

A Study of Cell-mediated Immune Response to Pancreatic Antigens in Patients with Fibrocalculous Pancreatic Diabetes

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In tropical countries, apart from insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM), there are special forms of diabetes confined to this part of the world, which have been designated malnutrition related diabetes mellitus (MRDM).¹ Two subtypes of diabetes mellitus, namely, fibrocalculous pancreatic diabetes (FCPD) and protein deficient diabetes mellitus (PDDM) are included in the category of MRDM. Both of these 2 subtypes are presumed to have a common denominator, protein-energy malnutrition; with additional characteristic pathological features of the disease in FCPD, the fibrosis of the pancreas and the presence of pancreatic calculi.¹

Clinical features of both FCPD and PDDM resemble those of IDDM in that the diagnosis of diabetes is made in the majority of patients with younger ages, rather low body mass index and most patients require insulin for control of their diabetes. Although FCPD and PDDM rarely develop ketoacidosis after withdrawal of insulin treatment, this can occasionally occur; and absence of ketosis in IDDM patients is not

SUMMARY In order to investigate whether there was any association between autoimmunity to pancreatic antigens with FCPD as well as IDDM, cell-mediated immune response to pancreatic antigens was studied by lymphoproliferation assay in 7 FCPD, 17 IDDM, 33 NIDDM patients and 102 normal controls. Optimal pancreatic antigen concentrations used were 100, 150 and 200 µg/ml. Positive results were considered for each concentration of antigens tested, at stimulation index (SI) > (mean ± 2 SD) SI obtained from normal age-matched controls with the use of the corresponding concentration of antigen. The one who gave positive result with any of these optimal antigen concentrations was considered to be the responder to pancreatic antigens. With this criterion, the responders were found to be 3/7 (42.9%) FCPD, 6/17 (35.3%) IDDM and 6/33 (18.2%) NIDDM patients; while there were 11 of all 102 (10.8%) normal controls.

uncommon.^{2,3} Thus, apart from the presence of pancreatic calcification found in the subtype FCPD, the manifestations of both FCPD and PDDM are difficult to distinguish from IDDM. Measurement of serum C-peptide levels during glucagon stimulation test gave no cut-off value to differentiate FCPD from IDDM.⁴ Such resemblances have made some authors questioning the very existence of PDDM, which has no characteristic pathological feature of the disease as found in FCPD.^{5,6}

While the pathogenesis of IDDM has been extensively studied and the results have indicated the

role of autoimmune process in the destruction of B-cells of islets of Langerhans,^{7,8} the pathogenesis of FCPD is still far from clear. Various pathogenetic factors have been suggested, including undernutrition and cyanide containing foodstuffs, *eg.* cassava.⁹⁻¹² However, some controversies still exist for the role of these factors.¹³⁻¹⁶

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Since FCPD has some characteristics of diabetes resembling those of IDDM, we have raised the question whether there would be any association between autoimmunity to pancreatic antigens, in FCPD similar to that found with IDDM. In this study, we have used a lymphoproliferation assay with the pancreatic antigens as the tool for this investigation.

MATERIALS AND METHODS

Patients

Three groups of diabetic patients were included in this study, *ie.* patients with FCPD, IDDM and NIDDM. FCPD patients were diagnosed according to the presence of pancreatic calcification on plain abdominal roentgenogram, requiring, insulin treatment since the onset of diabetes and having neither ketonuria nor ketosis despite the insulin withdrawal by themselves for several weeks. IDDM patients were those presenting with diabetic ketoacidosis without the history of insulin treatment, or were on insulin treatment and had a history of ketoacidosis on insulin withdrawal, or had deficient or absent C-peptide response following a glucagon stimulation test. Patients who did not meet these criteria for IDDM were classified as NIDDM.

During the period of 3 years study, 7 FCPD, 17 IDDM and 33 NIDDM patients were recruited.

The group of FCPD patients included 5 males and 2 females with ages ranging from 19 to 36 years. New cases and those with duration from onset of diabetic symptoms within a 2 years period were included in this group.

The group of IDDM patients included 9 males and 8 females with ages ranging from 15 to 30 years. These were newly diagnosed patients and those who had durations from onset of symptoms within a 1 year period.

The NIDDM patients comprised of 12 males and 21 females with ages ranging from 17 to 70 years. These patients were newly diagnosed and the ones who had durations from onset of symptoms within a 1 year period.

Normal controls

One hundred and two healthy individuals with a fasting blood glucose concentration of less than 120 mg/dl were included in this study. This group of subjects comprised of laboratory personnel and blood donors. Sixty-six of them were less than 40 years of age while 36 had ages greater than or equal to 40 years. Thirty-three males and 33 females were included in the group of normal controls aged < 40 years while 33 males and 3 females were included in the group \geq 40 years.

Lymphoproliferation assay

The antigen used in the lymphoproliferation assay¹⁷ was the pancreatic antigen prepared from the pancreas of a blood group O cadaveric donor.¹⁸ After removal of fat and connective tissue, the pancreas was cut into small pieces, homogenized in cold sterile 0.15 M phosphate buffered saline (PBS) pH 7.2, filtered through fine gauze and centrifuged at 700 \times g, in a refrigerated centrifuge (CRU-5000, International Equipments Co., Needham HTS., MA, USA) for 20 minutes. The supernatant was collected for use as the antigen and the protein content was measured by modified Lowry's method.¹⁹ This antigen preparation was stored in small aliquots at -20°C. Fresh aliquot was always used in each experiment.

Peripheral blood mononuclear cells (PBMC) obtained from sterile heparinized blood were adjusted to contain PBMC at the concentration of 1.0×10^6 cells/ml in complete medium, RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) heat inactivated

pooled normal human sera, 2 mM L-glutamine, 20 mM HEPES, 10mM NaHCO₃, 100 IU/ml penicillin and 100 μ g/ml streptomycin. This PBMC preparation was cultured in the presence of various doses of pancreatic antigens. The pancreatic antigen concentrations used were 100, 150 and 200 μ g/ml. Twenty five microlitres of the antigen were added to 100 μ l of PBMC in each well of round bottom 96-well tissue culture plate (Nunclon, Nunc, Roskilde, Denmark), then cultured at 37°C in humidified air with 5% CO₂ incubation (Forma Scientific, Marietta, Ohio, USA). The antigen at each concentration was tested with PBMC in quadruplicate wells and 25 μ l of medium was also used instead of the antigen, as the negative control. Four days later, 1 μ Ci of [³H]thymidine (New England Nuclear, Boston, MA, USA) was added to each well of the plate. After 18 hours incubation at 37°C, the cells were harvested by using cell harvester (Nunc Cell Harvester, Nunc). [³H]thymidine incorporations was counted in a liquid scintillation spectrometer (LS 1801, Beckman Instrument Inc., CA, USA) and read as counts per minute (cpm). The result was expressed as the stimulation index (SI) which was the mean cpm of test cultures divided by the mean cpm of the negative control cultures.

In each experiment, 10 μ l of 500 μ g/ml phytohemagglutinin (PHA) (Difco Laboratories, Detroit, Michigan, USA) was also used as the stimulant, of 100 μ l PBMC at a concentration of 1.0×10^6 cells/ml, for testing the viability of PBMC obtained from each subjects.

Statistical analysis

Chi-square test with Yates' correction for continuity was used for testing the significance of difference between the proportion of responders in each group of patients and the group of normal controls.

RESULTS

Standardization of optimal pancreatic antigen concentrations

Optimal pancreatic antigen concentrations for use in the lymphoproliferation assay were evaluated by testing PBMC obtained from 11 IDDM, 21 NIDDM, 4 FCPD and 65 normal controls with various pancreatic antigen concentrations, *ie.* 5, 10, 25, 50, 100, 150, 200, 250 and 300 $\mu\text{g/ml}$. In this experiment, the high magnitudes of SI (> 4.0) were found only in 2 IDDM patients, with the use of the pancreatic antigen at concentrations of 100 and 200 $\mu\text{g/ml}$, as shown in Fig. 1. Thus, the optimal pancreatic antigen concentrations were chosen to be within this range, *ie.* 100, 150 and 200 $\mu\text{g/ml}$. These antigen concentrations were used in the subsequent experiments for the investigation of lymphoproliferative responses in the rest of the subjects.

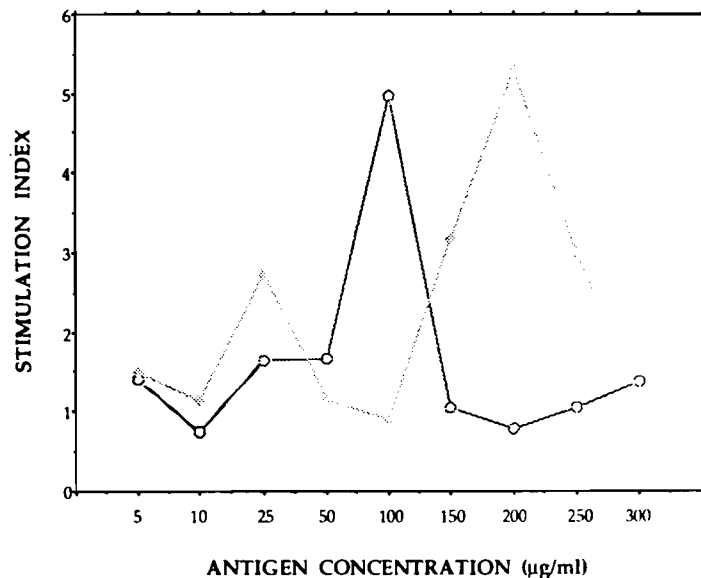


Fig. 1 Stimulation index obtained from the lymphoproliferation assay performed with the pancreatic antigens at various concentrations and peripheral blood mononuclear lymphocytes of 2 patients with IDDM.

Lymphoproliferative response

Lymphoproliferative response to the pancreatic antigens in all subjects studied, *ie.* 7 FCPD, 17 IDDM, 33 NIDDM and 102 normal controls are shown in Fig. 2, in which the response was expressed as stimulation index (SI). It can be seen that the response of lymphocytes obtained from each individual at different antigen concentrations are variable. Some responded with equal SI for all 3 antigen concentrations (100, 150 and 200 $\mu\text{g/ml}$) while others responded well to only 1 or 2 antigen concentrations. Thus, in order to identify the responders in these groups of subjects, the cut-off levels for positive results were investigated for every antigen concentration within the range of 100–200 $\mu\text{g/ml}$. In order to obtain the age-matched comparison, the cut-off level was considered separately for subjects with age < 40 years and those with age > 40 years old and used at mean $\pm 2\text{SD}$ of SI ob-

tained from normal controls in each corresponding age group at each antigen concentration used. For subjects with age < 40 years old, the cut-off levels for positive results were 1.98, 1.67 and 2.72 with the use of antigen concentrations, 100, 150 and 200 $\mu\text{g/ml}$, respectively, while they were 1.97, 1.55 and 2.14, respectively for those with ages > 40 years old. The one who gave SI value above the corresponding cut-off level at any antigen concentration within the range of 100–200 $\mu\text{g/ml}$ was considered to be the responder to the pancreatic antigens. By using this criterion, 3/7 (42.9%) of FCPD, 6/17 (35.3%) of IDDM, 6/33 (18.2%) of NIDDM patients and 11/102 (10.8%) of all normal controls were found to be the responders. Demographic study of these responders are shown in Table 1.

By statistical analysis, it was found that the proportion of the responders in IDDM groups was

significantly different ($p = 0.02$) from that in normal controls. However, this was not found in FCPD ($p = 0.06$) and NIDDM ($p = 0.42$) groups.

DISCUSSION

Among 3 types of diabetes mellitus, IDDM, NIDDM and MRDM, it is well known that MRDM and IDDM have some common clinical features *ie.* young age, low body mass index and insulin requirement.¹ Although ketosis resistance has been used to distinguish the different nature of diabetes between IDDM and MRDM, this is not universal.^{2,3} Thus, some authors have questioned the very existence of PDDM, the subtype of MRDM without pancreatic calcification.^{5,6}

Although FCPD, another subtype of MRDM, has pancreatic calcification as the characteristic pathological feature, there are some laboratory findings in this subtype

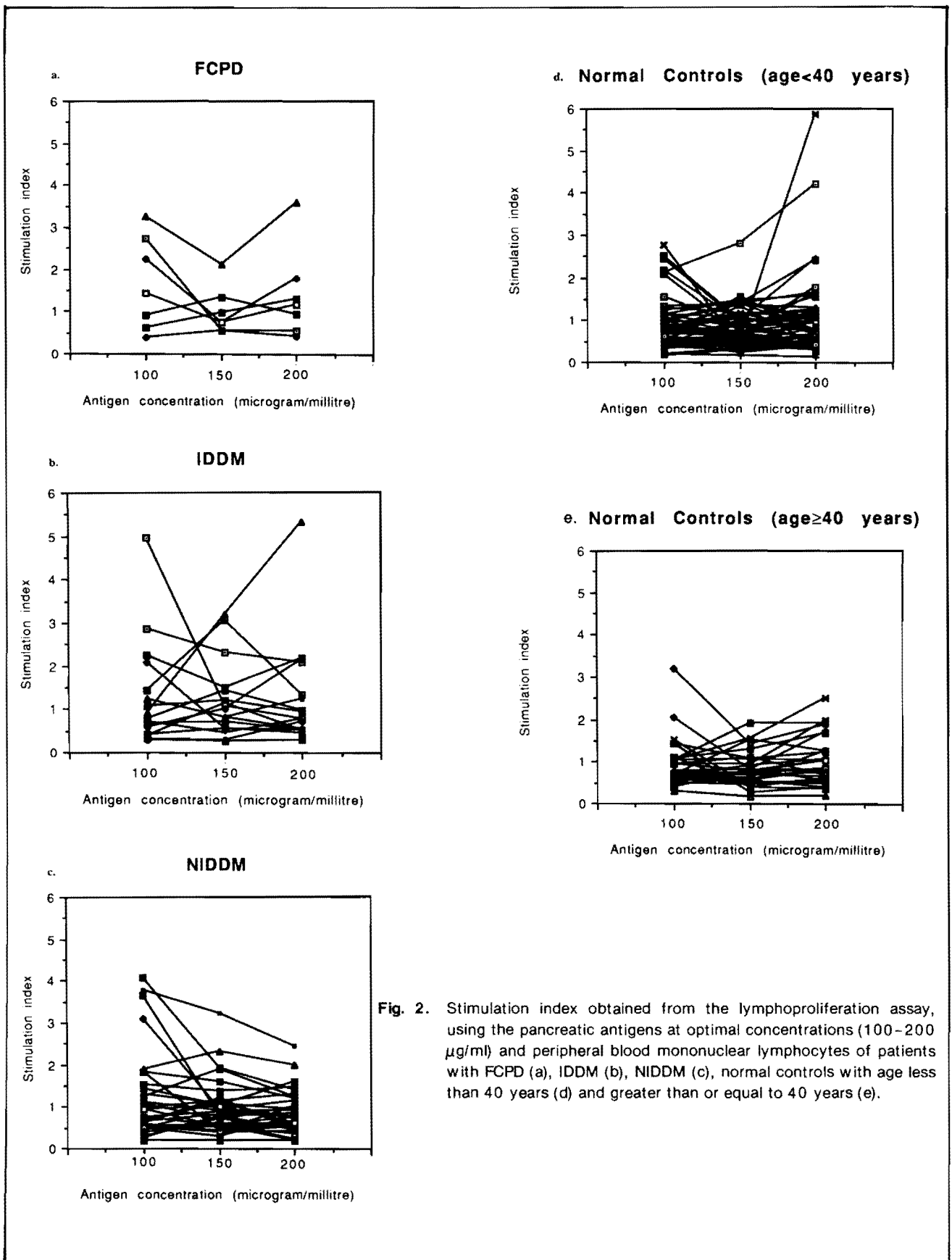


Fig. 2. Stimulation index obtained from the lymphoproliferation assay, using the pancreatic antigens at optimal concentrations (100-200 $\mu\text{g/ml}$) and peripheral blood mononuclear lymphocytes of patients with FCPD (a), IDDM (b), NIDDM (c), normal controls with age less than 40 years (d) and greater than or equal to 40 years (e).

Table 1. Responders to pancreatic antigens in each group of subjects, as determined by lymphoproliferation assay.

| Group of subjects | No. | Age | Sex | SI at each antigen concentration | | |
|-------------------|-----|-----|-----|----------------------------------|-----------|----------|
| | | | | 100 µg/ml | 150 µg/ml | 200µg/ml |
| FCPD | 1 | 26 | M | 2.74* | 0.54 | 0.56 |
| | 2 | 27 | F | 2.25* | 0.76 | 1.80 |
| | 3 | 19 | M | 3.27* | 2.11* | 3.58* |
| IDDM | 1 | 30 | M | 4.96* | 1.06 | 0.78 |
| | 2 | 26 | M | 2.09* | 0.54 | 0.53 |
| | 3 | 30 | F | 0.90 | 3.18* | 5.30* |
| | 4 | 15 | M | 1.45 | 3.06* | 1.35 |
| | 5 | 18 | F | 2.88* | 2.32* | 2.08 |
| | 6 | 22 | F | 2.25* | 1.51 | 2.18 |
| NIDDM | 1 | 24 | F | 3.11* | 0.96 | 1.13 |
| | 2 | 33 | F | 3.79* | 3.23* | 2.45 |
| | 3 | 63 | M | 4.09* | 1.91* | 1.41 |
| | 4 | 55 | F | 3.65* | 0.77 | 0.84 |
| | 5 | 49 | F | 1.25 | 1.88* | 1.25 |
| | 6 | 43 | F | 1.90 | 2.31* | 1.98 |
| Normal controls | 1 | 21 | F | 2.45* | 0.97 | 0.97 |
| | 2 | 25 | M | 2.77* | 0.40 | 1.06 |
| | 3 | 36 | M | 0.76 | 0.57 | 5.88* |
| | 4 | 32 | M | 2.13* | 2.80* | 4.20* |
| | 5 | 20 | M | 2.18* | 0.99 | 1.16 |
| | 6 | 24 | F | 2.10* | 0.71 | 1.16 |
| | 7 | 24 | F | 2.52* | 1.01 | 0.25 |
| | 8 | 54 | M | 2.07* | 0.82 | 1.74 |
| | 9 | 65 | M | 3.21* | 1.42 | 1.89 |
| | 10 | 50 | M | 1.04 | 1.91* | 1.94 |
| | 11 | 47 | F | 1.05 | 1.56* | 2.51* |

*The stimulation index (SI) which is higher than the cut-off value for positive results.

that resemble those in IDDM. Pancreatic B-cell function was found to be diminished both in IDDM and FCPD.⁴ Raised antibody titres against mumps virus, cytomegalovirus and *Mycoplasma pneumoniae* have been reported in FCPD patients.²⁰ An association between IDDM and viral infection, including that caused by Coxsackie B, cytomegalovirus and rubella has also been reported.^{7,8,21-24} Various immu-

nological changes have been described in FCPD patients, *ie*, reduction in T lymphocytes, elevation of IgG and IgM and presence of pancreatic haemagglutinating antibodies.^{25,26} Immunological changes have also been reported extensively for IDDM.^{7,8} In addition, several factors that have been suggested to play a role in the pathogenesis of FCPD, *ie*, undernutrition, cassava consumption, are not well esta-

blished for their roles in causing this subtype of diabetes.²⁶ Thus, we have raised the question of the distinction of FCPD from IDDM based on the pathogenesis of the diseases. Since data have indicated the role of an autoimmune process, particularly the cell-mediated immune response, in the destruction of B-cell of islets of Langerhans in IDDM,^{7,8} we were interested in the investigation of cell-mediated immune

response to pancreatic antigens in FCPD patients.

In this study, although we have been recruiting subjects for 3 years, only 7 FCPD patients who had onset within 2 years could be recruited. By using the lymphoproliferation assay, we found that 42.9% of FCPD patients responded to pancreatic antigens, while such a response was found in 35.3% of IDDM, 18.2% of NIDDM patients and 10.8% of normal controls. However, while the proportion of responders in the group of IDDM patients was statistically different ($p=0.02$) from that in normal controls, this was not the case of FCPD patients ($p=0.06$). This could be due to the small size of FCPD patients group, as the proportion of responders in the IDDM group was actually lower than that in the former (42.9% vs. 35.3%). Thus, if the proportion of responders in FCPD group proves to be constant at this rate when a large sample size is used, the difference of responder proportion between FCPD group and normal controls might be statistically significant.

Nevertheless, the percentages of responders found in each group of patients have suggested an association between autoimmunity to pancreatic antigens in FCPD as well as in IDDM but not in NIDDM patients. This response could well be autoimmunity evoked by beta cells which have altered antigen due to cause such as infection, or by some foreign antigens which have molecular mimicry with pancreatic antigens. On the other hand, the response could also be initiated as the response to normally sequestered pancreatic antigens which have been liberated as the result of pancreatic tissue damage by other causes, as has been suggested for IDDM *eg.* virus, chemical agents, interleukin-1,7,8,27. Recent studies of other investigators have also suggested a role of the immune response in the

pathogenesis of chronic pancreatitis. The presence of T lymphocytes around ducts and in the area of fibrous septa, the expression of HLA-DR antigen on duct cells and the possible involvement of TGF-beta 1 in the development of fibrosis in chronic pancreatitis have been reported.^{28,29} It has been found that β -cell loss in tropical calcific pancreatitis is related to exocrine loss and thus suggested that diabetes in tropical calcific pancreatitis is either secondary to pancreatitis or that a common factor(s) acts simultaneously on both components.³⁰

Although autoimmunity could possibly play role in the pathogenesis of FCPD as well as IDDM, the causative agents inducing this process or the involved antigen might be different between these two types of diabetes, as shown by the different areas of tissue destruction. While low exocrine pancreatic function was found in a higher proportion of FCPD as compared with IDDM,³¹ β cell involvement in IDDM was found to be lower than that in FCPD.³² Since the antigen used in our assay was the crude antigen, we could not confirm or exclude the same specificity of the autoimmune responses in FCPD and IDDM. The decreased concentration of lithostathine, the glycoprotein present in human pancreatic juice capable of inhibiting the growth of CaCO_3 crystals, in patients with chronic calcifying pancreatitis,³³⁻³⁶ may explain the difference of pathology found in IDDM and FCPD (the presence or absence of calcification). Since cases of FCPD patients are found in Northeastern part of Thailand more commonly than in other parts of the country (as occurs with patients with urinary bladder stones in whom the diet has been shown to contain aetiological factors),³⁷⁻³⁹ it would be interesting to see whether such dietary factors would play any role in relation to the biosynthesis of lithostathine.

In conclusion, our study results have suggested that there is an association of autoimmunity to pancreatic antigens in FCPD as well as in IDDM. Autoimmune process could plausibly be responsible for perpetuation of the pancreatic tissue destruction in FCPD as well, although there might be some differences between FCPD and IDDM in relation to the causative agents and/or other factors which contribute to the development of pancreatic calcification, resulting in some differences in pathological changes and clinical features between these 2 types of diabetes.

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