Characteristics of Experimental Autoimmune Hypophysitis in Rats: Major Antigens are Growth Hormone, Thyrotropin, and Luteinizing Hormone in this Model

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We produced experimental autoimmune hypophysitis (EAH) in rats and investigated its characteristics. Female Lewis rats were immunized by two injections with homologous pituitary homogenate and complete Freund's adjuvant. Blood was collected serially from the rats, and serum antibodies to pituitary antigens were examined. The rats were sacrificed 2 or 4 weeks after the final immunization, and histological examinations of the endocrine organs were carried out. Histological examination revealed slight, focal infiltration of mononuclear cells in the pituitary gland only in the rats immunized with the pituitary homogenate. Infiltration of mononuclear cells was not observed in the thyroid gland, pancreas, adrenal gland, or ovary. In the serological examination, antibodies to both cytosolic antigens and cytoplasmic particle antigens from the pituitary gland were detected by enzyme-linked immunosorbent assay (ELISA), and these antibody levels increased with time. Western blotting using the serum antibodies identified an immunoreactive protein of ~21.5 kDa among these antigens, and we confirmed that this protein was rat growth hormone (GH). Furthermore, antibodies to GH, thyrotropin (TSH), and luteinizing hormone (LH) were detected by ELISA. Antibodies to follicle stimulating hormone, prolactin, or adrenocorticotropic were not detected. These data suggest that several antigens from the pituitary gland are involved in EAH in rats, and that GH, TSH, and LH are major antigens among the pituitary antigens in this model.

Keywords: Autoimmune hypophysitis, Experimental hypophysitis, Anti-GH antibody, Pituitary hormone, Autoantibody

INTRODUCTION

Autoimmune hypophysitis, first described by Goudie et al.,[1] is a rare disease that usually occurs at the end of gestation or during the early postpartum period,[2–5] and this disease might be one of the postpartum autoimmune endocrine syndromes.[6] As serological diagnosis has not yet been established in this disease, the diagnosis is mainly based on biopsy of the pituitary gland or computed tomography.[7] One of the reasons for the lack of serological diagnosis might be that experimental autoimmune hypophysitis (EAH) has scarcely been investigated. There are many clinical reports for autoimmune hypophysitis, but so far as
we know, there are only six reports for EAH\(^{8-13}\)
Levine reported lymphocytic hypophysitis established in rats immunized with isologous, homologous, or heterologous pituitary glands;\(^{9,9}\) Klein et al. in rabbits immunized with homologous pituitary glands;\(^{10}\) and Yoon et al. in hamsters immunized with recombinant rubella virus glycoprotein.\(^{11}\) However, the pathogenic antigens were not determined in these studies. On the other hand, Onodera et al. reported polyendocrinopathy, including insulin-dependent diabetes mellitus (IDDM) and autoimmune hypophysitis, in mice infected with reovirus type 1.\(^{12,13}\) Mice infected intraperitoneally at 5 - 7 days of age with reovirus type 1 developed IDDM and autoimmune hypophysitis and produced autoantibodies to insulin and growth hormone (GH). However, the relationships between development of autoimmune hypophysitis and production of autoantibodies to GH were not clear in their model. Thus, it might be difficult to diagnose autoimmune hypophysitis serologically, because the pathogenic antigens have not been determined not only in humans, but also in experimental animals. Therefore, we produced EAH in female Lewis rats by immunizing them with rat pituitary homogenate and investigated the pathogenic antigens. Our results show that GH, thyrotropin (TSH), and luteinizing hormone (LH) might be major pathogenic antigens in this model, and we consider that these hormones might be pathogenic antigens in autoimmune hypophysitis in humans.

**MATERIALS AND METHODS**

**Animals, Induction of EAH, and Collections of Blood and Organs**

Female Lewis rats were obtained at 7 weeks of age from Charles River Japan (Yokohama, Japan). They were maintained under the specific pathogen-free conditions and given commercial chow pellets (Oriental Yeast, Tokyo, Japan) and drinking water ad libitum. They were used starting at 8 weeks of age for induction of EAH.

Rats (n=12) were immunized twice with a 7-day interval with 10 mg of homologous pituitary gland. Rat pituitaries were homogenized in phosphate-buffered saline (PBS, pH 7.4) at a concentration of 200 mg/ml (wet weight) with a Polytron mechanical blender (Kinematica, Luzern, Switzerland), and the suspension was emulsified with an equal volume of complete Freund's adjuvant (CFA, Difco, Detroit, MI) further supplemented with 10 mg/ml of *Mycobacterium tuberculosis* H37RA (Difco). The rats were injected subcutaneously in the each hind and each fore footpad with 0.05 ml of the emulsion on Days 0 and 7, respectively. Control animals (n=10) were injected with an emulsion of PBS and CFA supplemented with *Mycobacterium tuberculosis* H37RA alone.

Blood samples were collected partially by cardiac puncture under ether anesthesia on Days 0, 7, 21, and/or 28, and the half of the each treated animals were autopsied under ether anesthesia on Day 21 and Day 35, respectively. At autopsy, blood was collected from the abdominal aorta, and the pituitary gland, thyroid glands, pancreas, adrenal glands, and ovaries were removed. The organs were fixed with neutralized formaldehyde buffered solution and embedded in paraffin.

**Histology**

Five-micrometer-thick paraffin sections of the endocrine organs were stained with hematoxylin and eosin and observed microscopically.

**Preparations of Rat Pituitary Tissue Extracts**

Pituitary tissue antigens were prepared according to Crock et al.\(^{14}\) Briefly, pituitaries of female Lewis rats were homogenized in PBS with a Polytron mechanical blender. The homogenate was centrifuged at 400 xg (4°C, 20 minutes) to remove cell debris and nuclei. The supernatant was then centrifuged at 100,000 xg (4°C, 60 minutes) to obtain a cytosolic supernatant (fraction) and a cytoplasmic particle (pellet) fraction. The pellet was further washed with PBS and recentrifuged two times to obtain more purified particle proteins. The cytosolic fraction was then
depleted of IgG using protein G-coupled Sepharose (Pharmacia Biotech, Uppsala, Sweden). These preparations were stored at −80°C until use.

ELISA Analysis of Serum Antibodies to Rat Pituitary Tissue Extracts

Rat pituitary cytosolic fraction and cytoplasmic particle fraction were diluted with PBS to a concentration of 100 μg/ml of protein, and each well of a 96-well ELISA plate (Akita Sumitomo Bake, Akita, Japan) was incubated overnight with 100 μl of antigen solution at 4°C in order to coat the wells with antigens. After washing the plate five times with washing buffer (PBS containing 0.05% Tween 20), the wells were incubated with 200 μl of blocking buffer (PBS containing 0.5% bovine serum albumin (BSA, Sigma, St. Louis, MO) and 0.05% Tween 20) for 2 hours at room temperature. After the plate was washed five times, duplicate wells were incubated with 100 μl of rat serum diluted 1:100 in dilution buffer [PBS containing 0.5% BSA and 0.05% Tween 20] for 2 hours at room temperature. After the plate was washed five times, the wells were incubated with 100 μl of a 1:1,000 dilution (in dilution buffer) of affinity-purified rabbit anti-rat IgG antibodies conjugated with horseradish peroxidase (Zymed, San Francisco, CA) for 2 hours at room temperature. After the plate was washed five times, the wells were incubated with 100 μl of a 0.01% substrate (2,2′-azino-bis (3-ethylbenzthiazoline 6-sulfonic acid) diammonium, Sigma) solution for 1 hour at room temperature. The reaction was stopped by adding 100 μl of 1% sodium dodecyl sulfate (SDS) to each well. Using a plate reader (Multiscan Blicomatic, Labosystem, Helsinki, Finland) with measurement and reference wave-lengths of 405 nm and 492 nm, respectively, the absorbance ("absorbance at 405 nm" – "absorbance at 492 nm") of each well was measured.

Western Blotting Assay Using Serum Antibodies to Rat Pituitary Tissue Extracts

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli. [15]

Briefly, a 1:1 mixture of pituitary tissue extracts (10 mg/ml of protein) and sample buffer [80 mM Tris-HCl (pH 6.8) containing 100 mM dithiothreitol, 2% SDS, 15% glycerol, 0.006% bromophenol blue and 5% 2-mercaptoethanol] was heated at 94°C for 3 minutes, and 10-μl aliquots of the mixture were loaded per well of 4 to 20% and 15 to 25% gradient polyacrylamide gels (Daicichi Pure Chemicals, Tokyo, Japan). Electrophoresis was performed at 40 mA for 1 hour in electrophoresis buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.4). Separated proteins were transferred to a nitrocellulose membrane (Amersham, Buckinghamshire, England) using a semidry blotting apparatus with transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) at 6 V for 1 hour. The membrane was incubated overnight in blocking buffer (PBS containing 5% skim milk, 10% normal rabbit serum, and 3% BSA) at 4°C, and then in a 1:100 dilution of rat serum in dilution buffer (5% skim milk and 3% BSA in PBS). After it was washed with washing buffer (5% skim milk and 0.05% Tween 20 in PBS) for 10 minutes, the membrane was incubated for 1 hour at room temperature in a 1:2,000 dilution of biotinylated rabbit antibodies to rat IgG (Zymed) in dilution buffer, washed, and then incubated for 1 hour at room temperature in a 1:1,000 dilution of peroxidase-conjugated streptavidin (Zymed) in PBS containing 3% BSA. Immune complexes were finally detected by staining using 3,3′-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan).

To examine the nature of antibody, we performed absorption experiment. A 0.5 ml of a 1:5 dilution of serum in PBS from a pituitary-immunized rat was incubated with 15 μg of rat GH (Biogenesis, Poole, England) at 37°C for one hour, then the mixture was incubated overnight at 4°C. The mixture was centrifuged at 3,000 rpm (4°C, 20 minutes) in order to remove the immune complexes, and the Western blotting assay was performed using the supernatant as described above. Both the non-GH-absorbed serum and the GH-absorbed serum in the assay were used at the final dilution of 1:100.
ELISA Analysis of Serum Antibodies to Rat Anterior Pituitary Hormones

Rat GH and prolactin (PRL) were obtained from Biogenesis (Poole, England); rat TSH, LH, and follicle stimulating hormone (FSH) were from UCB-Bioproducts (Belgium); and synthetic rat adrenocorticotropic (ACTH) was from Sigma.

Rat GH, TSH, LH, FSH, PRL, and ACTH were prepared at concentrations of 1 μg/ml as coating antigens, the serum was diluted 1:10 and 1:100, and ELISA was performed as described above. Furthermore, in assays for FSH, PRL, and ACTH, chicken serum was used as blocking reagent to block non-specific binding of immunoglobulins to these hormones, and dilutions of the rat serum were prepared with PBS containing 50% chicken serum.

Statistical Analysis

The statistical significance of differences was evaluated by the Student's t-test when variances were equal, or by the Mann-Whitney U-test when variances were unequal.

RESULTS

Histological Changes in the Endocrine Organs

The rats were sacrificed 2 or 4 weeks after the final immunization, and histological examinations of the endocrine organs were carried out. Although it was only slight and focal, infiltration of mononuclear cells in the pituitary gland was observed in 1/6 and 4/6 of the pituitary-immunized rats autopsied on Day 21 and Day 35, respectively (Fig. 1B). Infiltration of mononuclear cells was observed only in the pituitary anterior lobe, not in the middle or posterior lobe. On the other hand, infiltration of mononuclear cells in the pituitary gland was not observed in any control rats (n=10) (Fig. 1A). We tried to produce severe EAH by immunizing rats 4 times with the pituitary homogenate at intervals of 7 days, but severe lymphocytic hypophysitis was not induced (data not shown) and the extents of infiltration of mononuclear cells in the pituitary gland in these animals were similar to those in the animals immunized 2 times. Infiltration of mononuclear cells in other endocrine organs, such as thyroid gland, pancreas, adrenal gland, and ovary, was not observed in the control rats or pituitary-immunized rats (data not shown).

ELISA Analysis of Serum Antibodies to Rat Pituitary Tissue Extracts

Serum antibodies to rat pituitary tissue extracts, cytosolic antigens and cytoplasmic particle antigens were examined serially by ELISA. In the pituitary-immunized rats, antibodies to both cytosolic and cytoplasmic particle antigens were detected, and these antibody levels increased with time (Fig. 2).

Western Blotting Analysis of Rat Pituitary Tissue Extracts Using Anti-pituitary Serum Antibodies

We then carried out Western blotting to determine the properties of the molecules recognized by sera from the pituitary-immunized rats, using the sera obtained 4 weeks after the final immunization. Antibodies reactive to a protein of ~21.5 kDa present in both cytosolic antigens and cytoplasmic particle antigens were detected in 6/6 sera from the pituitary-immunized rats (Fig. 3B). We confirmed that this protein was rat GH using antiserum to rat GH (UCB-Bioproducts) (data not shown). Some stained bands were observed in addition to the ~21.5 kDa protein, but these stained bands were thought to be non-specific, because they were also detected in the sera from the control rats (Fig. 3A).

Furthermore we examined the effects of absorption of serum antibodies with rat GH on the Western blotting analysis. Although stained band to a protein of ~21.5 kDa was observed in both the non-GH-absorbed serum and the GH-absorbed serum, the tone of a color of this band was much weaker in the GH-absorbed serum than in the non-absorbed serum (Fig. 4), indicating that antibodies to GH were markedly removed from the serum by incubation with GH.
depleted of IgG using protein G-coupled Sepharose (Pharmacia Biotech, Uppsala, Sweden). These preparations were stored at −80°C until use.

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To examine the nature of antibody, we performed absorption experiment. A 0.5 ml of a 1:5 dilution of serum in PBS from a pituitary-immunized rat was incubated with 15 µg of rat GH (Biogenesis, Poole, England) at 37°C for one hour, then the mixture was incubated overnight at 4°C. The mixture was centrifuged at 3,000 rpm (4°C, 20 minutes) in order to remove the immune complexes, and the Western blotting assay was performed using the supernatant as described above. Both the non-GH-absorbed serum and the GH-absorbed serum in the assay were used at the final dilution of 1:100.
FIGURE 1 Histological changes in the pituitary gland. A, a control rat (4 weeks after the final immunization); B, a pituitary-immunized rat (4 weeks after the final immunization). Arrows show infiltration of mononuclear cells and bars show 50 μm.

FIGURE 2 ELISA analysis of serum antibodies to rat pituitary tissue extracts. A, assay using pituitary cytosolic fraction; B, assay using pituitary cytosolic particle fraction. Values are means ± SD, and those of the pre-immunization (Week 0) are means from all animals. Statistical significances vs. control rats are indicated as *P<0.01 and **P<0.001.
FIGURE 3 Western blotting analysis of the reaction of serum antibodies with the 14.3- to 220-kDa proteins of rat pituitary tissue extracts. A, a control rat (4 weeks after the final immunization); B, a pituitary-immunized rat (4 weeks after the final immunization). Lane 1, molecular markers; Lane 2, pituitary cytosolic fraction; Lane 3, pituitary cytoplasmic particle fraction.

ELISA Analysis of Serum Antibodies to Rat Anterior Pituitary Hormones

As described above, antibodies to GH were detected in the sera from the pituitary-immunized rats by Western blotting; we therefore examined whether antibodies to other pituitary hormones were produced in these rats. In this assay, we used rat pituitary anterior hormones GH, TSH, LH, FSH, PRL, and ACTH and used the sera obtained 4 weeks after the final immunization. Antibodies to TSH and LH as well as to GH were detected in the sera from the pituitary-immunized rats by ELISA (Figs. 5A, 5B, and 5C), but antibodies to FSH, PRL, and ACTH were not detected (Figs. 5D, 5E, and 5F).

DISCUSSION

We produced EAH in female Lewis rats and investigated its characteristics. Our data suggest that several antigens from the pituitary gland were involved in the EAH in rats, and that at least GH, TSH, and LH are major antigens among the pituitary antigens in this model; although we could not produce severe lymphocytic hypophysitis.

So far as we know, there have been only six reports about EAH. In four of these studies, mainly histological examinations were performed and pathological antigens were not determined using serological techniques such as assays of serum antibodies.
On the other hand, Onodera *et al.* reported polyendocrinopathy, including IDDM and autoimmune hypophysitis, in mice infected with reovirus type 1. Mice infected intraperitoneally at 5–7 day of age with reovirus type 1 developed IDDM and autoimmune hypophysitis, and produced autoantibodies to insulin and GH. However, the relationships between development of autoimmune hypophysitis and production of autoantibodies to GH were not clear in their model.

Therefore, we examined serum antibodies to pituitary antigens in EAH in rats. Firstly, we examined serum antibodies to pituitary tissue extracts, pituitary cytosolic antigens and cytoplasmic particle antigens by ELISA. Pituitary-immunized rats produced antibodies to both cytosolic and cytoplasmic particle antigens, and the antibody levels to both types of antigens increased with time in these animals. Thus, it was confirmed that these rats produced antibodies to certain antigens derived from pituitary cells.

Secondly, we examined the properties of the antigens recognized by the antibodies by Western blotting. A protein of about 21.5 kDa present in rat pituitary extracts was detected by immunostaining with the sera from the pituitary-immunized rats, but not detected by staining with the sera from the control rats. We confirmed that this ~21.5 kDa protein was rat GH using antiserum to rat GH (data not shown).
Moreover this band was markedly diminished by absorption with GH. Nishiki et al. described the antibodies to 22 kDa protein of rat pituitary extracts in the serum from a patient with autoimmune hypophysitis associated with asymptomatic primary biliary cirrhosis.\textsuperscript{116} Furthermore, Yabe et al. reported that antibodies reactive to a 22 kDa protein of rat pituitary extracts were detected in sera from some patients with IIDD, Hashimoto's thyroiditis, Graves' disease, NIDDM, and pituitary dwarfism.\textsuperscript{17-19} Although it is unknown whether the patients having antibodies reactive to this 22 kDa protein were in the state of hypophysitis or not, our results suggest that this 22 kDa protein was GH. On the other hand, Crock et al., using human, monkey, and ovine pituitary extracts, detected 40, 45, or 49 kDa protein, but not 22 kDa protein, as an immunoreactive protein in the sera from patients with lymphocytic hypophysitis and suspected hypophysitis.\textsuperscript{15,18,20} We do not know the exact reason why there is a discrepancy between the results from Nishiki et al., Yabe et al. and us and those from Crock et al.

As antibodies to GH were detected in the sera from the pituitary-immunized rats by Western blotting, we examined whether antibodies to other rat anterior pituitary hormones in addition to GH were produced or not in these rats by ELISA. Antibodies to TSH and LH as well as to GH were detected in these sera, but antibodies to FSH, PRL, and ACTH were not. Although there is no direct evidence for it, it is thought that the epitope of TSH and LH recognized by antibodies from the pituitary-immunized rats might exist on the respective \(\beta\)-subunits, because the \(\alpha\)-subunits of TSH, LH, and FSH are common. However, proteins of \(~30\) kDa (rat TSH and LH) or \(~15\) kDa (their \(\beta\)-subunits) in rat pituitary extracts could not be detected by immunostaining with the sera from the pituitary-immunized rats in Western blotting. Although there is no evidence for it, it seems likely that the reduction of the disulfide bonds of TSH and LH by 2-mercaptoethanol, or denaturation of the protein by SDS and/or other agents used in Western blotting might prevent the detection of these proteins.
We detected anti-GH, -TSH, and -LH antibodies in the sera from the pituitary-immunized rats. Many investigators have reported the anti-pituitary hormone antibodies including anti-GH antibodies in sera from patients with endocrine disorders including autoimmune hypophysitits. Bottazzo et al. described anti-GH antibodies in a patient with Turner's syndrome; Poulard et al. anti-TSH, -LH/FSH, and -ACTH antibodies in patients with autoimmune hypophysitis; Mirakian et al. anti-GH and -PRL antibodies in patients with IDDM; Wild et al. anti-PRL antibodies in a patient with lymphocytic hypophysitis; Scherbaum et al. anti-GH, -LH, and -ACTH antibodies in patients with Cushing's disease; Sugiuara et al. anti-GH/PRL and -ACTH antibodies in patients with IDDM and ACTH deficiency; Komatsu et al. anti-GH/PRL and -ACTH antibodies in patients with the primary empty sella syndrome; Kajita et al. anti-GH/PRL and -ACTH antibodies in patients with hypopituitarism and their families; Ozawa et al. anti-GH/PRL and -ACTH antibodies in a patient with lymphocytic hypophysitis associated with painless thyroiditis; González-González et al. anti-GH/PRL and -ACTH antibodies in a patient with lymphocytic hypophysitis. Thus anti-pituitary hormone antibodies might have important roles in endocrine disorders including autoimmune hypophysitis. In our EAH model, anti-GH, -TSH, and -LH antibodies were detected, but anti-FSH, -PRL, or -ACTH antibodies could not be detected. There are several possibilities to explain the discrepancy between our animal experiment and human cases. First, our technique may not be sensitive enough to detect low levels of antibodies against FSH, PRL, or ACTH. Second, detection method is different from ours in the previous reports that showed positive antibodies against ACTH or PRL. They used ACTH or PRL secreting cell lines and positive reaction was identified by the indirect immunofluorescent technique. Immunofluorescent technique is subjective and requires experienced personnel. Also it may be possible that positive immunofluorescent reaction is not specific to ACTH or PRL. Third, reported cases with antibodies to FSH, PRL, or ACTH may be slightly different from pure lymphocytic hypophysitis. They included Cushing disease, IDDM, empty sella syndrome or hyperprolactinemia. Our findings suggest that neither neither FSH, PRL, nor ACTH is an important antigen in autoimmune hypophysitis, and also suggest that the patients having anti-GH, -TSH, or -LH antibodies described above were in the state of hypophysitis.

Our results show that GH, TSH, and LH might be major pathogenic antigens in EAH in Lewis rats, and suggest that it might be possible to diagnose autoimmune hypophysitis in humans by measuring serum antibodies to pituitary extracts or anterior pituitary hormones such as GH, TSH, and LH.

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References


