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Mini-Review

Detection of biotin-binding immunoglobulins in human sera

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ABSTRACT

Biotin-binding immunoglobulins (B-Igs) in sera from patients with atopic dermatitis, a dermatitis different from atopic dermatitis and allergic disorders, were first detected in 1993. B-Igs were first detected by modified immunoelectrophoresis: the combination of ordinary immunoelectrophoresis and avidin-biotin technique. The modified immuno-electrophoresis was time-consuming, thus, a simplified method (immunofixation method) was developed. The modified immunoelectrophoresis and the immunofixation method are qualitative methods. We developed quantitative methods in the avidin- or $F(ab')_2$anti-Ig-coated multiwell format. A more effective method for quantifying B-Igs in sera was established. The clinical significance of B-Igs is discussed in the text.

KEYWORDS: biotin-binding immunoglobulin, biotin, immunoelectrophoresis, immunofixation, avidin, streptavidin, $F(ab')_2$anti-immunoglobulin, multiwell microplate

INTRODUCTION

Biotin is formed in the prosthetic group in acetyl-CoA carboxylase (ACC), pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC) and 3-methyl-crotonyl-CoA carboxylase (MCC) in humans [1, 2]. In addition to these enzymes, there are other biotin-binding proteins [3]. Egg-white avidin and streptavidin derived from *Streptomyces avidinii* bind biotin very strongly [4]. Egg-yolk biotin-binding protein binds biotin with a lower affinity constant than that of avidin (streptavidin) [5]. Nuclear biotin-binding protein binds biotin in nuclei and the binding appears to be reversible [6]. Human serum biotinidase, which releases biotin from biocytin, binds biotin with high and low affinities [6]. Biotin protein ligase covalently attaches biotin to proteins such as apodecarboxylase and apotranscarboxylase [7, 8].

Apart from these proteins, immunoglobulins to which biotin is covalently linked were found in human sera for the first time in 1993 [9]. The symptoms (a red, inflamed and itchy rash) of a Japanese boy with atopic dermatitis but not biotin deficiency improved upon mass biotin administration. This indicated the presence of biotin not used *in vivo*, namely, protein-linked biotin (not free) for example. The patient serum was analyzed with ordinary electrophoresis using a cellulose acetate membrane and substances that bound to alkaline phosphatase (ALP)-labeled avidin were found in the γ-globulin fraction (unpublished data). We assumed that the substances were biotin-binding immunoglobulins (B-Igs).

On the basis of this assumption, we developed qualitative and quantitative methods detecting B-Igs in sera.

Modified immunoelectrophoresis

The sample to be analyzed was placed in wells on two agarose gel plates and electrophoresed, followed by immunodiffusion against anti-serum [10]. The gel plates were dried to be sheets. One gel sheet was stained with nigrosine for identification

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of the proteins. The other gel sheet was incubated with ALP-labeled avidin solution. After washing the gel sheet, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt solution was spread on the gel sheet and the enzyme reaction was stopped with acetic acid (Figure 1). This method was referred to as modified immunoelectrophoresis [9].

As shown in Figure 1, the modified immunoelectrophoresis could detect biotin-binding immunoglobulin G (B-IgG) in the serum of a patient with atopic dermatitis, but B-IgG was not detected in normal serum. This method was time-consuming (20 h), so a simpler method was desirable for a screening test.

Immunofixation

Anti-immunoglobulin (anti-Ig) was spotted on a cellulose acetate membrane. The membrane was incubated with the sample to be analyzed. After washing the membrane, it was soaked in ALP-labeled avidin solution for a constant time. After washing the membrane, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt was used as a chromogen and a positive reaction was visible as an indigo blue stain (Figure 2). As shown in Figure 2a, IgG (γ chain), IgA (α chain), IgM (μ chain), κ chain and λ chain in B-Igs were detected in the serum from a patient with atopic dermatitis, and the stains for IgM (μ chain) and κ chain were far weaker than those for IgG (γ chain), IgA (α chain) and λ chain. IgG (γ chain) and λ chain in B-Igs were detected in the serum from a patient with epilepsy (Figure 2b). On the other hand, B-Igs were not detected in the serum from a healthy subject (Figure 2c).

The immunofixation method could identify immunoglobulin classes and types of B-Igs using each antibody specific to class and type [9, 11], and subclasses of IgG (IgG1, IgG2, IgG3, IgG4) in B-IgG using each antibody specific to subclass [12]. The detection limit and the detection time of the immunofixation method were 0.2 μg mL^{-1} B-IgG (commercially available) and 5 h, respectively [12]. The immunofixation method was suitable for a screening test, but not a quantitative assay.

Avidin-coated microplate methods

B-IgG was caught by avidin supported on the solid phase (96-well microplate). After this step,

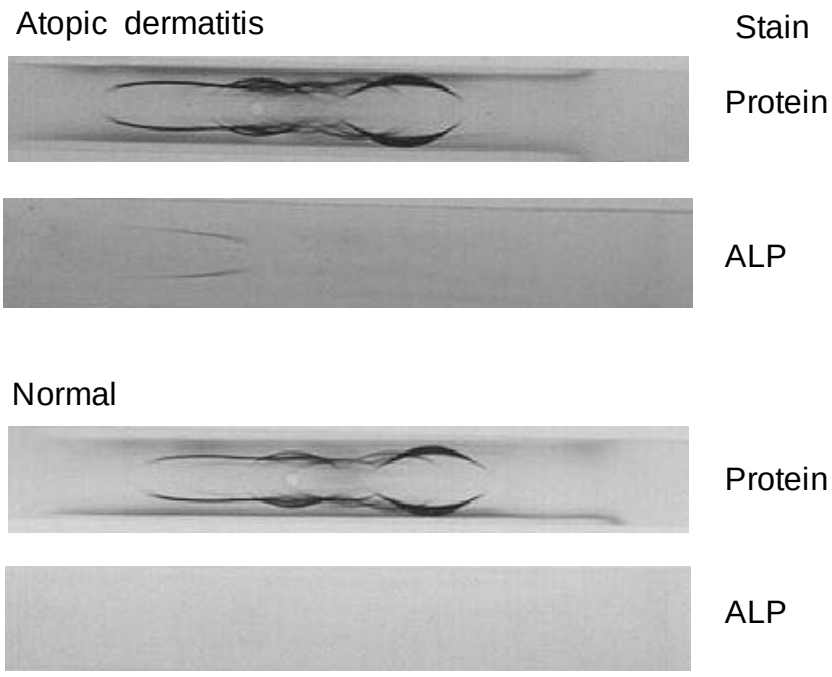

Figure 1. Modified immunoelectrophoresis. B-IgG is detected at the IgG precipitate line for the serum of a patient with atopic dermatitis, but not detected for that of a healthy subject (normal).

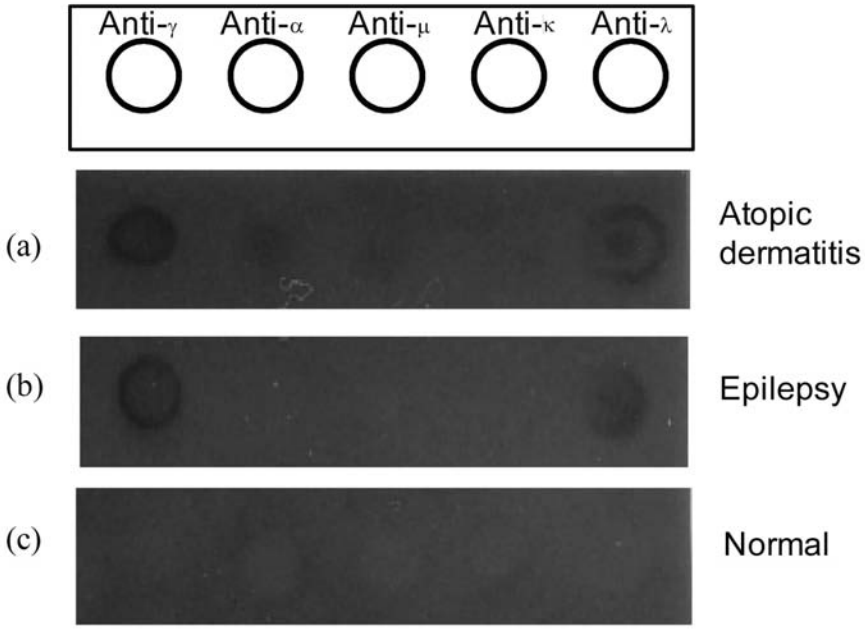

Figure 2. Immunofixation. Antibodies to γ, α, μ, κ and λ chain are spotted on the cellulose acetate membrane. (a) γ, α and λ chains in B-Igs are positive and μ and κ chains slightly positive for atopic dermatitis. (b) γ and λ chains are positive for epilepsy. (c) All chains are negative for a healthy subject (normal).

there were two different assays (Figure 3; methods A and B) [13]. In method A, B-IgG was detected by ALP-labeled anti-human IgG. *p*-Nitrophenyl phosphate was used as a substrate for ALP and absorbance was measured in a microplate reader. On the other hand, horseradish peroxidase (HRP)-labeled streptavidin instead of ALP-labeled anti-human IgG was used in method B. The substrate for HRP was *o*-phenylenediamine.

Method A. The standard curve was linear from 50 to 400 ng mL^{-1} B-IgG (commercially available) and the dose-response did not occur in the same range of IgG. When using bovine serum albumin (BSA)-coated plates, no dose-response relationship was observed. The sensitivity of the method was defined as the change in absorbance for each concentration of the unit per minute, since the intensity of absorbance depended on the reaction time. The sensitivity was 1.9×10^{-5} ng^{-1} mL min^{-1} (Table 1). Method A, however, could not distinguish B-IgG-positive and -negative human sera, indicating that IgG in sera may nonspecifically bind to the solid phase. The result suggested that a much more sensitive method was required to detect B-IgG in sera.

Method B. The sensitivity of method B was 5.3×10^{-4} ng^{-1} mL min^{-1} (Table 1), which was 28-fold higher than that of method A. The standard curve of method B was linear from 10 to 280 ng mL^{-1} B-IgG (commercially available) and the dose-response did not occur in the same range of IgG. When using BSA-coated plates instead of avidin-coated plates, no dose-response curve was seen. Method B was applied to the B-IgG-positive sera, but 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 and so on in sera. 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. Method B successfully measured the amount of B-IgG in sera. However, we could not obtain reliable assay values for some serum specimens, indicating that the amount of B-IgG in sera was

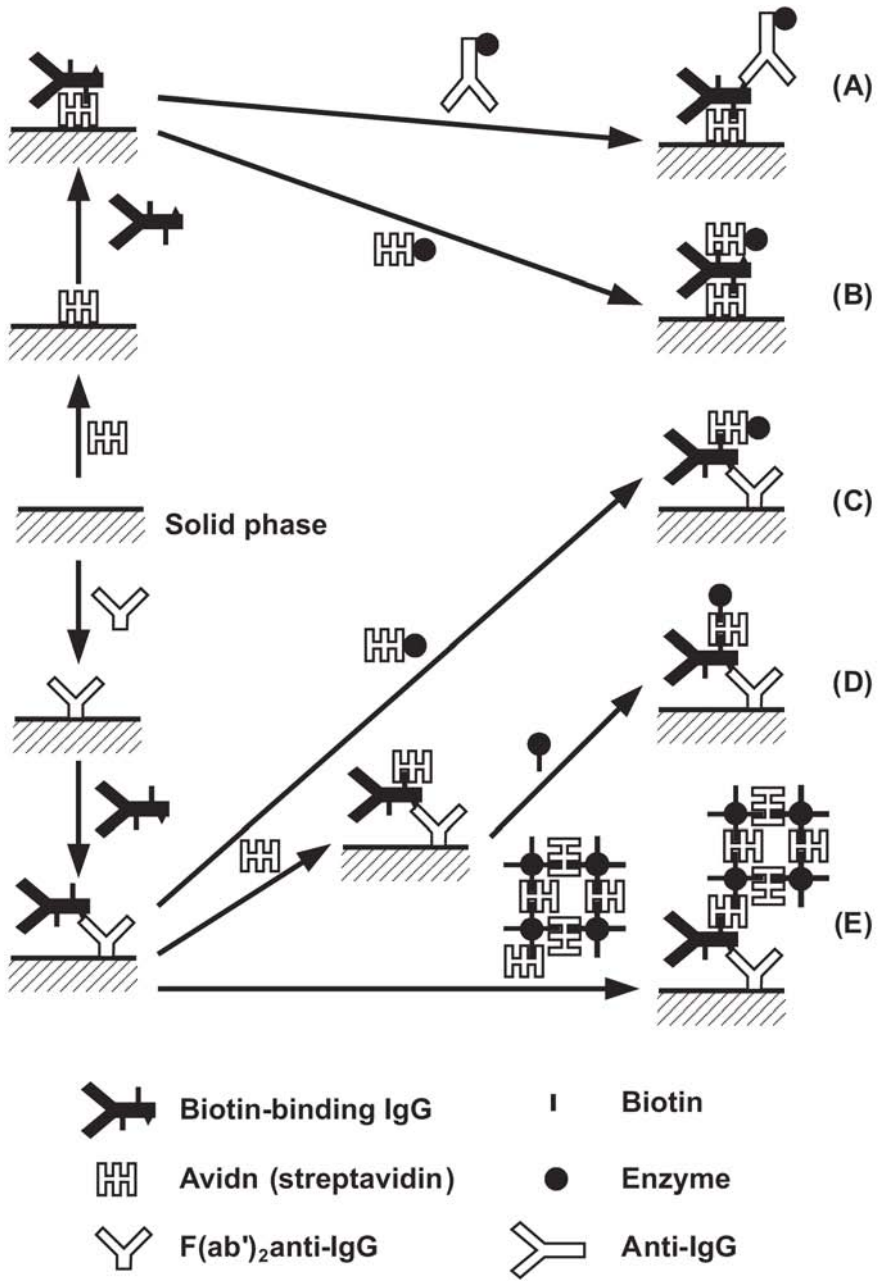

Figure 3. Concept for the detection of biotin-binding IgG in the multiwell-microplate format.

Table 1. The sensitivity of methods A - E.

Method	Sensitivity × 10^5/ng^{-1} mL min^{-1}	Relative sensitivity
A	1.9	1
B	53	28
C	100	53
D	310	163
E	960	505

very low and thus an improved method with higher sensitivity was required to measure concentrations of B-IgG less than 10 ng mL^{-1}. Method B was, however, valuable as the first assay procedure based on the multiwall microplate format, which enables the detection of B-IgG in human sera.

F(ab')$_2$anti-immunoglobulin-coated microplate methods

B-IgG was caught by F(ab')$_2$anti-IgG supported on the solid phase (96-well microplate). After this step, there were three different assays (Figure 3; methods C, D and E) [14]. Goat F(ab')$_2$anti-IgG and BSA used in the study did not contain biotinylated F(ab')$_2$anti-IgG and BSA. Methods C, D and E are often called the labeled avidin-biotin method [15], the bridged avidin-biotin method [16] and the avidin-biotin complex method [15], respectively. In method C, B-IgG was detected by HRP-labeled streptavidin. In method D, avidin was first reacted and then HRP-labeled biotin was reacted. In method E, B-IgG was detected by avidin-biotinylated HRP complex (HRP-ABC). *o*-Phenylenediamine was used as a substrate for HRP in each method and absorbance was measured in a microplate reader.

Method C. The standard curve was linear from 0 to 12.5 ng mL^{-1} B-IgG (commercially available), when multiwall microplates were coated with F(ab')$_2$anti-human IgG, while the dose-response did not occur in multiwall microplates coated with BSA and in the same range of IgG, instead of B-IgG. The sensitivity was 1.0×10^{-3} ng^{-1} mL min^{-1} (Table 1), which was higher than those of methods A and B, but method C could detect commercially available B-IgG but not detect B-IgG in the B-IgG-positive sera. The immunofixation method described above could detect B-IgG in sera, but method C could not, although the concept of method C is similar to that of the immunofixation method (Figure 3). This discrepancy may arise from differences in the procedure.

Method D. The standard curve was linear from 0 to 10 ng mL^{-1} B-IgG (commercially available) and the dose-response did not occur in the same range of IgG. When using BSA-coated plates, no dose-response relationship was observed. The sensitivity was 3.1×10^{-3} ng^{-1} mL min^{-1}, which was higher than those of methods A, B and C (Table 1), but method D could detect commercially available B-IgG but not detect B-IgG in the B-IgG-positive sera. Using streptavidin instead of avidin did not improve the sensitivity.

Method E. The standard curve was linear from 0 to 10 ng mL^{-1} B-IgG (commercially available) and the dose-response did not occur in the same range of IgG. When using BSA-coated plates, no dose-response relationship was observed. The sensitivity was 9.6×10^{-3} ng^{-1} mL min^{-1}, which was higher than those of methods C and D (Table 1). Method E could detect B-IgG in sera, while methods C and D could not. The level of B-IgG (30 - 84 ng mL^{-1}) was measured by method E in the B-IgG-positive sera (immunofixation), but it was lower than 4 ng mL^{-1} in the B-IgG-negative sera (immunofixation) [14].

Since F(ab')$_2$anti-IgG was immobilized on the solid phase matrix, both IgG binding no biotin and B-IgG can be bound to F(ab')$_2$anti-IgG. Thus, the ratio ([B-IgG]/[IgG]) of the concentration of B-IgG, [B-IgG], to that of IgG, [IgG], was important for detecting B-IgG in sera. In addition, the ratio (B/IgG) of biotin molecules to IgG molecules in a B-IgG molecule must affect detectability. The B/IgG of the B-IgG used in this study was 18; this value was obtained by a method described in the next section. The samples (IgG solution) were prepared by adding a different amount of B-IgG with the B/IgG of 18 to a constant amount of IgG, and were measured with method E. Method E could detect B-IgG in the IgG solution, when the B/IgG of B-IgG was 18 and [B-IgG]/[IgG] was more than about 5.5×10^{-5} (Figure 4). These results indicate that, if the B/IgG ratio of B-IgG in sera used in this study was 18, B-IgG levels in the sera could be measured in cases of a [B-IgG]/[IgG] ratio of more than approximately 5.5×10^{-5} [17]. We measured quantitatively the level of B-IgG in human sera for the first time without the purification of an IgG fraction from sera, although purification was required in method B.

Biotin-protein ratio of biotinylated Igs. As mentioned above, the biotin-protein ratio of biotinylated IgG, which was used as standard in the assay, was very important to quantitate the level of B-IgG in sera.

N-Hydroxysuccinimidobiotin (NHS-biotin) was used as a biotinylation reagent for labeling immunoglobulins (IgG, IgA and IgM). The biotinylated immunoglobulins synthesized were acid-hydrolyzed and the level of biotin molecules released from the biotinylated immunoglobulins was measured by an agar plate biotin bioassay method using *Lactobacillus plantarum* ATCC 8014 [17, 18]. The level of proteins in the biotinylated immunoglobulins was measured using BCA Protein Assay kit [17]. The biotin-protein ratios of biotinylated immunoglobulins $(B/Ig)_{af}$ were calculated and compared with the ratios of NHS-biotin molecules to immunoglobulin molecules (B/Ig) before synthesis of biotinylated immunoglobulins (Figure 5). The results indicated that biotinylated immunoglobulins with a different biotin-protein ratio, $(B/Ig)_{af}$, were successfully created by varying the ratio of NHS-biotin molecules to immunoglobulin molecules [17].

Interfering substance in method E. We measured quantitatively the levels of B-IgG in human sera, but they were higher than we predicted. We found that HRP-ABC nonspecifically bound to lipids like chylomicron in sera adsorbed onto both $F(ab')_2$anti-IgG-coated and BSA-coated wells [19]. Therefore, we thought that the difference between the absorbance obtained in $F(ab')_2$anti-IgG-coated well and the absorbance obtained in BSA-coated well corresponded to the level of B-IgG in sera [19].

Biotin-binding immunoglobulins in human sera

As described above, B-IgG could be measured using $F(ab')_2$anti-IgG- and BSA-coated wells together for a serum. Using $F(ab')_2$anti-IgA or $F(ab')_2$anti-IgM instead of $F(ab')_2$anti-IgG made it possible that B-IgA or B-IgM in a serum was also detected in method E. Standard curves for B-IgA and B-IgM were linear from 0 to 20 ng mL^{-1} [19]. The biotin-protein ratios of standard biotinylated IgG, IgA and IgM were 20, 23 and 100, respectively.

B-IgG, B-IgA and B-IgM in 100 sera from healthy specimens were analyzed using method E (Table 2). The reference value was defined as the mean + 2S.D. and the values of B-IgG, B-IgA and B-IgM were 1.42, 3.33 and 3.05 ng mL^{-1}, respectively [19].

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

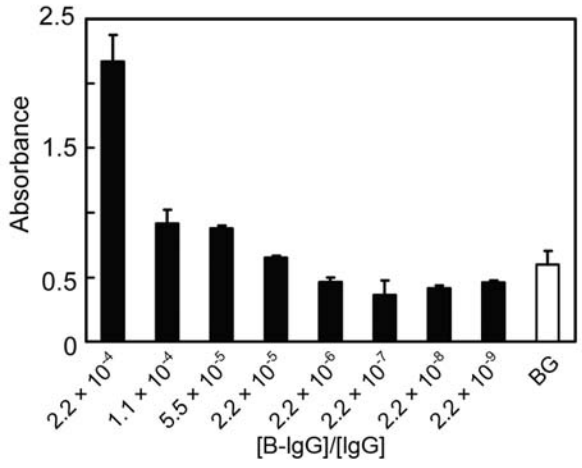

Figure 4. Influence of the ratio of B-IgG to IgG with Method E. BG shows the mean of absorbances for phosphate-buffered saline being added to $F(ab')_2$anti-IgG-coated wells. The vertical bars indicate the standard deviation for the mean of the experiments (n = 2).

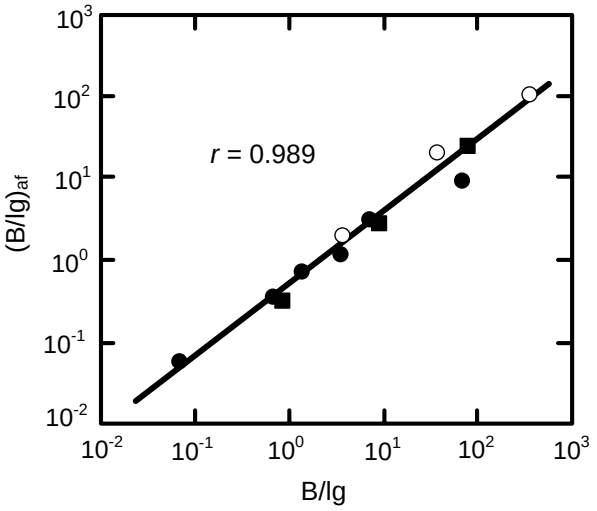

Figure 5. Biotin-protein ratio, $(B/Ig)_{af}$, *vs*. the ratio of NHS-biotin molecules to immunoglobulin molecules, B/Ig. ●, biotinylated IgG; ■, biotinylated IgA; ○, biotinylated IgM.

in bronchial asthma (28.3%), atopic dermatitis (48.7%), epilepsy (52.9%) and juvenile rheumatoid arthritis (62.5%) compared with those of healthy specimens ($p < 0.01$). Positive percentages for B-IgA were significantly increased in bronchial asthma (13.2%) and atopic dermatitis (20.5%) compared with those of healthy specimens ($p < 0.01$) [19]. B-Igs were detected in the sera of epilepsy using the qualitative method [9]. Nagamine *et al*. reported that the frequency of detection of B-Igs using the qualitative method was significantly higher in patients with bronchial

Table 2. The level and positive percentage of B-IgG, B-IgA and B-IgM in sera of healthy specimens and patients.

		B-IgG		
	n	Range	Positive	
		/ngmL^{-1}	*n*	Percentage/%
Normal	100	0.0 - 2.2	7	7.0
Bronchial asthma	53	0.0 - 10.3	15	28.3*
Atopic dermatitis	39	0.0 - 11.7	19	48.7*
Epilepsy	70	0.0 - 11.4	37	52.9*
Juvenile rheumatoid arthritis	8	0.3 - 12.4	5	62.5*

		B-IgA		
	n	Range	Positive	
		/ngmL^{-1}	*n*	Percentage/%
Normal	100	0.0 - 13.3	2	2.0
Bronchial asthma	53	0.0 - 12.7	7	13.2*
Atopic dermatitis	39	0.0 - 9.8	8	20.5*
Epilepsy	70	0.0 - 11.3	2	2.9
Juvenile rheumatoid arthritis	8	0.0 - 3.5	1	12.5

		B-IgM		
	n	Range	Positive	
		/ngmL^{-1}	*n*	Percentage/%
Normal	100	0.0 - 5.4	6	6.0
Bronchial asthma	53	0.0 - 2.1	0	0.0
Atopic dermatitis	39	0.0 - 4.1	1	2.6
Epilepsy	70	0.0 - 3.3	1	1.4
Juvenile rheumatoid arthritis	8	0.0 - 2.1	0	0.0

*$p < 0.01$.

asthma (25.0%), atopic dermatitis (43.8%) and rheumatoid arthritis (8.3%) than in healthy controls [12]. 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.

B-Igs in autoimmune thyroid disorders were also investigated using the qualitative method [11]. Prevalence of B-Igs was significantly higher in Graves' disease (47%) than in Hashimoto's disease (8%) and healthy controls (10%).

Clinical significance

Biotin is widely present in natural foodstuffs and most of the biotin is in a protein-bound form. Humans cannot synthesize biotin, and thus must obtain the vitamin from food [1, 20]. Biotin-bound proteins are hydrolyzed by various gastrointestinal proteases and biocytin and biotinyl peptides are yielded from the proteins. Biotinidase in pancreatic juice hydrolyzes biocytin and biotinyl peptides to release biotin. The released biotin is absorbed through an active transport system in the jejunum [21]. The biotin transported to blood binds to biotinidase, which occurs in liver, biotin-biotinidase complex is transported to cells, and biotin is released from the complex in cells [22, 23, 24]. Holocarboxylase synthetase attaches the biotin to various apocarboxylases to form holocarboxylases (ACC, PC, PCC and MCC). ACC is involved in the biosynthesis of fatty acids, PC in gluconeo-genesis, PCC in the catabolism of branched-chain amino acids and fatty acids of odd-carbon chain lengths and MCC in leucine catabolism [1, 25, 26]. The holocarboxylases are degraded in lysosome to be biocytin and biotinyl peptides, and biotinidase catalyzes the cleavage of the biocytin and biotinyl peptides to release free biotin, some of which is recycled. Most biocytin and biotinyl peptides are transported to blood, where free biotin, some of which is recycled, is liberated from the biocytin and biotinyl peptides by biotinidase in blood [24].

As previously described, B-Igs were detected in serum from a patient with atopic dermatitis. The biotin level and the activity of biotinidase in the serum were also investigated. These parameters were not abnormal and within the reference range, indicating no biotin deficiency. The administration of mass biotin to the patient improved the symptoms of atopic dermatitis. This seemed to indicate that, despite a normal level of plasma biotin, the level of biotin in cells decreased [9]. Therefore, we speculate that B-Igs might be related to the inhibition of transport of biotin from blood to cells. Nagamine *et al*. reported that B-Igs could be associated with autoimmune dysfunction in Graves' disease [11]. B-Igs were also detected in the sera from healthy subjects at a low frequency (Table 2). Thus, further studies are required to elucidate the clinical significance of B-Igs.

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