Amylase α-2A Autoantibodies
Novel Marker of Autoimmune Pancreatitis and Fulminant Type 1 Diabetes

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OBJECTIVE—The pathogenesis of autoimmune pancreatitis (AIP) and fulminant type 1 diabetes remains unclear, although it is known that immune-mediated processes severely compromise the endocrine and exocrine functions in both diseases.

RESEARCH DESIGN AND METHODS—We have screened a λTriplEx2 human pancreas cDNA library with serum from a patient with AIP and obtained positive clones. Sequence analysis revealed that 7 of 10 clones were identical to human amylase α-2A. Using a recombinant COOH-terminal amylase α-2A protein, we developed an enzyme-linked immunosorbent assay system to detect autoantibodies against human amylase α-2A.

RESULTS—All 15 serum samples from patients with AIP recognized the recombinant protein, whereas sera from 25 patients with chronic alcoholic pancreatitis and sera from 25 patients with a pancreas tumor did not. Interestingly, 88% (15/17) of patients with fulminant type 1 diabetes were positive for an autoantibody against amylase α-2A. These antibodies were detected in 21% of patients with acute-onset type 1 diabetes (9 of 42) and 6% of type 2 diabetic patients (4 of 67).

CONCLUSIONS—These results suggest that an autoantibody against amylase α-2A is a novel diagnostic marker for both AIP and fulminant type 1 diabetes and that, clinically and immunologically, AIP and fulminant type 1 diabetes are closely related. 

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Recently, autoimmune pancreatitis (AIP), a unique form of chronic pancreatitis, has been reported as a discrete disease entity (1). It is characterized by 1) irregular narrowing of the main pancreatic duct and swelling of the pancreas, both of which are due to abundant lymphoplasmacytic inflammation to the exocrine pancreas (2); 2) the increased serum level of IgG and IgG4; 3) positive autoantibodies such as lactoferrin autoantibody (3,4); and 4) a high prevalence of diabetes with complications (5).

We recently reported that a high proportion of pancreatic islets and exocrine pancreatic tissues were infiltrated by CD4+ or CD8+ T-cells in the inflammatory process, which might induce diabetes in AIP (6). In addition, treatment with prednisolone improved insulin secretion and glycemic control in AIP patients (5). These data support the concept that autoimmune mechanism(s) plays a pivotal role in the destruction of the endocrine and exocrine pancreas in AIP patients with diabetes.

Clinically, the most common initial symptom of AIP is jaundice, but in some patients, no symptoms or only mild symptoms, frequently without acute attacks of pancreatitis, may be present (7). It is difficult to distinguish AIP from other types of chronic pancreatitis or cancer of the pancreatic head (8). In such cases, detection of autoantibodies is useful for diagnosing AIP, but a proportion of patients with AIP are negative for autoantibodies against lactoferrin and CAII (3,4).

We encountered an AIP patient whose serum IgG and IgG4 levels were 3,498 and 2,430 mg/dl, respectively. It has been reported that median levels (5th and 95th percentiles) of IgG and IgG4 from patients with AIP were 2,389 mg/dl (1,349 and 4,310) and 742 mg/dl (265 and 1,150), respectively (3), so high concentrations of IgG in this case prompted us to search for new autoantigens associated with AIP. We also searched for the presence or absence of new autoantibodies in patients with abrupt onset and severe ketoacidosis-prone type 1 diabetes [called fulminant type 1 diabetes (9,10)], which involve the exocrine pancreas and the endocrine pancreas.

RESEARCH DESIGN AND METHODS

Serum used for screening the human pancreas cDNA library was obtained from a 67-year-old male patient (A.O.), who was admitted to our hospital complaining of slight abdominal pain and jaundice. Computed tomography revealed an enlarged pancreas, and laboratory findings showed high concentrations of IgG and IgG4. Tests for anti-lactoferrin and anti-CAII antibodies were both positive, but those for anti-nuclear antibody, anti-mitochondrial antibody, and rheumatoid factor were negative.

Additional AIP sera were obtained from 14 newly diagnosed patients at the University of Yamanashi Hospital and Toranomon Hospital, Tokyo. Diagnosis of AIP was based on criteria proposed by the Japan Pancreas Society (11). Our 15 patients filled criterion 1 (narrowing of the main pancreatic duct or enlargement of pancreas by imaging studies), together with criterion 2 (high serum γ-globulin, IgG, or IgG4 or the presence of autoantibodies, such as anti-nuclear antibodies and rheumatoid factor) and/or criterion 3 (marked interlobular fibrosis and prominent infiltration of lymphocytes and plasma cells in the periductal area). Serum samples were taken from 25 patients with chronic alcoholic pancreatitis, who were diagnosed according to a history of alcohol abuse, impaired exocrine pancreatic function, and the presence of calcified precipitates in the pancreas by imaging studies [Japan Pancreas
Society, criteria for chronic pancreatitis 2001 (12). Twenty-five serum samples were recruited from patients with pancreas tumor (cancer \( n = 8 \)) and intraductal papillary mucinous tumor (IPMT, \( n = 17 \)). Fulminant type 1 diabetes (\( n = 17, 13 \) cases at the onset and 4 cases after onset) was diagnosed by criteria (fasting C-peptide \( \leq 0.033 \) nmol/l and A1C \( \leq 8.0\% \) or C-peptide \( \leq 0.540 \) nmol/l and A1C \( \leq 8.0\% \)) as reported previously (13,14). Fulminant type 1 diabetes associated with pregnancy (15) was excluded from the present study. Acute-onset type 1 diabetes (\( n = 42 \)) and type 2 diabetes (\( n = 67 \)) samples were also recruited. The patients’ clinical characteristics are summarized in Table 1. Serum from patients with Hashimoto’s thyroiditis (\( n = 47 \)) were also studied. Diagnosis of the disease was made by elastic goiter and autoantibodies against both thyroglobulin and thyroid peroxidase. Control sera were obtained from 100 (59 male and 41 female) healthy volunteers.

**Immunoscreening.** The TrypEx2 human pancreas large insert cDNA library (HLS517a) and Escherichia coli XL-1 competent cells were obtained from BD Biosciences Clontech (Palo Alto, CA). The plaques on the plate were transferred to nitrocellulose filters presoaked with 10 mmol/l isopropyl-β-D-thiogalactopyranoside (IPTG), washed with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST), and blocked with TBST containing 1% BSA. The filters were incubated overnight at 4°C with the sera from the patients with AIP (A.O.) at a dilution of 1:500. After washing four times with TBST, the filters then reacted with goat horseradish peroxidase–conjugated anti-human IgG (American Qualex, San Clemente, CA) at a dilution of 1:2,000 for 45 min at room temperature. The filters were also washed four times with TBST; positive reaction was detected with 3,3′-diaminobenzidine.

**Preparation of the recombinant human AMY-2A.** A cDNA fragment of the positive clone was amplified by PCR with the sense primer, 5′-ATGGGATCTCTTGGGGTTCTGACAGA, and antisense primer, 5′-CTTCGAATTCCTCAATTTAGATTCAGCATGAATTGC, which added an ATG codon at the NH2-terminus. The PCR product was digested with BamHI and EcoRI and then ligated into pTrc His B (Invitrogen, Carlsbad, CA). After sequencing, the plasmid was transfected into E. coli BL-21 (Novagen, Darmstadt, Germany). The production of the recombinant protein was induced with 1 mmol/l IPTG and purified by His Bond column chromatography.

**Western blot analysis.** The 0.1% SDS–15% PAGE and transferring onto the nitrocellulose membrane was carried out as previously described (16) with slight modifications as follows: The membrane was blocked with 5% skim milk and 5% goat serum in TBS and then incubated with sera from the patients with AIP (1:500) overnight at 4°C. After washing five times with TBST, the membrane was reacted with goat horseradish peroxidase–conjugated anti-human IgG (1:2,000) for 30 min at room temperature. Positive reaction was detected by the same way as described in IMMUNOSCREENING.

**Enzyme-linked immunosorbent assay for detecting autoantibody against human AMY-2A.** Autoantibody against human AMY-2A was measured by enzyme-linked immunosorbent assay (ELISA) using methods previously described (5). In brief, a microtiter plate (Coster 3590; Corning, Horseheads, NY) was coated with 50 μl 0.1 μg recombinant human AMY-2A overnight at 4°C. After washing the plate three times with PBS, the plate was incubated with 200 μl 1% BSA in PBS for 30 min. Next, the patients’ sera were tested in triplicate at dilutions of 1:200 in 1% BSA for 1 h. The bound antibody was specially reacted with goat horseradish peroxidase–conjugated anti-human IgG (1:2,000) in 1% BSA for 30 min at room temperature. After washing, the plate was incubated with 100 μl 1-Step Slow TMB-ELISA (Pierce, Rockford, IL) for 30 min. The reaction was terminated by adding 100 μl 1 mol/l H2SO4, and absorbance was determined at an optical density of 450 nm. Intra- and interassay coefficient of variation, determined with the same lot of five ELISA plates, were 4.28 and 7.67%, respectively.

**Ethics.** An ethical committee approved all study protocols, and patients gave informed consent.

**Statistical analysis.** Statistical analysis was carried out using Fisher’s exact test (JMP, Cary, NC), in which we considered statistically significant if \( P \) values were <0.05. Receiver operating characteristic (ROC) analysis was carried out with MedCalc (MedCalc Software, Mariakerke, Belgium).

**RESULTS**

**Cloning of cDNAs from human pancreas.** We completely screened 2 × 104 plaques with the AIP patient’s serum (A.O.) and obtained 10 positive clones. Nucleotide sequencing of the insert cDNAs and a subsequent homology search revealed that 7 of 10 clones were identical to human amylase-2A (AMY-2A). When compared with the nucleotide sequence of the human AMY-2A cloned by Wise et al. (17), four of seven clones contained the full coding sequence, whereas the 5′ ends of the other three clones started from 61, 759, and 897 bp (in ATG is designated as 1) (Fig. 1). Other nonamylase clones were those of the housekeeping genes, such as the heat shock protein and the nuclear protein.
Western blot analysis, immunoprecipitation, and ELISA system for detecting anti-human AMY-2A.

Because IgG from the AIP patient used for screening recognized four different lengths of human AMY-2A clones, we hypothesized that the regions shared by these four clones, from codons 299 to 512, might contain a common epitope for the patient's IgG (Fig. 1). Therefore, we produced histidine-tagged human AMY-2A from codons 299 to 512 (AMY-2A/299–512) in E. coli BL21 and carried out Western blot analysis (Fig. 2A). Patient's serum (A.O.) recognized the 30-kDa recombinant protein (line 1), but sera from healthy volunteers did not (lines 3 and 4). When the patient's serum was preincubated with the recombinant protein, positive staining was abolished (line 2), suggesting that the autoantibody reacted with the recombinant protein, which contains the epitope.

Anti-human AMY-2A antibody produced in goat was bound to the in vitro–translated 35S–AMY-2A and was precipitated by protein G–sepharose (Fig. 2B). IgG from two patients with AIP also bound to the labeled protein and was precipitated, but the IgG from two healthy volunteers did not (Fig. 2B). This recombinant fluid phase autoantibody assay with in vitro transcription and translation of AMY-2A without additional amino acids, such as His-Tag, confirmed the specificity of the autoantibody against the protein.

Next, by coating the protein onto the plate, we developed an ELISA system for detecting anti-amylase antibodies in the serum. When compared with the normal serum, patient sera showed strong signals, which were well correlated with immunoprecipitated 35S–AMY-2A by protein G–sepharose (Fig. 2C). This positive reaction in ELISA was displaced in a concentration-dependent fashion by AMY-2A/299–512 (Fig. 2D). When the AIP patient’s serum (A.O.) was diluted, we could detect positive signals up to 1:1,000 dilution (Fig. 2E). To obtain a cutoff value for positivity, we carried out ROC analysis of the healthy volunteers and fulminant type 1 diabetic patients. We carried out ROC analysis of the healthy volunteers (n = 100) and fulminant type 1 diabetic patients (n = 17) with MedCalc.

FIG. 1. Cloning of human amylase α-2A cDNAs from λTriplEx2 human pancreas cDNA library. Seven clones of human amylase α-2A cDNAs. Their lengths and 5'-ends are shown (A in ATG is designated as 1). The top bar indicates human amylase α-2A cDNA as reported by Wise et al. (17), and the common regions shared by all seven clones, from codons 299 to 512, are shown in the bottom bar.

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![Figure 2](image-url)
volunteers (n = 100) and fulminant type 1 diabetic patients (n = 17) (Fig. 2F). Table 2 shows criterion values and coordinates of the ROC curve. When the value was set as 34 (area under the ROC curve 0.92; significance level P = 0.0001), sensitivity, specificity, and positive predictive value were 88.24, 99.0, and 93.7%, respectively.

### Prevalence of autoantibody against human AMY-2A in AIP patients

Using the ELISA system, we determined the prevalence of autoantibody against human AMY-2A in AIP patients and various pancreatic diseases (Fig. 3). All 15 IgGs from patients with AIP were positive for AMY-2A/299–512, whereas 1 of 100 IgGs from control subjects was positive for the antibody (P < 0.001, Fisher’s exact test). All the IgGs from the patients with chronic alcoholic pancreatitis (n = 25) or with pancreas tumor (pancreatic cancer, n = 8; IPMT, n = 17) were negative for the antigen. Antibodies were detected in 9% (4/47) of patients with Hashimoto’s thyroiditis, a representative organ-specific autoimmune disease (Fig. 3A).

Figure 3B shows the time course of the autoantibody titer from two AIP patients before and after prednisolone treatment. In patient A.O., IgG4 gradually increased and reached 5,540 mg/dl, but administration of prednisolone initiated a rapid decrease of IgG4 to 571 mg/dl. Before prednisolone treatment, the titer of the autoantibody against AMY remained high, and prednisolone treatment induced a rapid decrease of the titer of AMY-2A autoantibody to a normal level. The fall rate of the antibody titer seemed to be parallel to that of serum IgG4. In patient T.M., administration of prednisolone also rapidly decreased the titer of the autoantibody against AMY. The autoantibodies did not increase even at the drug maintenance dose in both cases.

### Prevalence of autoantibody against human AMY-2A in patients with fulminant type 1 diabetes and acute-onset type 1 diabetes

We next studied the prevalence of autoantibody against human AMY-2A in various types of diabetic patients (fulminant type 1 diabetes, n = 17; acute-onset type 1 diabetes, n = 67; Fig. 4). Interestingly, 88% of patients with fulminant type 1 diabetes were positive for the autoantibody, but 1% of control was positive for the antibody (P < 0.001, Fisher’s exact test). The autoantibody was detected with low frequency in patients with acute-onset type 1 diabetes.
Using 13 serum samples from our AIP patients, we carried
out the adoptive transfer experiment of amylase-specific
T-cells to rodents suggest that cellular and/or humoral autoimmunity against AMY-2A plays some role in the pathogenesis of AIP.

Furthermore, the adoptive transfer of amylase-specific CD4+ T-cells to rats was able to confer pancreatitis, whereas the transfer experiment with lactoferrin-specific or CAII-specific CD4+ T-cells failed to induce experimental pancreatitis (23). Our findings of a high prevalence of autoantibody against AMY-2A in human AIP and the results from the adoptive transfer experiment of amylase-specific CD4+ T-cells to rodents suggest that cellular and/or humoral autoimmunity against AMY-2A plays some role in the pathogenesis of AIP.

Approximately 80% of patients with chronic pancreatitis are alcoholic, the pathogenesis of which still remains unclear. However, it is well known that acute or chronic alcohol exposure suppresses all branches of the immune system (24), and none of our sera from patients with chronic alcoholic pancreatitis were positive for autoantibody against AMY-2A (Fig. 3). Therefore, an assay for autoantibody against AMY-2A is useful for distinguishing AIP from chronic alcoholic pancreatitis.

It is of particular interest that anti–AMY-2A autoantibody is detected in 88% of patients with fulminant type 1 diabetes. Fulminant type 1 diabetes is a recently proposed subtype of type 1B, nonimmune-mediated, or idiopathic type 1 diabetes (9,10). A nationwide survey revealed that fulminant diabetes accounted for ~20% of Japanese type 1 diabetes with ketosis or ketoacidosis and flu-like symptoms frequently observed at onset (25). Clinical characteristics of this subtype of type 1 diabetes are 1) remarkably abrupt onset of diabetes; 2) very short (<1 week) duration of diabetic symptoms; 3) severe ketoacidosis at diagnosis; 4) negative status of islet-related autoantibodies, such as GADAb and anti–IA-2 antibody; 5) virtually no C-peptide secretion (10 μg/day in urine); and 6) elevated serum pancreatic enzyme levels (26). These features and the absence of insulitis in patients’ pancreases have led some to hypothesize that an autoimmune mechanism does not contribute to the development of fulminant type 1 diabetes, but rather that viral infection plays a central role in the pathogenesis of the disease (27). However, we previously demonstrated CD4+ and CD8+ T-cell infiltration to pancreatic exocrine cells and to the islet in an autopsy case deceased immediately after the onset of fulminant type 1 diabetes (28).

Imagawa and Hanafusa (27) also confirmed cellular infiltration of pancreatic islets in patients with fulminant type 1 diabetes. Shimada et al. (29) described a fulminant type 1 diabetic patient with a high serum level of CXCL10, a chemokine that induces migration of activated T-cells to local lesions and GAD-reactive CD4+ cells in the periphery. These results, and the presence of an autoantibody against AMY-2A, suggest that the disease might be autoimmune-related, involving the exocrine and the endocrine pancreas (10,28).

Exocrine dysfunction and impaired glucose tolerance are common features for both AIP and fulminant type 1 diabetes. With regard to the HLA genotype, Kawamura et al. (30) demonstrated that the DRB1*0405–DQB1*0401 haplotype is closely associated with AIP in the Japanese population, and Tanaka et al. (31) revealed that the DQA1*0303–DQB1*0401 haplotype is strongly associated with fulminant type 1 diabetes in a homologous manner. When we studied the frequency of this allele in our patients with AIP, 5 of 15 patients were heterozygous for the DRB1*0405–DQB1*0401 haplotype. Although further study with larger sample sizes will be needed, these two reports and our own analysis suggest the importance of the DRB1*0401 allele in both diseases. Furthermore, we are able to detect autoantibody against AMY-2A in both with nearly the same prevalence. Although further investigation is needed, the present results suggest that clinically and immunologically, AIP and fulminant type 1 diabetes are closely related to one another.

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