Detection of Biotin-Binding Immunoglobulin G in Human Sera Using Avidin-Coated Multiwell Microplates

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Biotin-binding human immunoglobulin G (B-IgG) was detected by two different methods in the avidin-coated multiwell format for the first time. In the first method, B-IgG was caught by avidin supported on the solid phase and was detected by alkaline phosphatase (ALP)-labeled anti-human IgG (method A). This method, however, was not able to distinguish B-IgG-positive and -negative human sera. The second method included peroxidase-labeled avidin instead of ALP-labeled anti-human IgG (method B). The sensitivity of method B was 28 times higher than that of method A. Method B in the multiwell-microplate format could detect B-IgG in the IgG fraction purified from human sera for the first time. This suggests that the level of B-IgG in human sera is very low.

Key words —— biotin-binding immunoglobulin, biotin, avidin, multiwell microplate

INTRODUCTION

Biotin is a water-soluble vitamin that acts as a prosthetic group of the carboxylases in humans.[1] These enzymes are involved in lipogenesis, glucogenesis, and branched-chain amino acid metabolism and are biotin-binding proteins.[1] In addition to these enzymes, there are other biotin-binding proteins.[2] Egg-white avidin and streptavidin derived from *Streptomyces avidinii* bind biotin very strongly.[3] Egg-yolk biotin-binding protein binds biotin with a lower affinity constant than that of avidin (streptavidin).[4] Nuclear biotin-binding protein binds biotin in nuclei and the binding appears to be reversible.[5] Human serum biotinidase, which releases biotin from biocytin, binds biotin with high and low affinities.[5] Biotin protein ligase covalently attaches biotin to proteins such as apodecarboxylase, apotranscarboxylase, *etc*.[6,7]

Another class of biotin-binding protein, which differs from the biotin-binding proteins described above which incorporate biotin into their active site, was found for the first time in 1993.[8] That biotin-binding protein was immunoglobulin covalently linked with biotin *in vivo* and the prevalence of biotin-binding immunoglobulin was higher in patients with atopic dermatitis, a dermatosis different from atopic dermatitis and allergic disorders.[9] Subsequently, biotin-binding immunoglobulins were also found in patients with autoimmune diseases.[10] The biotin-binding immunoglobulins were qualitatively detected using modified immunoelectrophoresis and/or an immunofixation method.[8–10]

In this study, we successfully detected biotin-binding immunoglobulin G (B-IgG) in human sera using avidin-coated multiwell microplates.

MATERIALS AND MEHODS

Materials ——The following materials were obtained from the sources indicated: flat-bottomed multiwell microplates (Immulon II) from Dynatech Laboratories, Inc., Chantilly, U.S.A.; avidin from Wako Pure Chemical Industries, Ltd., Osaka, Japan; BCA Protein Assay Reagent Kit from Pierce Chemical Co., Rockford, U.S.A.; bovine serum albumin (BSA) and peroxidase-labeled streptavidin from Sigma Chemical Co., St. Louis, U.S.A.; alkaline phosphatase (ALP)-labeled goat anti-human IgG (ALP-anti-hIgG), E-Y Laboratories, Inc., San Mateo, U.S.A.; *p*-nitrophenyl phosphate from Nakarai Tesque, Inc., Kyoto Japan; diethanolamine from Katayama Chemical Industries, Co., Ltd., Osaka, Japan; *o*-phenylenediamine (OPD) tablet and diluent for OPD from Abbott Laboratories, Abbott Park, U.S.A.; biotinylated human IgG from Vector Laboratories, Inc., Burlingame, U.S.A.; Consera from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; MabTrap™ GII from Amersham Pharmacia Biotech AB, Uppsala, Sweden; Microcon-50 from Amicon, Inc., Beverly, U.S.A.; and MBL plate IgG from Medical & Biological Laboratories Co., Ltd.,

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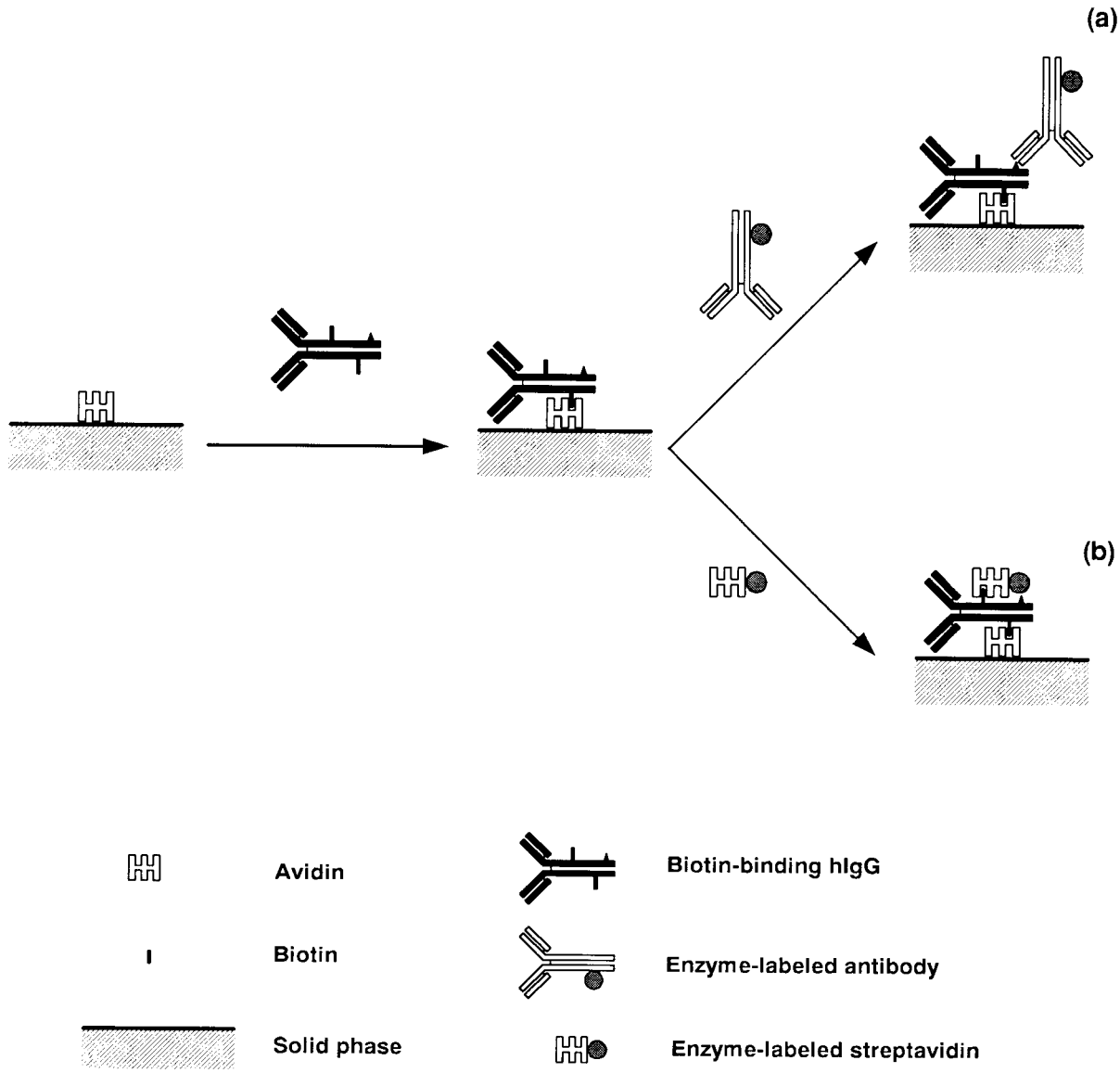

Fig. 1. Concept of the Detection of Biotin-Binding hIgG (B-IgG) in the Avidin-Coated Multiwell-Microplate Format

Nagoya, Japan. All other chemicals were of reagent grade or better. The water used was 17-Mohm grade.

Immunofixation ——B-IgG in sera was detected with the immunofixation method previously reported.[8–10] Briefly, B-IgG in sera was fixed to anti-human IgG coated on a cellulose acetate membrane and reacted with ALP-labeled avidin through the specific avidin-biotin interaction. 5-Bromo-3-indolylphosphate *p*-toruidine salt was used as a chromogen and a positive reaction was visible as an indigo blue stain.

Detection of B-IgG Using Multiwell-Microplate Methods —— Multiwell plates were coated with avidin by adding 100 μl of avidin 10 μg/ml in carbonate-bicarbonate buffer 0.05 mol/l plus 0.02% NaN_3 (pH 9.6) to each well and incubating them overnight at 6°C. After the wells were washed 3 times with Dulbecco's phosphate-buffered saline (PBS) (–) plus 0.02% NaN_3 (PBS-N), 1% BSA-PBS-N 300 μl was added to each well. The plates were incubated for 2 hr at 37°C and then washed 3 times with PBS-N. One hundred microliters of dilutions of commercially available B-IgG, test sera, or purified IgG fraction was added to each well. After incubation for 2 hr at 37°C, the plates were washed 3 times with PBS-N. The captured B-IgG was then detected using an enzyme-labeled antibody (method A) or enzyme-labeled streptavidin (method B). In method A (Fig. 1-(a)), 100 μl of a dilution of ALP-anti-hIgG was added to each well and

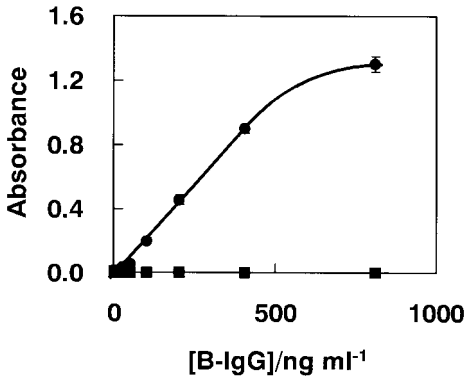

Fig. 2. Dose–Response Curves Using Method A

The closed circles are results from avidin-coated multiwell microplates and the closed squares from BSA-coated multiwell microplates. The vertical bars indicate the standard deviation for the mean of the experiments.

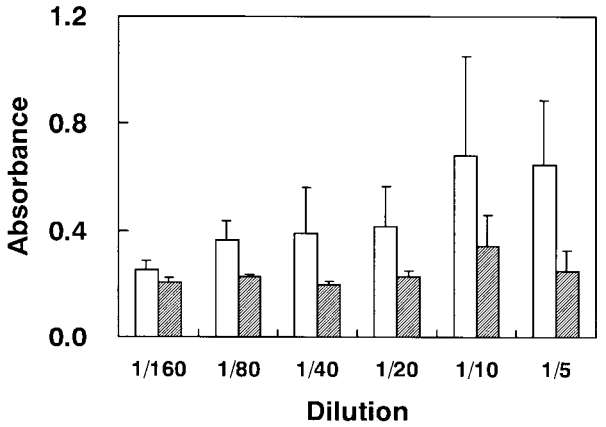

Fig. 3. Detection of B-IgG in Serum with Method A Using Avidin- (Open Bars) or BSA-Coated (Hatched Bars) Multiwell Microplates

The vertical bars indicate the standard deviation for the mean of the experiments.

the plates were incubated for 2 hr at 37°C. The wells were then washed 3 times with PBS-N and *p*-nitrophenyl phosphate 100 μl in a 10% diethanolamine buffer (pH 9.8) was added to each well before incubation at 37°C for the fixed time. The reaction was stopped by the addition of NaOH 3 mol/l to each well. Absorbance at 405 nm was then measured in a microplate reader (Wellreader SK6001, Seikagaku Corporation, Tokyo, Japan).

In method B (Fig. 1-(b)), 100 μl of a dilution of peroxidase-streptavidin was added to each well instead of ALP-anti-hIgG. After the plates were incubated for 1 hr at 37°C, the wells were washed 3 times with PBS-N and then 2 times with PBS. One hundred microliters of OPD 3 mg/ml containing 0.02% H_2O_2 was added to each well and the plates were incubated at 37°C for the fixed time. The reaction was terminated by the addition of 1 mol/l H_2SO_4 0.1 ml. Absorbance was measured at 492 nm in a microplate reader.

Purification of IgG Fraction from Human Sera —— The IgG fraction was purified from human sera using a protein G column (MabTrap™ GII kit). The IgG fraction was concentrated with Microcon-50 until its concentration reached the initial one. The radial immunodiffusion method (MBL plate IgG) was used for the quantitation of IgG.

RESULTS AND DISCUSSION

The concentrations of avidin and ALP-anti-hIgG used in the experiments were 10 μg/ml and 840 ng/ml, which were determined respectively prior to the following experiments (data not shown). Figure 2 shows a typical dose-response curve obtained using method A. Absorbance increased linearly in the range of 50 to 400 ng/ml B-IgG (commercially available) when multiwell microplates were coated with avidin, while absorbance did not change in the same range of IgG (data not shown). When using BSA-coated plates instead of avidin-coated plates, no dose-response relationship was observed (Fig. 2). These results indicate that ALP-anti-hIgG can specifically bind to B-IgG fixed to avidin on the solid phase. Since method A could detect commercially available B-IgG, the method was applied to the detection of B-IgG in human sera. Prior to this study, these sera were determined to be positive or negative for B-IgG using the immunofixation test previously developed.[8–10] The B-IgG-positive sera were collected from patients with atopic dermatitis. An initial serum sample (1 : 5 dilution with PBS) was serially diluted and each dilution was measured using method A. The absorbance in avidin-coated plates was significantly higher than that in BSA-coated plates (Fig. 3), showing the possible detection of B-IgG in sera. However, when absorbance obtained for the B-IgG-positive serum was compared with that for a commercially available (Consera) B-IgG-negative serum, there were no significant differences between the positive and negative sera (Fig. 4), indicating that IgG in sera may nonspecifically bind to the solid phase. This result suggests that a much more sensitive method is required to detect B-IgG in sera. The sensitivity of the method was defined as the change in absorbance for each concentration of the unit per minute, since the

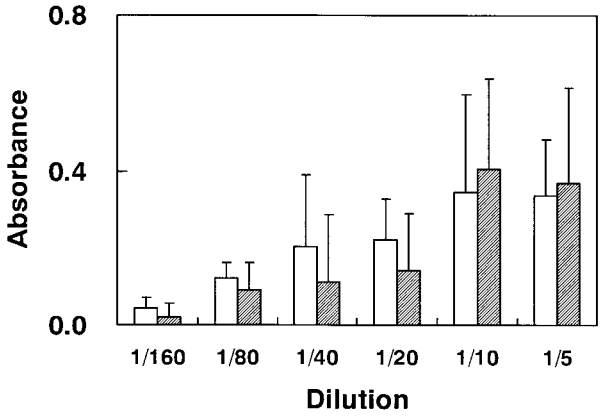

Fig. 4. Dose–Response for B-IgG-Positive (Open Bars) and B-IgG-Negative (Hatched Bars) Sera Using Method A
The vertical bars indicate the standard deviation for the mean of the experiments.

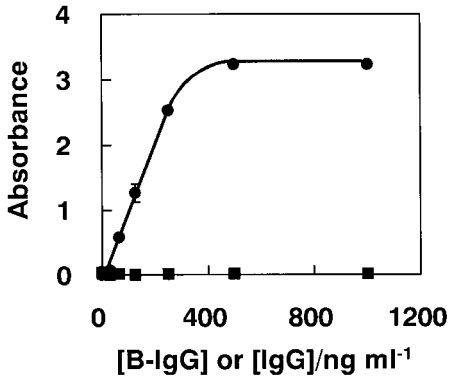

Fig. 5. Dose–Response Curves Using Method B
The closed circles are for B-IgG and the closed squares for IgG in avidin-coated multiwell microplates. The vertical bars indicate the standard deviation for the mean of the experiments.

intensity of absorbance depended on the reaction time in this study. The sensitivity of method A was 1.9×10^{-5} ml/ng/min.

In the final step of method B, peroxidase-labeled streptavidin was used instead of ALP-anti-hIgG. A 1 ng/ml peroxidase-labeled streptavidin concentration was found to be optimal to obtain higher absorbance. Figure 5 shows a typical dose-response curve obtained using method B. Absorbance increased linearly in the range of B-IgG 10 to 280 ng/ml and reached a plateau when multiwell microplates were coated with avidin, while absorbance did not change in the same range of IgG. In addition, absorbance changed little even when a solution with much higher concentration (5 mg/ml) was added to the wells. When using BSA-coated plates instead of avidin-coated plates, no dose-response curve was seen (data

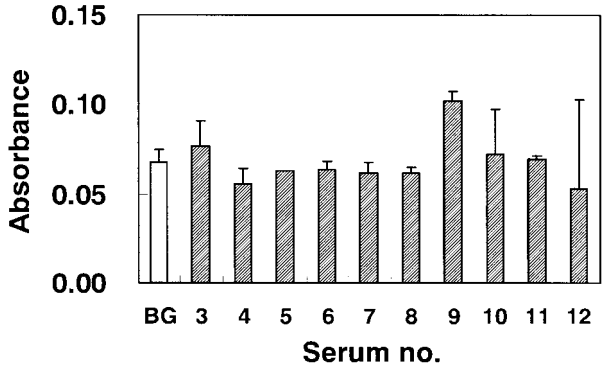

Fig. 6. Detection of B-IgG in Sera Using Method B
BG shows the mean of absorbances for diluted sera added to BSA-coated wells. The vertical bars indicate the standard deviation for the mean of the experiments.

not shown). The sensitivity of method B was 5.3×10^{-4} ml/ng/min, which was higher than that of method A. Thus method B was applied to the B-IgG-positive sera (Fig. 6). However, the absorbance due to the B-IgG-positive sera was similar to that obtained with the BSA-coated wells (background signal; BG). This undesirable result may depend on the presence of biotin-binding protein, biotinyl peptide, biotin-biotinidase complex, *etc*. in sera.[1] The IgG fraction was therefore purified from B-IgG-positive sera using a protein-G Sepharose column to remove these substances. The IgG fraction was concentrated until the IgG level returned to the initial level before purification, since the chromatographic technique dilutes samples. The prepared samples were analyzed using method B and the results are depicted in Fig. 7. The absorbances for B-IgG-positive sera (10 samples) were significantly higher than those for B-IgG-negative sera (6 samples), indicating that method B can detect B-IgG in sera. It should be noted that method B failed to detect B-IgG in 8 samples (serum nos. 5–12) before purification (Fig. 6).

To detect B-IgG in sera, a longer enzyme reaction time was required than that for detecting commercially available B-IgG. Because method B lacked sufficient sensitivity and the detection limit was about 10 ng/ml, we could not obtain reliable assay values for some serum specimens. These results show that the amount of B-IgG present in sera was very low and thus an improved method with higher sensitivity must be developed to measure concentrations of B-IgG less than 10 ng/ml. Method B is, however, valuable as the first assay procedure based on the multiwell-microplate format, which enables the detection of B-IgG in human sera.

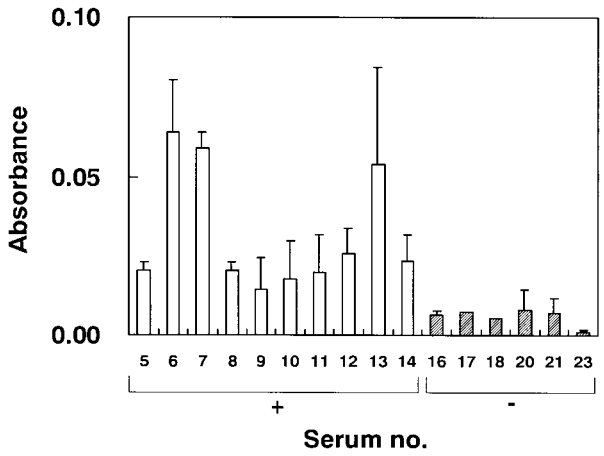

Fig. 7. Detection of B-IgG in the Fractions from B-IgG-Positve (Open Bars) and B-IgG-Negative (Hatched Bars) Sera Using Method B

+ and – indicate the sera in which B-IgG was detected and not detected using the immunofixation technique, respectively.

In this study, B-IgG in human sera was detected for the first time using a method with a multiwell-microplate format other than immunofixation or immunoelectrophoresis, which was previously developed by our colleagues,[8–10] confirming the existence of B-IgG in the sera of patients with atopic dermatitis. In addition, the results in this study indicate that the amount of B-IgG present in sera is extremely low. To measure B-IgG in human sera quantitatively, a more sensitive method that employs the multiwell-microplate format and does not require a sera purification step is required. We are now developing such an improved method for use as a routine method.

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