Western Blot Analysis of Rat Pituitary Antigens Recognized by Human Antipituitary Antibodies

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Abstract. Antipituitary antibodies have been reported to exist in sera of patients with autoimmune endocrine disorders. In order to investigate the pituitary antigens recognized by antipituitary antibodies, we studied the autoantigens in rat pituitary membrane and cytosolic fractions recognized by human antipituitary antibodies and anti-human pituitary hormone antibodies. Sera from 6 patients which showed positive antipituitary antibodies by immunofluorescence methods were studied. Each serum identified some proteins with 14.5, 22, 47, 49, 65, 84 and 97.5 kDa. Anti-GH antibodies and anti-PRL antibodies identified a positive band with 22 kDa, suggesting that anti-GH and anti-PRL antibodies may be present in patients' sera. These results indicate that Western blot analysis of rat pituitary antigens recognized by human antipituitary antibodies is a useful method to elucidate the pathophysiology of autoimmune endocrine disorders.

Key words: Western blot analysis, Rat pituitary, Antipituitary antibodies

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ANTIPITUITARY antibodies have been reported to exist in sera of patients with ACTH deficiency, Sheehan's syndrome, empty sella syndrome, Hashimoto's thyroiditis, Graves' disease and insulin dependent diabetes mellitus [1-7], which suggests the pathophysiological roles of the antibodies in these diseases. Although antipituitary antibodies are currently measured by sensitive immunofluorescence (IF) methods by using rat pituitaries [1] or cultured pituitary derived cell lines [2], the pituitary antigens recognized by antipituitary antibodies have not been identified.

In this report, antigens in rat pituitary membrane and cytosolic fractions were identified by human antipituitary antibodies by Western blot analysis.

Materials and Methods

Serum samples

Human sera which showed positive antipituitary antibodies when studied by immunofluorescence methods (supplied by BML Laboratories, Kawagoe, Japan) were studied.

Materials

Rat pituitaries were obtained from RKL (Gilbertsville, PA). Polyacrylamide gel (14%) and polyvinyliden difluoride (PVDF) membrane were from TECO (Tokyo, Japan). Protein standards were purchased from Bio-Rad Laboratories (Richmond, CA). Biotinylated anti-human IgG rabbit

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polyclonal antibodies were purchased from Sigma (St. Louis, MO). Streptavidin-biotin complex peroxidase, biotinylated anti-rabbit IgG swine polyclonal antibodies, anti-human ACTH rabbit antibodies, anti-human GH rabbit antibodies, anti-human PRL rabbit antibodies, anti-human FSH rabbit antibodies, anti-human LH rabbit antibodies and anti-human TSH rabbit antibodies were from DAKO (Glostrup, Denmark). The POD Immunostain Set was from Wako Pure Chemicals (Kyoto, Japan).

Preparation of rat pituitary antigens

Twelve rat pituitaries were homogenized in 3 ml of homogenizing buffer (0.25 M sucrose, 0.1 mM EDTA and 3 mM Tris-HCl buffer, pH 7.4) by a Polytron homogenizer (5,000 rpm, 1 min), centrifuged at 10,000 × g at 4 °C for 10 min and the resultant supernatants were used as pituitary antigens.

Western blot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli’s method [8]. In brief, 50 μl of samples (200 μg protein) were mixed with 50 μl of sample buffer (0.1 M dithiothreitol, 2% SDS, 15% glycerol, 0.006% bromophenol blue, 2-mercaptoethanol, 0.08 M Tris-HCl, pH 6.8), heated at 56 °C for 10 min, and electrophoresed in 14% polyacrylamide gel for 1.5 h at 18 mA (running buffer 25 mM Tris, 192 mM glycine, 0.1% SDS). Separated proteins were transferred to a PVDF membrane by means of a semi-dry blotting apparatus (Bio-Rad Laboratories, Richmond, CA) at 6 V for 60 min. The membrane was incubated in blocking buffer (5% skim milk, 10% normal rabbit serum, 3% BSA in PBS, pH 7.2) at 4 °C for 16-20 h. The membrane was then incubated in 1:101 diluted human serum in dilution buffer (5% skim milk, 3% BSA in PBS, pH 7.2) at room temperature for 2 h, washed in washing buffer (5% skim milk, 0.05% Tween 20 in PBS, pH 7.2), then incubated in 1:500 diluted biotinylated anti-human IgG rabbit polyclonal antibodies in dilution buffer at room temperature for 1 h and washed in washing buffer. The membrane was then incubated in 1:10 diluted streptavidin-biotin complex peroxidase in 3% BSA in PBS, pH 7.2 at room temperature for 1 h, washed in washing buffer and visualized by 5 min reaction with POD Immunostain Set. To analyze rat pituitary antigens with antibodies to human pituitary hormones, primary rabbit antibodies and biotinylated anti-rabbit IgG swine polyclonal antibodies were used. Other procedures were the same as described above.

Antipituitary antibodies measurement by immunofluorescence (IF) methods

Antipituitary antibodies were measured by IF methods with rat pituitaries as previously described [1, 2]. Positive staining patterns are divided into eight different patterns as shown in Table 1.

Table 1. Positive staining patterns obtained with immunofluorescence methods using rat pituitaries.

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Results

Analysis of pituitary antigens recognized by positive sera to antipituitary antibody

Western blot analyses of rat pituitary antigens were performed with sera from six patients which showed various types of positive antipituitary antibodies by IF methods (Fig. 1). Serum 1, which had type 6 sera identified a wide range of bands. Serum 3, 4 and 5, however, immunoreacted with less antigens than 1, 2 and 3, which showed the types 2, 3, and 4 sera, respectively.

Analysis of sera antibodies to immunoprotocols

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had type C antipituitary antibodies (Table 1), identified a weak band with a molecular weight of 97.5 kDa. Serum 2, which had type D antipituitary antibodies, identified positive 65 and 47 kDa bands. Serum 3, which also had type D antipituitary antibodies, identified positive 65, 49, 47 and 22 kDa bands. Serum 4, which had type G antipituitary antibodies, identified a positive 22 kDa band. Serum 5, which had type H antipituitary antibodies, identified positive 22 and 14.5 kDa bands. Serum 6, which had positive antibodies to surface antigens of GH producing pituitary cells, identified positive 84 and 22 kDa bands.

**Analysis of pituitary antigens recognized by antibodies to human pituitary hormones**

Analysis of rat pituitary membrane and cytosol antigens recognized by antibodies to human pituitary hormones (ACTH, GH, PRL, FSH, LH and TSH) were performed by a peroxidase anti-peroxidase method (Fig. 2). Although no positive bands were identified by means of anti-FSH antibodies, anti-LH antibodies or anti-TSH antibodies, an apparent band with an approximate molecular weight of 22 kDa was identified by both anti-human GH antibodies and anti-human PRL antibodies. Several positive bands between 22 and 110 kDa were identified by anti-human ACTH antibodies.

**Discussion**

A number of autoantibodies have been reported to be detected in sera of patients with autoimmune diseases. Autoantibodies in autoimmune diseases are divided into two groups: some are organ specific autoantibodies which recognize specific cells or organs, and the others are organ non-specific autoantibodies which recognize serum components or cell components distributed in various sites throughout the body. Since organ specific antibodies including antipituitary antibodies may play a role in related diseases associated with clinical significance, it is of interest to elucidate the mechanism of their autoimmune responses. Since antipituitary antibodies have been detected in sera of patients with ACTH deficiency, Sheehan’s syndrome, empty sella syndrome, Hashimoto’s disease, Graves’ disease, and insulin dependent diabetes mellitus [1-7], it has been suggested that antipituitary antibodies are involved in the pathogenesis of autoimmune multi-endocrine disorders, but little information has been available on specif-

![Western blot analysis of sera which showed positive antipituitary antibodies by immunofluorescence (IF) methods.](image1)

![Western blot analysis of rat pituitary antigens by antibodies to human pituitary hormones. Lane 1: anti-human ACTH. Lane 2: anti-human GH. Lane 3: anti-human PRL. Lane 4: anti-human FSH. Lane 5: anti-human LH. Lane 6: anti-human TSH. Lane 7: serum 2 (IF type D). Lane 8: serum 3 (IF type D). Lane 9: serum 4 (IF type G). Lane 10: serum 5 (IF type H).](image2)
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![Fig. 1. Western blot analysis of sera which showed positive antipituitary antibodies by immunofluorescence (IF) methods. Lanes 1, 2: serum 1 (IF type C). Lanes 3, 4: serum 2 (IF type D). Lanes 5, 6: serum 3 (IF type D). Lanes 7, 8: serum 4 (IF type G). Lanes 9, 10: serum 5 (IF type H). Lanes 11, 12: serum 6 (IF positive GH1, cells).](image)

![Fig. 2. Western blot analysis of rat pituitary antigens by antibodies to human pituitary hormones. Lane 1: anti-human ACTH. Lane 2: anti-human GH. Lane 3: anti-human PRL. Lane 4: anti-human FSH. Lane 5: anti-human LH. Lane 6: anti-human TSH. Lane 7: serum 2 (IF type D). Lane 8: serum 3 (IF type D). Lane 9: serum 4 (IF type G). Lane 10: serum 5 (IF type H).](image)
ic pituitary antigens recognized by antipituitary antibodies. In this study, following the preparation of crude antigens from rat pituitary membrane and cytosolic fractions, the molecular weight of antigens recognized by antipituitary antibodies was determined by Western blot analysis.

Human antipituitary antibodies which were used in the present study had different staining patterns for IF methods (Table 1). As shown in Fig. 1, serum 1, which had a type C pattern, identified a positive band with a molecular weight of 97.5 kDa. Serum 2, which had a type D pattern, identified positive 65 and 47 kDa bands. Serum 3, which also had a type D pattern, identified positive 65, 49, 47 and 22 kDa bands. Serum 4, which had a type G pattern, identified a positive 22 kDa band. Serum 5, which had a type H pattern, identified positive 22 and 14.5 kDa bands. Serum 6, which had positive antipituitary antibodies to surface antigens in pituitary derived GH producing cells, identified positive 84 and 22 kDa bands. These results support the concept that various patterns of antipituitary antibodies detected by IF methods reflect different pituitary antigens.

Bottazzo et al. [3] reported the existence of autoantibodies to anterior pituitary cells secreting GH, LH and FSH in patients’ sera. In order to study whether antipituitary antibodies recognize specific anterior pituitary hormones, we identified the antigens in rat pituitary membrane and cytosolic fractions recognized by antibodies to human pituitary hormones (ACTH, GH, PRL, FSH, LH and TSH) employing Western blot analysis (Fig. 2). Anti-human FSH antibodies, anti-human LH antibodies and anti-human TSH antibodies did not recognize rat pituitary antigens, and anti-human ACTH antibodies identified several bands between 22 and 110 kDa. These results suggest that these antibodies used in this study may not recognize specific rat pituitary hormones. In contrast, both anti-human GH antibodies and anti-human PRL antibodies identified a positive band with a molecular weight of 22 kDa, which was also recognized by human antipituitary antibodies. These observations suggest, at least in part, the existence of anti-GH antibodies and anti-PRL antibodies in patients’ sera, which may play a role in the pathophysiology of autoimmune pituitary disorders.

Recently, Crock et al. [9] reported the detection of antipituitary autoantibodies by immunoblotting with human pituitary tissues as antigens. They showed that a 45 kDa pituitary specific membrane protein was identified as an autoantigen in one of 19 patients with idiopathic growth hormone deficiency and the empty sella syndrome and a 43 kDa membrane protein in pituitary and brain was identified as an autoantigen in another patient with idiopathic growth hormone deficiency and in one of 14 patients with secondary growth hormone deficiency. Although the reasons for the discrepancies between the results obtained at the two laboratories are not yet known, they are possibly due to the different sources of pituitary antigens and different methods utilized in the preparation of pituitary antigens. Further studies are needed to elucidate the interrelationship between antibodies to cytosol fractions in cells and those to cell membranes, the latter being considered to cause cell damage in target organs.

In conclusion, the identification of pituitary antigens recognized by human antipituitary antibodies employing Western blot analysis is of importance in studying the pathophysiology of autoimmune mechanisms in autoimmune endocrine disorders. We are in process of determining antipituitary antibodies in a large number of patients by using Western blot analysis and investigating the relationships between specific pituitary antigens and clinical features. It is also necessary to further characterize rat pituitary antigens recognized by human antipituitary antibodies.

Acknowledgment

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References


