Quantitation of Biotin-Binding Immunoglobulins G, A, and M in Human Sera Using F(ab’)_2 Anti-human Immunoglobulin-Coated Microplates

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Biotin-binding IgG (B-IgG) in human sera was quantified using previously developed F(ab’)_2 anti-human IgG-coated multiwell microplates (Muratsugu M. et al., 2003, Biol. Pharm. Bull., 26, 1605—1608). The levels of B-IgG in sera, however, were higher than those we predicted. In this study, we modified the assay using F(ab’)_2 anti-human IgG-coated multiwell microplates and successfully quantified the levels of B-IgG in sera. The cause of the unpredicted results was discussed in the text. In addition, the levels of biotin-binding IgA (B-IgA) and IgM (B-IgM) in sera could be measured using F(ab’)_2 anti-human IgA- or IgM-coated multiwell microplates. We quantified B-IgG, B-IgA, and B-IgM in sera from healthy specimens and patients with bronchial asthma, atopic dermatitis, epilepsy, and juvenile rheumatoid arthritis.

Key words biotin-binding immunoglobulin; biotin; F(ab’)_2 anti-human immunoglobulin; multiwell microplate; nonspecific binding

Biotin is formed in the prosthetic group in acetyl-CoA carboxylase (EC 6.4.1.2), pyruvate carboxylase (EC 6.4.1.1), propionyl-CoA carboxylase (EC 6.4.1.3), and 3-methylcrotonyl-CoA carboxylase (EC 6.4.1.4) in humans.1,2) In addition to these enzymes, there are other biotin-binding proteins such as avidin, streptavidin, egg-yolk biotin-binding protein, nuclear biotin-binding protein, biotinidase (EC 3.5.1.12), biotin protein ligase (EC 6.3.4.15), etc.3)

Apart from these proteins, immunoglobulins to which biotin was covalently linked were found in human sera for the first time in 1993.4) The prevalence of biotin-binding immunoglobulin was higher in patients with atopic dermatitis, other dermatitis, allergic disorders, and autoimmune disease.4—6) Recently, biotin-binding IgG (B-IgG) was detected in a multiwell microplate format for the first time.7) We developed a quantitative assay for B-IgG without any purification of samples using F(ab’)_2 anti-human IgG-coated multiwell microplates.8) We showed that the ratio of biotin to protein molecules (biotin-protein ratio) in B-IgG in human sera affected the detection limit in this assay9) and how to determine the biotin-protein ratio of B-IgG as a standard for the measurement of samples using F(ab’)_2 anti-human IgG-coated multiwell microplates.8) We showed that the ratio of biotin to protein molecules (biotin-protein ratio) in B-IgG in human sera affected the detection limit in this assay9) and how to determine the biotin-protein ratio of B-IgG as a standard for the measurement of samples using F(ab’)_2 anti-human IgG-coated multiwell microplates.8)

MATERIALS AND METHODS

Materials The following materials were obtained from the sources as indicated: flat-bottomed multiwell microplates (Immulon II) from Dynatech Laboratories, Inc., U.S.A.; 1,1,1-trichloro-2,2,2-trifluoroethane (TCTFE), from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan; N-hydroxyxyscinimidobiotin (NHS-biotin) and the BCA Protein Assay Reagent kit from Pierce Chemical Co., Rockford, U.S.A.; biotin, dimethyl sulfoxide (DMSO), human IgG, human IgA, human IgM, and bovine serum albumin (BSA) from Sigma Chemical Co., St. Louis, U.S.A.; goat F(ab’)_2 anti-human IgG, goat F(ab’)_2 anti-human IgA, and goat F(ab’)_2 anti-human IgM from Cappel, Organo Teknika Corp., Durham, U.S.A.; the VECTASTAIN Elite ABC kit (ABC kit) from Vector Laboratories Inc., Burlingame, U.S.A.; the ELISA Color Reagent kit (Type O) (CR kit) from Sumitomo Bakelite Co., Ltd., Tokyo, Japan. All other chemicals were of reagent grade or better. The water used was 17-Mohm grade.

Serum samples were provided by Dr. Y. Fujita, Department of Pediatrics, Nihon University School of Medicine, Dr. O. Nishihara, National Hospital Organization Okayama Medical Center, Dr. T. Watanabe, School of Human Science and Environment, University of Hyogo, Dr. T. Taketani, Kurobe City Hospital, and Dr. H. Yaoi, Yaoi Clinic. The patients were classified according to the clinical diagnosis and informed consent was obtained from all participants at each laboratory.

Preparation of Standards for B-IgG, B-IgA, and B-IgM

Twenty-five microliters of 13.3 mg ml⁻¹ NHS-biotin DMSO solution was added to 1 ml of 2 mg ml⁻¹ IgG, IgA, or IgM in 50 mMol l⁻¹ sodium bicarbonate/HCl buffer (pH 8.6). The mixture was gently stirred at room temperature for 30 min. The reaction solution was dialyzed against 0.15 mol l⁻¹ NaCl and finally against Dulbecco’s phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS). The amounts of standard biotinylated IgG (sB-IgG), sB-IgA, and sB-IgM were expressed as the amounts of IgG, IgA, and IgM, respectively, which were determined using a BCA Protein Assay kit with human IgG as standard.

The biotin–protein ratio of each standard biotinylated protein was determined using the previous method10); the ratios of sB-IgG, sB-IgA, and sB-IgM were 20, 23, and 100, respectively.

Quantitation of Biotin-Binding Immunoglobulins

Multiwell plates (96 wells) were coated with F(ab’)_2 anti-
human immunoglobulin by adding 100 μl of 75 μg ml⁻¹ F(ab')₂-anti-human IgG, 76 μg ml⁻¹ F(ab')₂-anti-human IgA, and 42 μg ml⁻¹ F(ab')₂-anti-human IgM or 1% BSA (background) PBS plus 0.02% NaN₃ (PBS-N) to each well, then incubating overnight at 6°C. After the wells were washed 3 times with PBS-N, 300 μl of 1% BSA in PBS-N (PBS-NB) was added to each well. The plates were incubated for 2 h at 37°C and then washed 3 times with PBS-N. One hundred microliters of dilutions of standard biotinylated immunoglobulins or diluted test sera (serum : PBS-NB 1:9) was added to F(ab')₂ antibody or BSA-coated wells. After incubation for 2 h at 37°C, the plates were washed 3 times with PBS-N and then twice with PBS containing 0.05% merthiolate (PBS-M). One hundred microliters of avidin-biotinylated horse radish peroxidase complex (HRP-ABC), which was prepared according to the directions in the ABC kit with slight modifications before use, was added to each well. After 1 h of incubation, five washes in PBS-N and two washes in PBS-M, 100 μl of substrate solution in the CR kit was added to each well. After the plate was incubated at room temperature for a fixed time, the color reaction was terminated by the addition of the reactive stop solution in the kit to each well. Absorbance was measured at 490 nm in the microplate reader (Model 450, Bio-Rad Labs., Richmond, U.S.A.) and the data were transferred to a computer using a home-made program for data analysis. 10) Absorbances in F(ab')₂ ant-immunoglobulin-coated wells and BSA-coated wells were designated A₀ and Aₐ, respectively, and ΔA was assigned to the difference between these absorbances (A₀ - Aₐ). This method is referred to as the F(ab')₂ ABC method in this study.

Trichlorotrifluoroethane-Treated Sera TCTFE was added to the serum (1:1 in volume), and then the mixture was vigorously agitated with the vortex mixer. After standing the mixture for 10 min, it was centrifuged at 900 × g for 10 min. The level of B-IgG in the supernatant was measured with the F(ab')₂ ABC method.

Statistics Significance was examined using Fisher’s exact probability test.

RESULTS AND DISCUSSION

Quantitation of B-IgG in Sera We investigated the sera of healthy specimens or patients using the F(ab')₂ anti-human IgG-coated multiwell microplates developed previously, and we found that the levels of B-IgG in sera were higher than those we predicted. Therefore, 100 μl of sera diluted with PBS-NB was added to F(ab')₂ anti-human IgG-coated wells and simultaneously to BSA-coated wells. The absorbance in each well was measured with the F(ab')₂ ABC method. The absorbances in F(ab')₂ anti-human IgG-coated wells were almost the same as those in BSA-coated wells for Serum No. 12, 27, 31, 36, 93, and 100 (Fig. 1A). Since the absorbance in the BSA-coated wells meant that HRP-ABC nonspecifically adsorbed onto the well, these sera did not contain B-IgG. The absorbances in F(ab')₂ anti-human IgG-coated wells were higher than those in BSA-coated wells for Serum No. 17, 50, 51, 57, 63, and 88 (Fig. 1B). The positive difference between absorbances in F(ab')₂ anti-human IgG-coated wells and those in BSA-coated wells (ΔA > 0) meant that B-IgG existed in the sera (Serum No. 17, 50, 51, 57, 63, 88), because the absorbance obtained for BSA-coated wells implied the nonspecific binding of HRP-ABC.

One hundred microliters of 2.5, 5.0, or 10 ng ml⁻¹ sB-IgG was added to F(ab')₂ anti-human IgG-coated wells and BSA-coated wells. The absorbance in each well was measured with the F(ab')₂ ABC method. The absorbances in F(ab')₂ anti-human IgG-coated wells elevated on increasing the concentration of sB-IgG added to each well (Fig. 2). On the other hand, the absorbances in BSA-coated wells were lower than those in F(ab')₂ anti-human IgG-coated wells and did not depend on the concentration of sB-IgG (Fig. 2). The results indicated that HRP-ABC did not adsorb onto the BSA-coated wells.

The higher absorbance in BSA-coated wells was observed when only sera were added to the wells, but not when sB-IgG solution was used. The result indicated that matter in sera nonspecifically adsorbed onto the F(ab')₂ anti-human IgG and/or BSA layers, and then HRP-ABC bound to the known/unknown substances in sera.

We treated the sera with TCTFE to investigate what caused the nonspecific adsorption of HRP-ABC.

Quantitation of B-IgG in the Trichlorotrifluoroethane-Treated Sera We examined the sera which showed the
same absorbance in F(ab')2 anti-human IgG-coated wells as that in BSA-coated wells (Group 1) or a higher absorbance in the former compared to the latter (Group 2). The supernatant consisting of the mixture of the serum and TCTFE was applied to the F(ab')2 ABC method to measure the level of B-IgG, and the results were summarized in Table 1. The absorbance ($A_{F(a b')}$) was calculated, and the levels of B-IgG, B-IgA, and B-IgM were obtained using the standard curves.

B-IgG, B-IgA, and B-IgM in 100 sera from healthy specimens were analyzed using the F(ab')2 ABC method (Table 2). The reference value was defined as the mean $\pm$ S.D. and the values of B-IgG, B-IgA, and B-IgM were 1.42, 3.33, and 3.05 ng ml$^{-1}$, respectively.

The ranges and positive percentages for B-IgG, B-IgA, and B-IgM are listed in Table 2. Positive percentages for B-IgG were significantly increased in bronchial asthma (28.3%), atopic dermatitis (48.7%), epilepsy (52.9%), and juvenile rheumatoid arthritis (62.5%) as compared to healthy specimens ($p<0.01$). Positive percentages for B-IgA were significantly increased in bronchial asthma (13.2%) and atopic dermatitis (20.5%) as compared to healthy specimens ($p<0.01$). Biotin-binding immunoglobulin was first detected in the sera of epilepsy using the qualitative method. Nagamine et al. reported that the frequency of detection of biotin-binding immunoglobulin using the qualitative method was significantly higher in patients with bronchial asthma (25.0%), atopic dermatitis (43.8%), and rheumatoid arthritis (8.3%) than in healthy controls. The results for bronchial asthma and atopic dermatitis in this study were similar to their results. No significant correlations were found by regression analysis between B-IgG and B-IgA, B-IgG and B-IgM, or B-IgA and B-IgM in bronchial asthma, atopic dermatitis, epilepsy, and juvenile rheumatoid arthritis (data not shown). The clinical significance of biotin-binding immunoglobulin is not clear at the present time, but the quantitative method we developed will be useful to elucidate the significance.

CONCLUSION

B-IgB, B-IgA, and B-IgM in human sera were successfully quantified using F(ab')2 ABC method in which sB-IgB, sB-

### Table 1. Effect of TCTFE on Sera

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum No.</th>
<th>Before TCTFE treatment</th>
<th>After TCTFE treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$A_{F(a b')}$</td>
<td>$A_{F(a b')}$</td>
<td>$A_{F(a b')}$</td>
</tr>
<tr>
<td>1</td>
<td>0.390±0.186</td>
<td>0.393±0.111</td>
<td>0.035±0.093</td>
</tr>
<tr>
<td>2</td>
<td>0.175±0.105</td>
<td>0.186±0.117</td>
<td>0.023±0.076</td>
</tr>
</tbody>
</table>

### Table 2. The Level and Positive Percentage of B-IgG, B-IgA, and B-IgM in Sera of Healthy Specimens and Patients

<table>
<thead>
<tr>
<th></th>
<th>B-IgG</th>
<th>B-IgA</th>
<th>B-IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range/ng ml$^{-1}$</td>
<td>Positive Percentage</td>
<td>Range/ng ml$^{-1}$</td>
</tr>
<tr>
<td>Normal</td>
<td>0.0—2.2</td>
<td>7</td>
<td>7.0</td>
</tr>
<tr>
<td>Bronchial asthma</td>
<td>0.0—10.3</td>
<td>15</td>
<td>28.3</td>
</tr>
<tr>
<td>Atopic dermatitis</td>
<td>0.0—11.7</td>
<td>19</td>
<td>48.7</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>0.0—11.4</td>
<td>37</td>
<td>52.9</td>
</tr>
<tr>
<td>Juvenile rheumatoid arthritis</td>
<td>0.3—12.4</td>
<td>5</td>
<td>62.5</td>
</tr>
</tbody>
</table>

* $p<0.01$.
IgA, and sB-IgM with a confirmed biotin–protein ratio were used for the standard, respectively. When measuring biotin-binding immunoglobulins in human sera, we must use BSA-coated wells to avoid the nonspecific binding of HRP-ABC. Lipids like chylomicron in sera appeared to cause the nonspecific binding.

REFERENCES