Studies on Circulating Anti-Pituitary Antibodies in NIDDM Patients

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Abstract. The clinical significance of anti-pituitary antibodies (APA) was examined by enzyme-linked immunosorbent assay (ELISA) in individuals with non-insulin dependent diabetes mellitus (NIDDM). Serum samples were obtained from 150 NIDDM patients and 45 normal subjects. Urinary C-peptide (U-CPR) was also measured for the NIDDM patients. APA-positive serum was incubated with porcine pancreas, liver, kidney, or spleen powder and analyzed by immunoblot. The prevalence of APA was found to be significantly ($P<0.05$) higher in NIDDM patients (24.7%) than in the controls (6.7%) by ELISA. The index values for APA were inversely related to the levels of U-CPR ($P<0.005$). The levels of U-CPR were significantly ($P<0.001$) lower and the prevalence of insulin deficiency was significantly ($P<0.05$) higher in NIDDM patients who were APA positive than in those who were APA negative. The presence of APA may therefore be related to reduced secretion of insulin in NIDDM patients. In Western blot analysis, preincubation of APA-positive sera with porcine pancreas powder prevented recognition of the 22-kD protein (APA). The possibility of a common autoantigenicity in the pancreas and the pituitary was indicated.

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APA have been detected in the serum of individuals with ACTH deficiency, Sheehan's syndrome, empty sella syndrome, Hashimoto's thyroiditis, Graves' disease and insulin-dependent diabetes mellitus (IDDM) [1-3, 6-10]. We previously detected APA in the serum of patients with non-IDDM (NIDDM) and IDDM by Western blot analysis [11]. In the present study, intending to quantify the degrees of APA, we have tested serum samples from individuals with NIDDM by ELISA assay. On the other hand, our previous report suggested the possibility that the presence of APA may be related to reduced secretion of insulin in NIDDM patients [11]. In this study, in order to show common autoantigenicity in the pancreas and pituitary, we analyzed APA-positive serum by Western blot with and without preincubation with porcine pancreas powder before analysis. Moreover, to examine as to whether APA recognize human GH, GH was measured by
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radioimmunoassay after mixing with APA positive serum.

Materials and Methods

Subjects

Serum samples were obtained from 150 individuals with NIDDM and 45 normal controls without diabetes (Table 1). Serum samples were analyzed for APA by ELISA. As a measure of insulin deficiency, urinary C-peptide (U-CPR) was also assayed in samples from NIDDM patients. APA-positive serum was incubated with porcine pancreas, liver, kidney or spleen powder before analysis by immunoblotting.

Materials

Porcine pituitaries were obtained from COSMO Bio. (Tokyo, Japan); microtiter plates from Nunc-Immuno Module (A/S Nunc, Roskilde, Denmark); polyacrylamide gels (8 to 16%) and polyvinylidene difluoride membranes from Tefco (Tokyo, Japan); protein standards from Bio-Rad (Richmond, CA); biotinylated rabbit polyclonal antibodies to human immunoglobulin G (IgG), as well as acetone-treated porcine liver, pancreas, kidney, and spleen powder, from Sigma (St. Louis, MO); streptavidin-biotin complex peroxidase from Dako (Glostrup, Denmark); a POD Immunostain kit from Wako (Kyoto, Japan); and aprotinin from Teikoku Zoki (Tokyo, Japan).

Preparation of porcine pituitary antigens

Two porcine pituitaries were homogenized in 3 ml of homogenization buffer [0.25 M sucrose, 0.1 mM EDTA, and 3 mM Tris-HCl (pH 7.4)] with a Pyltron homogenizer (5000 rpm, 1 min). The homogenate was centrifuged at 10,000 × g at 4 °C for 10 min, and the resulting supernatant was used as a source of pituitary antigen.

ELISA for APA

Microtiter plates were coated with porcine pituitary antigens (25 µg of protein/ml diluted in 0.05 M PBS) by incubation overnight at 4 °C, and were then incubated with 3% BSA in PBS at 37 °C for 30 min. After washing with 0.05% Tween 20 in PBS, to each well was added 100 µl of reaction buffer [0.1 M Tris-HCl (pH 8.0) containing 0.25% BSA, 0.05% Tween 20, and 0.5% each of normal mouse and rabbit serum] followed by 50 µl of human serum or APA standard. The plates were incubated, with shaking, at 25 °C for 1 h and then washed with PBS. Subsequently 100 µl of peroxidase-labeled rabbit polyclonal antibodies to human IgG were added to each well and the plates were incubated for 30 min at 25 °C. After washing with PBS, 100 µl of substrate solution [0.11 M sodium acetate-citrate buffer (pH 5.5) containing 0.006% H2O2 and TMB (0.2 µg/ml) was added to each well and the plates were incubated at 25 °C for 30 min. The colorimetric reaction was stopped by adding of 100 µl of 0.5 M H2SO4. The absorbance at 450 nm was measured and the cut-off index was determined. Assays were performed in duplicate. The index value was determined as follows.

$$\text{Index value} = \frac{\text{absorbance at 450 nm of test serum}}{\text{absorbance at 450 nm of the serum of APA-negative normal subject}}$$

Western blot analysis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [12]. In brief, 100 µl of porcine pituitary antigens (200 µg of protein) were mixed with 100 µl of sample buffer [0.1 M dithiothreitol, 2% (w/v) SDS, 15% (v/v) glycerol, 0.006% bromphenol blue, 2-mercaptoethanol, and 80 mM Tris-HCl (pH 6.8)].
and the mixture was heated at 94 °C for 3 min and subjected to electrophoresis in 8 to 16% polyacrylamide gels for 1 h at 40 mA (electrophoresis buffer; 25 mM Tris, 192 mM glycine and 0.1% SDS). Separated proteins were transferred to a polyvinylidene membrane by means of a semidy blotting apparatus (Bio-Rad), at 6 V for 1 h. The membrane was incubated at 4 °C for 16 to 20 h in blocking buffer [5% (w/v) skim milk, 10% (v/v) normal rabbit serum, 3% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (pH 7.2)], and then at room temperature for 2 h in 1:101 dilution of human test serum in dilution buffer (5% skim milk and 3% BSA in PBS). After washing in washing buffer (5% skim milk and 0.05% Tween 20 in PBS), the membrane was incubated for 1 h at room temperature with a 1:500 dilution of biotinylated rabbit antibodies to human IgG in dilution buffer. After another wash the membrane was then incubated at room temperature for 1 h with a 1:10 dilution of streptavidin-biotin complex peroxidase (in PBS containing 3% BSA). Immunocomplexes were finally visualized by staining for 5 min with a POD Immunostain kit. We have previously shown [5] that both antibodies to GH and to PRL react with a band appearing at 22 kD. The presence of APA was indicated by the detection of such an immunoreactive band.

**Effect of antibody preadsorption on Western blot analysis**

APA-positive serum (10 μl) was incubated for 2 h at room temperature with 500 μl of dilution buffer and 500 μl of homogenized acetone-treated porcine pancreas, liver, kidney or spleen powder (10 mg/ml) before analysis by Western blotting. As well as this, 10 μl of APA-positive serum and 500 μl of dilution buffer were incubated for 2 h with 500 μl of homogenized acetone-treated porcine pancreas powder (10 mg/ml), or the homogenate supernatant (10,000 × g, 10 min), in the absence or presence 500 U of aprotinin (or with 500 U of aprotinin alone in 500 μl of dilution buffer). The treated antiserum was then subjected to Western blot analysis.

**Determination of U-CPR**

The daily excretion of U-CPR was measured with radioimmunoassay kits, purchased from Mitsubishi Kagaku (Tokyo, Japan), according to the manufacturer’s procedures.

**Assay of pituitary hormones and other antibodies**

The basal serum concentrations of thyroid-stimulating hormone (TSH), LH, FSH, PRL, GH and ACTH were measured with RIA kits from Mitsubishi Kagaku (Tokyo, Japan). Anti-thyroid peroxidase, anti-nuclear and anti-thyroglobulin antibodies were determined with ELISA kits from Nissui Kagaku (Tokyo, Japan).

**Statistical analysis**

Data are expressed as means ± SEM. Difference in U-CPR (μg/24 h), body mass index (BMI) (kg/m²), postprandial plasma glucose (mg/dl), glycohemoglobin A1c (HbA1c) and 1,5anhydroglucitol (1,5AG) were assayed post hoc by Fisher’s PSLD analysis after analysis of variance. The chi-square test with Yates correction was used to determine the significance of differences between groups. A level of *P*<0.05 was considered statistically significant.

**Results**

**APA by ELISA in normal subjects and in patients with NIDDM**

The APA index values determined by ELISA in normal subjects were distributed in the 0.45 to 5.93 range (1.48 ± 1.01). The index values >2.7 (mean ± 2 SD) were diagnosed as positive in APA by ELISA. The cut off value was less than 2.7. A histogram of APA index values within the normal range in normal subjects is shown in Fig. 1. The receiver operating characteristic (ROC) curve of APA index values determined by ELISA as a classifier of insulin deficiency in patients with NIDDM is also shown in Fig. 2.

To assess intraassay and interassay variation in samples, independent repeats for 3 different sera were selected and examined by ELISA. The results for each sample was correlated closely for 10 of seven ELISAs. The reproducibility of APA samples was good and their coefficient of variation (CV)
was 4.15 to 9.58% in intraassay variation (n=10) and 7.60 to 9.58% in interassay variation (n=7).

To examine as to whether APA recognize human GH, GH measurement by radioimmunoassay was done after absorption by APA positive sera. GH positive samples were mixed with APA positive samples for 12 h at 4 °C. GH value in serum which was treated with APA positive serum reduced to 9.5–86.9% compared to the value in pretreated serum (n=3).

**Prevalence of APA**

APA were detected in 24.7% (37/150) of individuals with NIDDM and in 6.7% (3/45) of healthy controls by ELISA (Fig. 3). The prevalence of APA was significantly higher in patients with NIDDM than in nondiabetic subjects (P<0.05).

**Insulin deficiency in patients with NIDDM**

Insulin deficiency was examined in NIDDM patients classified as APA positive or APA negative by ELISA. The amount of U-CPR was significantly lower in the APA-positive group (28.2 ± 3.6 μg/day) than in the APA-negative group (63.9 ± 5.24 μg/day) of NIDDM patients (P<0.0001) (Fig. 4). Classification of patients with NIDDM on the basis of U-CPR levels into non-insulin-deficient (U-CPR ≥ 20 μg/day) and insulin-deficient (U-CPR <20 μg/day) groups, revealed that 10.7% (16/150) were insulin deficient. The prevalence of insulin deficiency was significantly higher in the APA-positive group (27.0%, 10/37) than in the APA-negative group (5.3%, 6/113) (P<0.05) (Fig. 4). No significant differences between APA-positive and APA-negative NIDDM patients in BML
postprandial plasma glucose, HbA1c or 1,5AG were apparent (Table 2). No correlation between the APA and BMI index values, duration after onset and HbA1c was observed. Only the levels of U-CPR were still inversely related with the APA index value in NIDDM patients (Fig. 5).

Effect of antibody preadsorption on immunoblot analysis

An immunoreactive protein of 22 kD was no longer detected on immunoblot analysis when APA-positive sera were preincubated with homogenized acetone-treated porcine pancreas powder (Fig. 6). In contrast, preincubation of APA-positive sera with homogenized acetone-treated porcine liver, kidney or spleen powder did not prevent recognition of the 22-kD protein (Fig. 6). Figure 7 shows that detection of the 22-kD protein was prevented by preincubation of APA-positive sera not only with homogenized acetone-treated porcine pancreas powder in the absence or presence of aprotinin, but also with homogenate supernatant
Fig. 7. Further characterization of the effect of preadsorption of anti-pituitary antibody (APA)-positive serum with porcine pancreatic antigens on Western blot analysis. Western blot analysis was performed with untreated APA-positive serum (lanes 1 and 2; lane 1: strong positive serum, lane 2: weak positive serum) or APA-positive serum that had been preincubated with homogenized acetone-treated porcine pancreas powder in the absence (lanes 3 and 4) or presence (lanes 7 and 8) of aprotinin, with homogenate supernatant in the absence (lanes 5 and 6) or presence (lanes 9 and 10) of aprotinin, or with aprotinin alone (lane 11; weak positive serum).

Fig. 8. Time course of anti-pituitary antibody (APA) detected by ELISA and Western blotting in one case with diabetic ketoacidosis.

Discussion

In this study, with the aim of quantifying the degree of APA, serum samples were analyzed for APA by ELISA in NIDDM patients. The prevalence of APA was significantly higher in patients with NIDDM than in non-diabetic subjects, both determined by ELISA. U-CPR was significantly lower and the prevalence of insulin deficiency was significantly higher in NIDDM patients who were APA positive than in those who were APA negative. A further finding that deserves comment is the inverse correlation observed between the APA index values by ELISA and the levels of U-CPR in NIDDM patients. These findings indicate that the presence of APA is related to insulin deficiency in patients with NIDDM. The time course of APA in our patient with diabetic ketoacidosis is compatible with this hypothesis.

To examine the mechanism of the presence of APA related to insulin deficiency in NIDDM patients we made a co-incubation study in APA-positive NIDDM patients' serum by Western blot analysis. Our observation that preincubation of APA-positive serum with porcine pancreas homogenate, but not with homogenates of porcine...
liver, kidney or spleen, prevented recognition of the 22-kD porcine pituitary protein by immunoblot analysis suggests that the pancreas and the pituitary share the antigenic determinant. The fact that the effect of prevention with pancreatic homogenate was still apparent in the presence of aprotinin indicates that it was not attributable to the degradation of APA caused by pancreatic basic trypsin.

Coxsackie B virus has been isolated from the pancreas of patients with virus-induced diabetes mellitus and the virus has been shown to induce inflammation in islets of Langerhans, β-cell necrosis, and pituitary gland injury in mice [13]. Virus-infected cells have also been observed in the pancreas and pituitary glands of reovirus-infected diabetic mice [14]. The mRNA that encodes islet cell antigen 512 (ICA512), a standard autoimmune marker for IDDM, was detected in both brain and pancreas by Northern blot analysis [15]. Antibodies to pancreatic islet and brain glutamic acid decarboxylase are also likely to be cross-reactive [16]. Together, these observations are consistent with the possibility of common auto-antigenicity in the pancreas and pituitary. This common autoantigenicity may contribute to insulin deficiency in APA-positive NIDDM patients.

Recently humoral islet-cell antigen ICA 69 was identified in prediabetic individuals [17]. This protein was found to be expressed in human pancreas, heart and brain. Approximately 25% of recent-onset IDDM patients have detectable levels of antibodies to this protein [18]. APA also seem to be expressed in the pituitary and the pancreas, and are very frequently detected in patients with IDDM [11]. APA and ICA 69 seem to have something in common with each other. The results obtained thus far on autoimmune T-cells reactive with β-cell antigen in humans imply that multiple antigens may be involved in the disease process [19]. T-cell responsiveness to islet autoantigens is not IDDM specific, and the expression or distribution of target antigens of such autoreactive T-cells is never limited to β-cell. The mechanism by which β-cell-reactive T-cells are involved in the β-cell destruction process is an enigma [19]. Similarly, the mechanism by which the prevalence of insulin deficiency was significantly higher in the APA-positive NIDDM patients remains unknown. The significant reduction of GH values observed after mixing with APA-positive serum suggests that specific epitopes of APA may be found on GH and related hormones in the pituitary gland.

By ELISA we showed a higher prevalence of APA, lower levels of U-CPR and higher prevalence of insulin deficiency in NIDDM patients who were APA positive than in those who were APA negative, and an inverse correlation between the APA index values and the levels of U-CPR in NIDDM patients. These findings indicate the possibility of insulin deficiency in APA-positive NIDDM patients. Our co-incubation study of APA-positive serum with pancreas homogenate suggested a common autoantigenicity, which may be attributable to the insulin deficiency in APA-positive NIDDM patients.

In conclusion, ELISA assay has the advantage of quantifying the degrees of APA, and is recommended for screening, because many samples can be analyzed simultaneously. The ELISA as well as Western blot analysis would appear to be advisable for patients with endocrine abnormalities such as NIDDM.

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References


