assay (ELISA)
Serum samples were collected from 298 healthy controls and 197 patients with liver dysfunction (＞35U/ml glutamic pyrvic transaminase) and HCV infection, and the results for the this assay and the ELISA method were compared. Qualitative agree-
Table I  Recovery test of GOR antibody assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (GOR U/ml)</th>
<th>Found (GOR U/ml)</th>
<th>Recovery (%)</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>0.37</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.17</td>
<td>0.46</td>
<td>97.3</td>
</tr>
<tr>
<td></td>
<td>1.17</td>
<td>1.34</td>
<td>106.0</td>
</tr>
<tr>
<td>B</td>
<td>1.17</td>
<td>1.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.34</td>
<td>2.64</td>
<td>98.3</td>
</tr>
<tr>
<td></td>
<td>3.34</td>
<td>4.85</td>
<td>100.6</td>
</tr>
<tr>
<td>C</td>
<td>3.34</td>
<td>9.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>13.06</td>
<td>97.0</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>22.46</td>
<td>101.1</td>
</tr>
<tr>
<td>D</td>
<td>33.0</td>
<td>82.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>116.1</td>
<td>115.9</td>
<td>102.7</td>
</tr>
</tbody>
</table>

A recovery of 100% was obtained in these 495 samples. The values were blotted using a cut-off index (C.O.I.) in the case of ELISA and by GOR antibody concentration in the case of the this assay. Values over the cut-off values were judged positive. Furthermore, values over the quantitative determination range were set at a C.O.I. of over 10 and blotted as shown in Fig. 4.

The two methods showed good correlation in the positive range, although small deviations were seen in some samples. This assay can measure samples with high levels of GOR antibody from patients with liver dysfunction or HCV infection. This enables quantitative assay in the high abnormal range, and together with its previously mentioned good linearity, confirms the usefulness of this assay in clinical use.

Sample Distribution

The values for the 298 normal control samples, with a C.O.I. of less than 1, showed a normal curve (mean ± SD: 0.34 ± 0.08) as shown in Fig. 5a. The results of screening for the 98 liver dysfunction samples (4.83 ± 14.30) and 99 HCV patients samples (9.29±20.94) are shown in Figs. 5b and 5c. The minimum value for clear classification was confirmed at about 1 GOR unit/ml. This assay produced no data higher than the C.O.I. from the normal samples but much higher values than the C.O.I. from liver dysfunction and HCV samples. From this result, we believe that this assay will not produce false positive results in normal specimens. The frequencies of false positive are screening of GOR antibody, which results in more effective screenings. Furthermore, because GOR antibody level is higher in the patients infected with HCV than in those with liver dysfunction, it seems that higher levels of GOR antibody are produced in HCV infection.

Application to time course observation of patients receiving interferon (IFN) therapy

Figure 6 shows the correlation of time course between administration of IFN and changes in GOR antibody in patients with HCV. IFNa-2b (10 million units) was
Liver dysfunction

Fig. 5 Sample distribution of GOR antibody in serum from (a) normal controls, (b) patients with liver dysfunction, and (c) patients with HCV infection

administered by single daily administration for two weeks and thereafter three times per week for 12 weeks.

Cases in which therapy was ineffective are marked with a circle (○). In these cases, GOR antibody levels decreased temporarily on administration, but increased again after administration and HCV-RNA became positive. Cases in which treatment was effective are indicated with a triangle (△) or square (□). The triangle indicates that GOR antibody level decreased soon after the start of administration, with HCV-RNA becoming negative and GOR antibody level decreasing still further after the end of administration. The square shows that GOR antibody level decreased at the start of administration but did not change much thereafter, and that HCV-RNA was negative after the end of administration. These results indicate that measurement of

Fig. 6 Application to time course observation of IFN therapy patients

GOR antibody levels is useful in determining therapeutic efficacy over time of IFN treatment in patients with HCV.

Discussion

We have developed an anti-GOR assay using chemiluminescence and applied this method to the screening and monitoring of IFN treatment in patients infected with HCV. This method requires only 45 min. from the dispensing of specimen into sample cups until the output of test data. Its full automation reduces biohazard risk in infectious disease assay. The wide dynamic range of Luminomaster™ is about 30 times greater than that of the existing ELISA. This is due to the use of a combined double channel (high and low sensitivity) detection system. High sensitivity based on chemiluminescence enables detection of lower concentrations of antibody and quantitative assay at negative levels, which is impossible with the existing method. Moreover, because it has sufficient reproducibility, it is not necessary to obtain a second calibration curve within a week and real time data
management becomes possible. These advantages reduce reagent cost and labor. In GOR antibody screening, this method produced a mean value and SD of GOR antibody-negative samples of 0.34 and 0.08 units/ml. It was also confirmed that mean value plus 8SD of negative specimens reaches Cutoff Value 1. Because this assay is unlikely to produce false negative results, we consider it to be suitable for use in GOR antibody screening. In addition, the marked difference in mean GOR antibody level value between patients with liver dysfunction and those with HCV allows the differential diagnosis of these conditions. It was been confirmed that changes in GOR antibody levels in the monitoring of IFN treatment can show not only the presence of HCV-RNA but also the results of IFN treatment. Whereas HCV-RNA detection by PCR takes about one day, the present system requires only 45 min. This assay therefore offers advantages in both speed and cost. Further study is planned on the relationship between GOR antibody level and RNA genotype in the treatment of GOR from these application examples to clarify the mechanism of GOR antibody.

Conclusion

This anti-GOR assay system in Lumimaster™ offers the following advantages over the present assay:
1. Detection can be made within a shorter time and with good reproducibility
2. The detection range is wide, allowing a reduction in retesting after the dilution of sample
3. This system allows the determination of the therapeutic efficacy over time of IFN treatment in patients with HCV.
4. This system can be used in screening for HCV.
5. The full automation of this system reduces biohazard risk to health care workers.

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References

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