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Pretreatment of Test Sera with Three Kinds of IgG-Adsorbents Did Not Improve Sensitivity in the Indirect ELISA to Detect Anti-Dengue IgM Antibodies

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Abstract: Sera from clinical dengue cases in Malaysia were examined to detect IgG- as well as IgM-class anti-dengue antibodies by the indirect ELISA with or without treatment by 3 kinds of IgG-adsorbents. Relative adsorption factor (AF) as determined by the decreased ELISA-OD ratio was 0.42–0.58 for IgG and 0.33–0.39 for IgM, respectively. After IgG-adsorbents treatment, however, the number of anti-dengue IgM-positive serum specimen decreased from 11–20% of the untreated control. The result indicated that pretreatment of test sera with IgG-adsorbents did not improve or even reduced the sensitivity to detect anti-dengue IgM antibodies in the test sera.

Key words: IgM-ELISA, dengue, IgG-adsorbents

INTRODUCTION

Dengue fever (DF) and dengue hemorrhagic fever (DHF), acute febrile diseases caused by dengue viruses of 4 serotypes (D1, D2, D3, D4), have been major health problems in many tropical countries especially in southeast Asia (Halstead, 1966; 1980; 1992). For laboratory diagnosis in DF/DHF, hemagglutination-inhibition (HI) test (Clarke and Casals, 1958) has long been used as a gold standard. Recently, however, detection of IgM-class antibodies by hemadsorption immunosorbent test (Gunasegaran et al., 1986), or ELISA (Bundo and Igarashi, 1985; Lam et al., 1987; Innis et al., 1989) has been used to replace or supplement the HI test. In the IgM antibody assay, IgM-capture ELISA (MAC-ELISA) has frequently been used (Duermeyer and van den Veen, 1978), because of the following advantages. As discussed by Burke and Nisalak (1982) for the IgM-capture radioimmunoassay on JE, IgM-capture method can eliminate competitive binding of IgG and IgM antibodies to the assay antigen which leads to false negative result. Secondly, crude antigens can be used in the IgM-capture method. However, in the anti-dengue MAC-ELISA, enzyme-conjugated anti-
flavivirus antibodies has to be prepared to detect viral antigens which have been bound to the captured IgM-antibodies. Although this conjugation can be replaced by already available anti-dengue monoclonal antibodies followed by enzyme-conjugated anti-mouse IgG, which should be free from cross-reaction with human immunoglobulins (Lam et al., 1987). Although the indirect ELISA requires relatively purified antigen to coat the microplate to capture antibodies, the method is simpler and straightforward, if competitive binding of IgG and IgM antibodies can be eliminated.

In this communication, 3 kinds of available IgG-adsorbents were used to pretreat dengue patients sera, and their effect to remove IgG and IgM anti-dengue antibodies was measured by the indirect ELISA. The effect of such treatment on the detection of anti-dengue IgM antibodies was discussed in terms of diagnostic efficacy.

**MATERIALS AND METHODS**

**Test sera:** One hundred serum specimens were selected out of the stock of patients’ sera sent to the Division of Virology, Institute for Medical Research (IMR), Kuala Lumpur, Malaysia, for laboratory confirmation. An aliquot of each specimen was carried by the first author to the Department of Virology, Institute for Tropical Medicine, Nagasaki University. The sera were diluted 1:100 in PBS-T (phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 and 0.02% NaN₃) for the ELISA.

**Cell culture:** *Aedes albopictus* clone C6/36 cell line was grown in Eagle’s medium in Earle’s saline supplemented with 9% heat-inactivated fetal calf serum and 0.2mM each of nonessential amino acids at 28°C (Igarashi, 1978).

**Preparation of purified dengue virion antigen:** Seed of dengue virus type 2 (D2) New guinea B strain was inoculated to the stationary culture C6/36 cells in 500ml volume bottles. After 7 days incubation at 28°C using the maintenance medium (cell growth medium from which serum concentration was reduced to 2%), the infected fluid was harvested and inoculated into spinner culture C6/36 cells in 1 liter volume (Morita and Igarashi, 1989). The culture was incubated at 28°C for 7 days, and infected culture fluid was harvested. Virion was concentrated by 6% polyethylene glycol precipitation and purified by 30–50% sucrose gradient sedimentation (Srivastave et al., 1987). Aliquots of the purified virion were stored at −70°C until use.

**Indirect ELISA:** The principle was described by Conroy et al. (1991). The flat-bottom 96-well ELISA plate (Nunc) was coated with purified D2 virion diluted in a coating buffer (0.05M sodium carbonate-bicarbonate buffer, pH 9.6), at 4°C overnight. The optimal dilutions of the D2 antigen and horseradish peroxidase (HRPO)-conjugated anti-human IgG and IgM were predetermined by the checkerboard titration. The plate was inactivated by incubation with Block Ace (Yukijirushi) at 37°C for 1 hr. The plate was emptied and washed 4 times with PBS-T using Microplate Washer (Titertek, M96V, Flow). The plate was reacted with diluted test sera along with standard positive and negative controls by incubating at 37°C for 1 hr. The plate was emptied and washed as above and reacted with HRPO-con-
jugated anti-human IgG or IgM (Cappel) diluted in PBS-T, at 37°C for 1 hr. The plate was emptied and washed as above followed by the incubation with substrate solution (0.5 mg/ml o-phenylenediamine dihydrochloride and 0.02% H₂O₂) at room temperature for 1 hr in the dark. The HRPO reaction was stopped by adding 1 N H₂SO₄ and OD₄₇₄ was recorded by an ELISA Analyzer (ETY-96, Tohyoh) using OD₆₃₀ as a reference wavelength. The cut-off value of the ELISA-OD was defined as the double of the average OD of 8 negative control wells. The positive/negative ratio (P/N ratio) was calculated by dividing ELISA-OD of each test specimen by the cut-off value. Any specimen showing P/N ratio equal to or greater than 1.0 were considered as positive.

IgG adsorbents: Three preparations were used in the test: (1) RF Adsorbent (Behringer), the product of protein G, (Bjork and Kronvall, 1984), (2) Absorb G (Kaketsuken), the formalin-fixed Staphylococcus aureus Cowan I strain containing protein A, (Forsgren and Sjoquist, 1966), and (3) Streptococcus pyogenes, mixture of AR1 and AW43 strains (Kronvall et al., 1984). The RF-Adsorbent was commercially obtained, while Absorb G and Str. pyrogenes preparations were kindly supplied by Chemoserothapeutic Institute (Kaketsuken). All preparations were used according to the manufacturer’s instruction. The efficiency of adsorption (AF) was calculated by the following formula:

\[
AF = \frac{\text{ELISA-OD (control)} - \text{ELISA-OD (treated)}}{\text{ELISA-OD (control)}}
\]

RESULTS

Comparative efficacy to remove IgG and IgM anti-dengue antibodies by IgG adsorbents

Indirect IgG- and IgM-ELISA were performed for the serum specimens treated and untreated with 3 kinds of IgG-adsorbents. The results were summarized to see the efficacy of IgG-adsorbents to remove anti-dengue IgG and IgM antibodies, as shown in Fig. 1 and Table 1.

In Fig. 1, each dot represents each test serum and its AF of IgG is shown on the X-axis, while AF of IgM on the Y-axis, respectively. Since relative amount of anti-dengue IgG and IgM antibodies is different in each test serum, it is not surprising that relatively low correlation coefficient (R) was observed between IgG-AF and IgM-AF [0.106 for RF-Adsorbent, 0.139 for Absorb G, and 0.096 for Str. pyrogenes, respectively]. Therefore, the inclination of the regression line calculated for each IgG-adsorbent [0.641 for RF-Adsorbent, 0.388 for Absorb G, and 0.426 for Str. pyrogenes, respectively] cannot be considered to represent relative efficacy to remove IgG compared with IgM antibodies.

Table 1 shows the average and standard deviation of IgG-AF and IgM-AF calculated for these 3 reagents. The average IgG-AF was highest for RF-Adsorbent (0.589), followed by Str. pyrogenes (0.472), then Absorb G (0.424). These figures were higher than the IgM-AF for each of the 3 IgG-adsorbents: RF-Adsorbent (0.393), Absorb G (0.354), and Str. pyrogenes (0.330), respectively. Statistical test showed that the difference between IgG-AF and IgM-AF
Fig. 1. Correlation between the absorption factor (AF) for anti-dengue IgG and IgM antibodies from dengue patients' sera determined by the indirect ELISA. IgG adsorbents used were RF Adsorbent (Behringer) in panel A, Absorb G (Kaketsuken) in panel B, and *Str. pyrogenes* in panel C. Each dot represents each test serum with IgG-AF on the X-axis and IgM-AF on the Y-axis, respectively. Simple curve fitting was used to obtain regression line with correlation coefficient (R).
Table 1. Average and standard deviation (SD) of AF for IgG and IgM treated with IgG adsorbents

<table>
<thead>
<tr>
<th>IgG adsorbents</th>
<th>Immunoglobulin</th>
<th>Adsorption factor (AF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average</td>
</tr>
<tr>
<td>RF Adsorbent</td>
<td>IgG</td>
<td>0.589</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>0.393</td>
</tr>
<tr>
<td>Absorb G</td>
<td>IgG</td>
<td>0.424</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>0.354</td>
</tr>
<tr>
<td>Str. pyrogenes</td>
<td>IgG</td>
<td>0.472</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>0.330</td>
</tr>
</tbody>
</table>

was significant for RF-adsorbent and Str. pyrogenes. (P<0.05). Also significant was the difference between the IgG-AF for RF-adsorbent and Absorb G (P<0.05).

Detection of anti-dengue IgM antibodies by the indirect ELISA with and without treatment with IgG adsorbents.

Next, the data was analyzed to see whether the anti-dengue indirect IgM ELISA result was affected by treatment of test sera with IgG-adsorbents, as shown in Fig. 2 and Table 2.

In Fig. 2, each dot represents each test serum with its P/N ratio of the indirect IgM ELISA in the control specimen on the X-axis and the P/N ratio of the treated specimen on the Y-axis, respectively. Correlation coefficient (R) between these 2 parameters [0.377 for RF-Adsorbent, 0.526 for absorb G, and 0.701 for Str. pyrogenes] showed that there were certain degree of correlation between the P/N ratio with and without IgG-adsorbent treatment. The inclination of the regression line was 0.371 for RF-Adsorbent, 0.205 for Absorb G, and 0.695 for Str. pyrogenes, respectively. The figure can be considered to represent relative amount of anti-dengue IgM antibodies remaining after treatment with IgG-adsorbent.

Table 2 shows average and standard deviation of the P/N ratio of the anti-dengue indirect IgM ELISA with or without treatment with 3 kinds of IgG-adsorbent. The average P/N ratio became always lower in the treated specimen [0.524–0.547] compared with the control [0.756–1.194]. However, the difference was not statistically significant [P>0.1]. Also the difference between the average P/N ratio obtained by 3 kinds of IgG adsorbents were not statistically significant [P>0.1].

The cut-off value of the negative control serum was not significantly reduced by the treatment with IgG-adsorbents. Therefore, the number of positive sera with anti-dengue IgM antibodies decreased by treatment of IgG adsorbents. The number of positives decreased from 37 to 4 (10.8%) in the experiment with RF-Adsorbent, from 44 to 8 in the experiment with Absorb G, and from 25 to 5 in the experiment with Str. pyrogenes, respectively.
Fig. 2. Correlation between the P/N ratio of anti-dengue indirect ELISA with or without treatment with IgG adsorbents
IgG adsorbents used were RF Adsorbent (Behringer) in panel A, Absorb G (Kaketsuken) in panel B, and Str. pyrogenes in panel C. Each dot represents each test serum with P/N ratio of the control on the X-axis and P/N ratio of the treated specimen on the Y-axis, respectively. Simple curve fitting was used to obtain regression line with correlation coefficient (R).
Table 2. Average and standard deviation (SD) of the P/N ratio in the indirect dengue IgM ELISA with and without treatment of IgG adsorbents

<table>
<thead>
<tr>
<th>IgG adsorbent</th>
<th>treatment</th>
<th>P/N ratio of IgM ELISA</th>
<th>Average</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RF Adsorbent</td>
<td>control</td>
<td>1.109</td>
<td>0.970</td>
<td></td>
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<tr>
<td></td>
<td>treated</td>
<td>0.524</td>
<td>0.587</td>
<td></td>
</tr>
<tr>
<td>Absorb G</td>
<td>control</td>
<td>1.194</td>
<td>0.925</td>
<td></td>
</tr>
<tr>
<td></td>
<td>treated</td>
<td>0.547</td>
<td>0.261</td>
<td></td>
</tr>
<tr>
<td>Str. pyrogenes</td>
<td>control</td>
<td>0.756</td>
<td>0.525</td>
<td></td>
</tr>
<tr>
<td></td>
<td>treated</td>
<td>0.525</td>
<td>0.455</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Assay on IgM class antibodies after treatment of IgG adsorbents was successfully reported for rubella (Ankerst et al. 1974; Handsher and Fogel, 1977; Yoshikawa, 1978a, b; Kawano et al., 1986) as well as for human immunodeficiency virus (Weihlen et al., 1990). Although we did not test these viruses in parallel to monitor our experimental procedures, the data obtained in this study indicate that treatment of dengue patient’s sera with presently available IgG adsorbents followed by the indirect ELISA could lead to false negative results. As described in the accompanying paper, the selection of the assay antigen and specificity of the enzyme-conjugated detecting antibodies in the MAC-ELISA will also provide different results in the IgM-ELISA.

**ACKNOWLEDGMENTS**

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