Detection of Antipituitary Antibodies in Patients with Autoimmune Thyroid Disease

MICHIKO NISHINO, SHIGEKI YABE, MASAMI MURAKAMI*, TSUGIYASU KANDA** AND ISAO KOBAYASHI

Department of Laboratory Medicine, Gunma University School of Medicine, 3–39–15 Showa-machi, Maebashi 371–8511, Japan
* First Department of Internal Medicine, Gunma University School of Medicine, 3–39–15 Showa-machi, Maebashi 371–8511, Japan
** Department of General Medicine, Gunma University School of Medicine, 3–39–15, Showa-machi, Maebashi 371–8511, Japan

Abstract. The aim of the present study was to investigate the prevalence of antipituitary antibodies (APA) in patients with autoimmune thyroid disease as determined by Western blot analysis and enzyme-linked immunosorbent assay (ELISA). Results by Western blot analysis showed positivity for APA in serum of 22.4% of patients with Graves' disease (n=143, p<0.05) and 18.5% of patients with Hashimoto's thyroiditis (n=54, p<0.05), which were significantly higher than 6.2% in healthy controls (n=97). Similar results were obtained with ELISA. The titers of APA measured by ELISA (APA/ELISA) remained unchanged before and after therapy with antithyroid drug for Graves' disease, while thyrotropin-binding inhibitor immunoglobulins (TBI) decreased significantly. Similarly, no changes in APA by Western blot analysis were observed after therapy. In patients with Graves' disease, APA were not associated with thyroid status. There was no difference in APA between patients with positive and negative thyroid autoantibodies. A significant but weak positive correlation between APA/ELISA and anti-human GH measured by ELISA (anti-hGH/ELISA) was observed in patients with Graves' disease (r=0.601 p<0.001) and Hashimoto's thyroiditis (r=0.428 p<0.005). These findings have demonstrated the existence of APA detected by Western blot analysis and ELISA in some cases of autoimmune thyroid disease. The present results suggest that hGH and other antigens may be involved in APA in patients with Graves' disease and Hashimoto's thyroiditis.

Key words: Anti-human GH antibodies, Enzyme-linked immunosorbent assay, Western blot analysis, Graves' disease, Hashimoto's thyroiditis

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SEVERAL investigators have reported the prevalence of circulating antipituitary antibodies (APA) using different techniques in various autoimmune diseases [1–8]. As early as 1975, Bottazza et al. [1] showed the existence of APA to prolactin secretor cells of human pituitary in insulin-dependent diabetes mellitus, using an indirect fluorescent antibody technique [2]. In 1986, Sugiura et al. [3] developed a sensitive assay for APA, based on avidin-biotin detection technique and rat pituitary antigens. They [4] further developed a sensitive assay to detect antibodies to anterior pituitary cell surface membrane by indirect immunofluorescence for APA using mouse AT20 cells and rat GH3 cells.

Using the method described by Sugiura et al. [3, 4], we reported the existence of APA detected in patients with Hashimoto's thyroiditis and Graves' disease [5]. Subsequently, Hansen et al. [6] confirmed pituitary cell autoantibody in sera from patients with untreated Graves' disease.

However, there is no detailed information concerning the changes in APA during the course of therapy for Graves' disease, or the relationship between APA and thyroid status. It is not known whether antigens are recognized by APA detected in patients with autoimmune thyroid disease. In the
present study, we attempted to determine the existence of APA in patients with autoimmune thyroid disease, particularly Graves' disease, using Western blot analysis and enzyme-linked immunosorbent assay (ELISA), which were recently developed in our laboratory [7, 8].

Patients and Methods

Patients

Serum samples were obtained from 143 patients with Graves' disease (26 males and 117 females, aged 18 to 75 years; duration of disease varied from 8 months to 30 years), and 54 patients with Hashimoto's thyroiditis (11 males and 43 females, aged 23 to 76 years). Control sera were also obtained from 97 healthy volunteers (48 men and 49 women, aged 21 to 56 years). None of the patients or control subjects had any pituitary dysfunctions. All patients with Graves' disease received antithyroid drug (methylmercaptoimidazole or propylthiouracil) for at least 2 years. Remission was defined when thyroid hormone levels could be maintained within normal limits for at least 2 years after withdrawal of antithyroid drug. All patients gave their written informed consent.

Preparation of rat pituitary antigens

Preparation of rat pituitary antigens was performed, as previously described [7-9]. Rat pituitary glands (RKL, Gilbertsville, PA) were sliced and homogenized in 0.25 mol/L saccharose, 0.1 mmol/L ethylenediamine tetraacetic acid (EDTA), 3 mmol/L Tris (hydroxymethyl) aminomethane-HCl, pH 7.4 in a Polytron homogenizer. The homogenate was centrifuged at 10,000 × g at 4°C for 10 min and the supernatant was used as the source of pituitary antigen. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis

Western blot analysis was performed to determine the presence of APA in serum samples as previously described [7]. In brief, 50 µl of the rat pituitary antigens (approximately 200 µg protein) were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins blotted onto a polyvinylidene difluoride membrane (TEFCO, Tokyo, Japan) by means of a semidy-sed blotting apparatus (Bio-Rad Laboratories) at 6 V for 1.5 h. After incubation in blocking buffer, the membrane was incubated in 101-fold diluted patient's serum at 4°C for 24 h. Membranes were then washed and incubated in 1:500 diluted biotinylated rabbit anti-human IgG polyclonal antibodies (Sigma, St. Louis, MO) in dilution buffer at room temperature for 1 h. The membrane was then washed and incubated in streptavidin-biotin complex peroxidase reagent at room temperature for 1 h, and the positive bands were visualized by chemiluminescence using a peroxidase immunostain kit (Wako Pure Chemicals, Kyoto, Japan).

APA measurement by ELISA (APA/ELISA)

ELISA measurement was performed using rat pituitary gland as an antigen as previously described with minor modifications [8-10]. Briefly, microtiter plates were coated with 100 µl rat pituitary antigen (supernatant diluted to 25 µg/ml) in each well followed by incubation overnight at 4°C. After the samples of serum were diluted 201-fold, 100 µl of diluted serum was added to each well and incubated at room temperature for 2 h. Plates were then washed five times with phosphate-buffered saline (PBS), 100 µl of peroxidase-labeled rabbit anti-human IgG polyclonal antibody (Sigma) was added to each well and incubated at room temperature for 1 h. After washing with PBS, 100 µl of substrate solution (0.1 mol/L sodium acetate-citrate containing 0.006% H2O2 and 0.2 mg/ml 3,3',5,5'-tetramethylbenzidine dihydrochloride, pH 5.5) was added to each well, followed by incubation at room temperature for 30 min. The reaction was stopped by addition of 100 µl of 0.5 N H2SO4 to each well and the absorbance was measured at 450 nm. The results of ELISA were expressed as a cutoff index (C.I.) in which the absorbance values obtained for each sample of serum was divided by the absorbance of antibody-negative pooled sera. The distribution of cutoff index values obtained in control subjects was 0.33–4.05 (1.42 ± 0.643). Samples which exceeded the mean value + 2 S.D. (C.I. > 2.7) of control values were regarded as
positive for APA/ELISA.

_Anti-hGH measurement by ELISA (Anti-hGH /ELISA)_

ELISA, using human GH (hGH) (Biogenes Ltd, Poole city, England, UK) [11] as an antigen, was performed as described above. HGH was purified from human pituitary glands and contaminants of other pituitary hormones were 0.01 to 0.02%. Each well of the microtiter plates was coated with 100 µl hGH (5 µg/ml). To set the anti-hGH antibody titer, negative control sera were obtained from 150 healthy volunteers. The distribution of cutoff index values obtained in control subjects was 0.31–2.36 (0.79 ± 0.45). Samples which exceeded the mean value +2 S.D. (C.I. > 1.7) of control values were regarded as positive for anti-hGH/ELISA.

_Thyroid-related laboratory tests_

Serum free T₄ (FT₄), freeT₃ (FT₃) and TSH were measured by enzyme immunoassay (EIA) kit (Wako, Osaka, Japan). Thyrotropin-binding inhibitor immunoglobulins (TBI) were measured using a commercially available kit (Cosmic Corp. Tokyo, Japan). Serum antibody titers against microsomal antigen and thyroglobulin were measured by the latex-agglutination technique (Sorodia AMC and Sorodia ATG, Fuji Rebio, Tokyo, Japan).

_Statistical analysis_

Data were expressed as mean ± S.D. and statistical analysis was performed by unpaired Student’s t-test. Percent positive analysis was performed by χ² tests. Correlation coefficients were calculated by Pearson’s correlation formula.

_Results_

_Detection of APA in autoimmune thyroid disease_

APA were demonstrated to be positive by Western blot analysis in 32 of 143 (22.4%) patients with Graves’ disease, in 10 of 54 (18.5%) patients with Hashimoto’s thyroiditis and in 6 of 97 (6.2%) healthy controls. The prevalence of positive APA were significantly higher in patients with Graves’ disease or Hashimoto’s thyroiditis compared to healthy controls (p < 0.05).

By APA/ELISA analysis, APA were positive in 28 of 143 (19.6%) patients with Graves’ disease, in 10 of 54 (18.5%) patients with Hashimoto’s thyroiditis and in 5 of 97 (5.2%) healthy controls. Again, the prevalence of positive APA/ELISA were significantly higher in patients with Graves’ disease or Hashimoto’s thyroiditis compared to healthy controls (p < 0.05). Furthermore, there were no significant differences in APA positive rate among varying ages with patients of Graves’ disease and between males and females with Graves’ disease.

_Changes in APA before and after therapy for Graves’ disease_

Western blot analysis was performed to detect APA in sera from 12 patients with Graves’ disease.

_1 2 3 4 5 6 7 8_

Fig. 1. Western blot analysis of rat pituitary antigen on SDS-PAGE gels with sera from patients with Graves’ disease.

before and after therapy. APA, which were determined by Western blot analysis, were positive in 3 patients and negative in 9 patients. As shown in Fig. 1, positive APA and negative APA persisted throughout the observation period. APA/ELISA were found to be positive in 4 patients and negative in 8 patients. The mean values of APA/ELISA, before therapy, after therapy and sustained remission, were 2.96±2.02 (C.I.), 2.84±1.94 (C.I.) and 2.78±2.06 (C.I.), respectively. No significant changes were found throughout the observation period.

Before therapy for Graves’ disease, FT3 and FT4 levels were 12.60±3.63 pg/ml and 4.03±0.69 ng/dl in the APA-positive cases and 11.49±4.24 pg/ml and 3.83±0.80 ng/dl in the APA-negative cases, which were not different between the two groups. Serum FT3, FT4 and TBI1 levels decreased significantly during antithyroid drug therapy and remission after the discontinuation of antithyroid drug.

Duration of Graves’ disease and APA

Patients with Graves’ disease were divided into 4 groups according to the duration of antithyroid drug therapy: up to 4 years, 5 to 9 years, 10 to 19 years and 20 years or more. No significant difference was observed in the prevalence of positive APA among 4 groups (Table 1).

Relationship between APA and thyroid autoantibodies

Patients with Graves’ disease were divided into 4 groups according to the duration of antithyroid drug therapy: up to 4 years, 5 to 9 years, 10 to 19 years and 20 years or more. No significant difference was observed in the prevalence of positive APA among 4 groups (Table 1).

Table 1. Prevalence of APA after therapy in 143 patients with Graves’ disease

<table>
<thead>
<tr>
<th>Group</th>
<th>APA (+)</th>
<th>APA (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of therapy (years)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>~4</td>
<td>9 (22.5)</td>
<td>31 (77.5)</td>
</tr>
<tr>
<td>5~9</td>
<td>6 (25.0)</td>
<td>18 (75.0)</td>
</tr>
<tr>
<td>10~19</td>
<td>14 (21.9)</td>
<td>50 (78.1)</td>
</tr>
<tr>
<td>20~</td>
<td>3 (20.0)</td>
<td>12 (80.0)</td>
</tr>
</tbody>
</table>

Abbreviation
APA (+): antipituitary antibodies positive. APA (-): antipituitary antibodies negative.
There was no significant difference among 4 groups of duration of therapy for APA (+) or APA (-).

Table 2. Relationship between APA in 143 patients with Graves’ disease and 54 patients with Hashimoto’s thyroiditis

<table>
<thead>
<tr>
<th>Autoantibodies</th>
<th>APA (+) n (%)</th>
<th>APA (-) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCHA positive</td>
<td>23 (23.2)</td>
<td>76 (76.8)</td>
</tr>
<tr>
<td>MCHA negative</td>
<td>10 (22.7)</td>
<td>34 (77.3)</td>
</tr>
<tr>
<td>TGHA positive</td>
<td>10 (19.2)</td>
<td>42 (80.8)</td>
</tr>
<tr>
<td>TGHA negative</td>
<td>23 (25.3)</td>
<td>68 (74.7)</td>
</tr>
</tbody>
</table>

b. Hashimoto’s thyroiditis

<table>
<thead>
<tr>
<th>Autoantibodies</th>
<th>APA (+) n (%)</th>
<th>APA (-) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCHA ≥25,600</td>
<td>4 (15.4)</td>
<td>22 (84.6)</td>
</tr>
<tr>
<td>MCHA &lt;25,600</td>
<td>6 (21.4)</td>
<td>22 (78.6)</td>
</tr>
<tr>
<td>TGHA positive</td>
<td>5 (17.2)</td>
<td>24 (82.8)</td>
</tr>
<tr>
<td>TGHA negative</td>
<td>5 (20.0)</td>
<td>20 (80.0)</td>
</tr>
</tbody>
</table>

Abbreviations
APA (+): antipituitary antibodies positive. APA (-): antipituitary antibodies negative.
MCHA: microsomal antibody. TGHA: thyroglobulin antibody.

microsomal antibody (MCHA) positive group and MCHA negative group. The prevalence of positive APA was no different between the two groups (Table 2a). Similarly, patients with Graves’ disease were divided into thyroglobulin antibody (TGHA) positive and TGHA negative group. The prevalence of positive APA was no different between the two groups (Table 2a).

Patients with Hashimoto’s thyroiditis were divided into two groups, according to the MCHA titer: MCHA ≥25,600 and MCHA <25,600. The prevalence of positive APA was no different between the two groups (Table 2b). Patients with Hashimoto’s thyroiditis were also divided into TGHA positive and TGHA negative groups. The prevalence of positive APA was no different between the two groups (Table 2b).

Correlation between APA/ELISA and anti-hGH/ELISA

The correlation between APA/ELISA and anti-hGH/ELISA was studied to investigate the antigen recognized by APA in patients with autoimmune
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Discussion

Previous studies reported that APA were frequently detected in patients with pituitary diseases including ACTH deficiency [12], lymphocytic adenohypophysitis [13], hypopituitarism [14], empty sella syndrome [15], Sheehan’s syndrome [16] and pituitary tumor [17]. These observations suggest that the presence of APA may be at least in part related to the autoimmune process that affects pituitary functions. Komatsu et al. [15] suggested that pituitary antibodies detected by a sensitive assay with immunofluorescence methods might be related to the development of pituitary atrophy and pituitary empty sella syndrome.

In 1989, we reported that APA were considerably more prevalent in Hashimoto’s thyroiditis than in Graves’ disease, using unfixed cryostat sections of rat pituitaries [5]. Subsequently, Hansen et al. [6] reported that antibodies reactive with cytoplasmic components of pituitary GH/PRL cells, might be present in sera from patients with Graves’ disease, more than patients with Hashimoto’s thyroiditis who had a similar frequency of APA compared to that of healthy subjects. Thus, due to inconsistent results previously obtained, we attempted to determine an interrelationship between APA and autoimmune thyroid disease by newly established methods.

In the present study, prevalence of a 22k Da band detected by Western blot analysis was significantly higher in patients with Graves’ disease and Hashimoto’s thyroiditis than in healthy subjects. Similar results were obtained in patients with such autoimmune thyroid diseases by ELISA. We also reported high prevalence of antibodies to GH3 cells and AtT-20 cells in patients with autoimmune thyroid disease [18]. However, APA were prevalent in Hashimoto’s thyroiditis than in Graves’ disease in our previous study [5]. The contradictory results in the prevalence of APA may be explained by the different assay methods and different antigens used for APA. In addition, it appears that these differences could reflect different prevalence of Graves’ disease and Hashimoto’s thyroiditis.

Ozawa et al. [13] reported that the recovery from lymphocytic hypophysitis associated with painless thyroiditis was followed by changes in circulating APA determined by immunofluorescence method. Kobayashi et al. [19] reported a case of diabetic coma

thyroid disease. As shown in Fig. 2a, a positive correlation between APA/ELISA and anti-hGH/ELISA was observed in patients with Graves’ disease (r=0.601, p<0.001). Positive APA/ELISA was found in 28 of 143 patients (19.6%), and positive anti-hGH/ELISA was found in 21 patients (14.7%) (Fig. 2a).

As shown in Fig. 2b, there was a positive correlation between APA/ELISA and anti-hGH/ELISA in patients with Hashimoto’s thyroiditis (r=0.428, p<0.005). Positive APA/ELISA was found in 10 of 54 patients (18.5%), and showed 7 positive anti-hGH/ELISA (13.6%) (Fig. 2b).

Fig. 2. a: Correlation between the APA value and anti-hGH detected by ELISA in patients with Graves’ disease. b: Correlation between the APA value and anti-hGH detected by ELISA in patients with Hashimoto’s thyroiditis.
due to type I diabetes mellitus who showed changes in APA during therapy for diabetes mellitus. Furthermore, Kobayashi et al. [19] reported an inverse relationship between urinary C-peptide levels and APA titers measured by ELISA in patients with type II diabetes mellitus, suggesting that the presence of APA might be related to a reduced secretion of insulin. More recently, we showed a significant decrease in APA levels detected by ELISA in patients with GH deficiency after GH therapy [9]. In the present study, the changes in APA before and after antithyroid drug therapy for Graves' disease were then studied. However, there were no significant changes in APA by either Western blot analysis (Fig. 1) and ELISA. These results, with regard to previous reports [13, 19], suggested that positive APA detected in patients with Graves' disease are not closely associated with thyroid function.

We also investigated whether there were any differences in APA between thyroid autoantibodies positive and negative patients with Graves' disease in the present study. There was no difference in the presence of APA between the two groups, suggesting that APA and thyroid autoantibodies appeared to exist independently.

We have demonstrated a 22 kDa band of APA as a soluble cytoplasm in GH-related protein in previous studies [7]. We have recently identified a binding protein involved in 22 kDa band recognized by sera from patients with positive APA [9]. It was demonstrated that the first domain from the N terminal of 22 kDa protein had a 67% homology to human GH and 100% homology to rat GH [9]. In the present study a relatively weak positive correlation between anti-hGH/ELISA and APA/ELISA was observed in patients with Graves' disease and in patients with Hashimoto's thyroiditis.

These results suggest that hGH is, at least in part, related to the antigen for APA detected in patients with autoimmune thyroid diseases. However, some cases of patients showed dissociation between anti-hGH/ELISA and APA/ELISA. Thus, a positive but relatively weak correlation between anti-hGH/ELISA and APA/ELISA suggest that other antigens may be involved in APA detected in the present study.

Many antibodies appear to have no harmful effect even though they are detected in high concentrations in patients' sera and thus are of diagnostic value, although some of the patients with positive antibodies are closely related to the functional disorders. The basal pituitary hormone levels including GH were not affected by the presence of APA in patients with Graves' disease at sustained remission after withdrawal of antithyroid drug (data not shown). Whether the presence of APA may be associated with genetic factors or acquired defects in regulation of immune response is not known. Thus, the APA detected in patients with autoimmune thyroid disease in the present study may not affect pituitary functions. However, the possibility of development of impaired pituitary functions in the future should not be excluded in the cases of positive APA, including thyroid disease and normal subjects. To the best of our knowledge, such information has not been reported to date.

In conclusion, APA were detected in certain patients with Graves' disease and Hashimoto's thyroiditis by Western blot analysis and ELISA, suggesting that the presence of APA is related to the pathophysiology of patients with autoimmune thyroid disease, although APA appeared to be unrelated to thyroid functional abnormalities. The present results suggested that hGH and other antigens might be involved in APA detected in patients with autoimmune thyroid disease.

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