Anti-pituitary Antibody-induced Multiple Endocrine Disorders in Mice

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Circulating anti-pituitary antibodies (APAs) have been detected in patients with autoimmune diseases, although the role of autoantibodies in the pathogenesis of autoimmune diseases is still unclear. With the aim of elucidating the autoimmune mechanisms involved in patients with multiple endocrine disorders, we evaluated the pathological changes in the pituitary gland, thyroid, pancreas and adrenal gland of mice, both wild-type and using a murine model of autoimmune thyroid disease [MRL-lpr/lpr] that had been immunized with murine, rat, porcine or human pituitary glands. In four of seven mice, a 22 kD band corresponding to APA was detected by Western blotting in the serum from mice that had been immunized with human pituitary tissues but not in the serum from mice immunized with rat or pig tissue. Inflammatory changes were detected in all groups of mice, occurring in the hypophysis, pancreas and adrenal glands but not in the thyroid. In conclusion, APA-induced autoimmune endocrine disorders are likely to be important for studying the mechanisms involved in autoimmune syndromes.

KEY WORDS: AUTOIMMUNE DISEASE; MULTIPLE ENDOCRINE DISORDER; ANTI-PITUITARY ANTIBODIES (APAS)

Introduction

The role of autoantibodies in the pathogenesis of autoimmune diseases is still unclear.¹² Anti-pituitary antibodies (APAs) have been detected in the serum of patients with autoimmune diseases, and certain antibodies have been proven to cause tissue damage; however, many antibodies appear to have no harmful effect.

Recently, we detected APAs in patients with autoimmune thyroiditis, insulin-dependent diabetes mellitus and pituitary dwarfism.³ Circulating APAs were detected in 18% of patients with autoimmune thyroiditis. Western blot analysis confirmed that APAs were present in the serum of 36% of patients with Hashimoto’s disease and in 29% of those with Graves’ disease.⁴ Autoimmune hypophysitis in rats and rabbits can be induced by injections of pituitary gland homogenates in Freund’s adjuvant.⁵⁶ Histological examination of the pituitary gland reveals focal lymphoid cell infiltration.⁵⁶ In addition, viral antigens have been shown to induce autoimmune hypophysitis in rodents.⁷⁸ Haspel et al.⁷ demonstrated, in a murine model, virus-induced autoimmune pancreatitis and gastritis in addition to hypophysitis.
To explore the likely autoimmune mechanisms involved in patients with multi-endocrine disorders, we investigated the pathological status of the pituitary gland, thyroid, pancreas and adrenal gland in mice immunized by murine, rat, porcine and human pituitary gland. Wild-type mice and also the murine model of autoimmune thyroid disease (MRL-lpr/lpr) were used in this study.

Subjects and methods

PREPARATION OF RAT PITUITARY ANTIGEN

Rat pituitaries (n = 3, 60 mg) obtained from RKL (Gilbertsville, PA, USA) were homogenized in 3 ml buffer (0.25 M sucrose, 0.1 ml EDTA, 3 mM TRIS–HCl buffer, pH 7.4), using a Polytron homogenizer (Hitachi, Japan) (5000 rpm for 1 min), and centrifuged at 10 000 g at 4 ºC for 10 min. The resultant supernatant was used as pituitary antigen. Murine, porcine and human pituitaries (n = 2 each) were prepared.

ANIMALS

Mice (MRL-lpr/lpr and wild type) were immunized by intraperitoneal injection of homogenized murine, rat, porcine and human pituitary gland (0.1 ml antigen including 2 mg pituitary tissue and 0.1 ml conjugated adjuvant per mouse). After 2 weeks, a second immunization (0.2 ml antigen per mouse) was performed.

The mice were killed, by neck torsion, 4 weeks after the second immunization and, as detailed below, the endocrine organs were examined for inflammatory changes and their serum analysed for the presence of APAs using Western blot analysis.

WESTERN BLOT ANALYSIS

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli.9 In brief, 50 µl serum samples (200 g protein) were mixed with 50 µl buffer, 1 µl sample buffer (0.1 dithiothreitol, 2% SDS, 15% glycerol, 0.006% bromophenol blue, mercaptoethanol, 0.08 M TRIS–HCl, pH 6.8) heated at 56 ºC for 10 min, and electrophoresed in 14% polyacrylamide gel for 1.5 h at 18 mA in running buffer (25 mM TRIS, 192 mM glycine, 0.1% SDS). Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using semi-dry blotting apparatus (Bio-Rad Laboratories, Richmond, CA, USA) at 6 V for 60 min. The membrane was incubated in blocking buffer (5% skimmed milk, 10% normal rabbit serum, 3% bovine serum albumin [BSA] in 0.1 M phosphate buffer solution [PBS], pH 7.2) at 4 ºC for 16 – 20 h. The membrane was then incubated in 1:101 diluted human serum in dilution buffer (5% skimmed milk, 3% BSA in PBS, pH 7.2) at room temperature for 2 h, washed in washing buffer (5% skimmed milk, 0.05% Tween 20 in PBS, pH 7.2), then incubated in 1:500 diluted biotinylated anti-human immunoglobulin G (IgG) rabbit polyclonal antibodies in dilution buffer at room temperature for 1 h and washed in washing buffer. Subsequently, the membrane was 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 a 5-minute reaction with POD Immunostain Set (Vector Laboratories, Inc., Burlingame, CA, USA). Primary rabbit antibodies and biotinylated anti-rabbit IgG swine polyclonal antibodies were used to analyse rat pituitary antigens with antibodies to human pituitary hormones.

Anti-pituitary antibodies were measured by immunofluorescence techniques, with rat pituitary antigens as described previously.10

HISTOPATHOLOGICAL EXAMINATION

The hypophysis, pancreas, pituitary gland and thyroid (removed from immunized rats...
or mice) were fixed in 10% buffered formalin and stained with haematoxylin–eosin. Tissues were evaluated blindly by experienced pathologists, who were familiar with autoimmune diseases, but who had no knowledge of the study design.

Results

DETECTION OF APA
In four of seven mice, a 22 kD band corresponding to APA was detected in the serum from mice that had been immunized with human pituitary tissue, but not in the serum from mice immunized with rat or porcine tissue (Fig. 1).

HISTOPATHOLOGICAL EXAMINATION
Two different pathologists confirmed hypophysitis, pancreatitis and adrenitis in all immunized mice, but no thyroiditis was observed (Fig. 2). The inflammatory changes were detected in all immunized mice, although there was some variation in the frequency of positive inflammatory changes between the different groups (Table 1).

Discussion

This study demonstrates the presence of APAs by Western blot analysis in mice with pituitary antigen-induced hypophysitis, pancreatitis and adrenitis. This suggests that autoimmune regulation is mediated through the pituitary–endocrine axis, which controls the secretion of pancreatic and adrenal hormones.

Previously, APA protein has been detected, by Western blotting, in 36.4% of patients with Hashimoto’s disease and in 28.6% of patients with Graves’ disease. Most of the patients displayed a band of 22 kD. The frequency of anti-nuclear antibody, however, was low at < 6%. These results indicate that the APA antigen has organ-specific epitopes.

The thyroid gland is regulated by pituitary hormones, such as thyroid-stimulating hormone and adrenocorticotropic hormone. In Graves’ disease, APAs may interact with regulatory hormones in the pituitary gland during the development of hyperthyroidism. By contrast, thyroiditis in Hashimoto’s disease
appears to result from organ-specific autoimmunity.\(^\text{12}\) Although the pituitary gland is not the specific organ involved in thyroiditis, the hypothalamus–pituitary–thyroid axis would thoroughly regulate thyroid hormone. Further investigation is needed to elucidate the relationship between APAs and the thyroid gland.

Anti-pituitary antibodies have been detected, at relatively high frequency, in the sera of patients with insulin-dependent diabetes mellitus (IDDM).\(^\text{13,14}\) For example, Sugiura et al.\(^\text{13}\) detected APA, using immunofluorescence, in 24 of 81 patients with IDDM. Previously, using enzyme-linked immunosorbent assay (ELISA), we found

### TABLE 1:
Frequency of hypophysitis, thyroiditis, pancreatitis and adrenitis induced by pituitary antigens of different animal origin, in wild-type and lpr mice

<table>
<thead>
<tr>
<th>Antigen origin</th>
<th>Hypophysitis</th>
<th>Thyroiditis</th>
<th>Pancreatitis</th>
<th>Adrenitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>2/3</td>
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<tr>
<td>Pig</td>
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<td>2/4</td>
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<tr>
<td>Human</td>
<td>1/3</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
</tr>
<tr>
<td>Total (%)</td>
<td>6/13 (46%)</td>
<td>0/13 (0%)</td>
<td>6/13 (46%)</td>
<td>6/13 (46%)</td>
</tr>
</tbody>
</table>

**FIGURE 2:** Inflammatory changes detected in the hypophysis (A), but not in the thyroid gland (B), in wild-type mice that had been immunized with human pituitary tissue. Lpr mice showed similar thyroiditis regardless of the presence (C) or absence of APAs (D). Magnification: (A), × 400; (B), (C) and (D), × 200.
that 39.1% of 64 patients with IDDM were positive for APA. Western blotting revealed a 22 kD APA band in 64% of the patients that were found to be APA-positive by ELISA. These data may help to elucidate the pathophysiologic role of APAs in IDDM.

Using ELISA, APA positivity has been detected in 5.3% of 38 patients with non-insulin-dependent diabetes mellitus (NIDDM). Further, virus-infected cells have been reported in both the pancreas and pituitary gland of the reovirus-infected diabetic mouse. In addition, an association has been reported between hypopituitarism and diabetes. These findings indicate the possibility that the pancreas and pituitary gland may have a common antigenicity and, therefore, the presence of APAs may be related to both insulin deficiency in NIDDM and the onset of IDDM.

Tartaglia et al. identified a high-affinity receptor for leptin, which was found to be expressed in several areas of the brain, including the hypothalamus. Leptin is secreted by adipocytes and is an important circulating signal for weight control. One of the recently suggested mechanisms of weight control is that the hypothalamic receptor receives the leptin signal and controls energy expenditure through the hypothalamus–pituitary–thyroid axis, which regulates the secretion of insulin and glucocorticoids. Both insulin and glucocorticoids stimulate leptin secretion. There may, therefore, be a link between the pituitary gland and insulin secretion by the pancreas. This supports further the hypothesis that APAs play a role in the pathogenicity of both the onset of IDDM and insulin deficiency in patients with NIDDM.

Anti-pituitary antibodies have been detected in only 8.0% of 25 patients with pituitary dwarfism and in none of 12 patients with pituitary adenoma. Western blotting showed no positive band for APA in these pituitary gland disorders. Marjorie et al. reported the presence of APAs in 45% of patients with pituitary disorders, such as pituitary adenoma and empty sella syndrome. These APAs included anti-growth hormone (anti-GH) antibody, anti-thyroid-stimulating hormone antibody and anti-adrenocorticotropic hormone antibody. Bottazzo et al. also found autoantibodies to anterior pituitary cells that were secreting GH, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in patients’ sera. These patients had empty sella syndrome. Previously, a 22 kD band positive for APA was detected in a cytosolic fraction containing growth hormone. These results suggest that the target antigen of APA may be a growth factor.

In conclusion, APA-induced autoimmune endocrine disorders are likely to be important for studying the pathophysiology of the mechanisms involved in autoimmune multi-endocrine disorders. The pathogenesis of autoimmune thyroiditis may involve a different mechanism to the other types of endocrine autoimmune syndromes examined in this study. It is possible that autoimmune thyroiditis is induced by a unique antigen. We are currently studying autoimmune thyroiditis and anti-pituitary antibody production in response to a large number of rodent pituitary antigens, and examining the relationship between specific pituitary antigens and clinical features. Further investigation is also needed to characterize in greater detail the pituitary antigens recognized by determining amino acid sequence of the antigen epitopes.
References


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