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A NOVEL SUBTYPE OF TYPE 1 DIABETES MELLITUS CHARACTERIZED BY A RAPID ONSET AND AN ABSENCE OF DIABETES-RELATED ANTIBODIES

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ABSTRACT

Background and Methods Type 1 diabetes mellitus is now classified as autoimmune (type 1A) or idiopathic (type 1B), but little is known about the latter. We classified 56 consecutive Japanese adults with type 1 diabetes according to the presence or absence of glutamic acid decarboxylase antibodies (their presence is a marker of autoimmunity) and compared their clinical, serologic, and pathological characteristics.

Results We divided the patients into three groups: 36 patients with positive tests for serum glutamic acid decarboxylase antibodies, 9 with negative tests for serum glutamic acid decarboxylase antibodies and glycosylated hemoglobin values higher than 11.5 percent, and 11 with negative tests for serum glutamic acid decarboxylase antibodies and glycosylated hemoglobin values lower than 8.5 percent. In comparison with the first two groups, the third group had a shorter mean duration of symptoms of hyperglycemia (4.0 days), a higher mean plasma glucose concentration (773 mg per deciliter [43 mmol per liter]) in spite of lower glycosylated hemoglobin values, diminished urinary excretion of C peptide, a more severe metabolic disorder (with ketoacidosis), higher serum pancreatic enzyme concentrations, and an absence of islet-cell, IA-2, and insulin antibodies. Immunohistologic studies of pancreatic-biopsy specimens from three patients with negative tests for glutamic acid decarboxylase antibodies and low glycosylated hemoglobin values revealed T-lymphocyte–predominant infiltrates in the exocrine pancreas but no insulitis and no evidence of acute or chronic pancreatitis.

Conclusions Some patients with idiopathic type 1 diabetes have a nonautoimmune, fulminant disorder characterized by the absence of insulitis and of diabetes-related antibodies, a remarkably abrupt onset, and high serum pancreatic enzyme concentrations. (N Engl J Med 2000;342:301-7.)

TYPE 1 diabetes mellitus is caused by loss of insulin-secreting capacity due to selective autoimmune destruction of the pancreatic beta cells.[1,2] Insulitis (i.e., mononuclear-cell infiltration of the pancreatic islets) is the direct result of the autoimmune process. Antibodies to the cytoplasm of islet cells, glutamic acid decarboxylase, insulin, and tyrosine phosphatase–like protein (IA-2 or IA-2β), which appear before the clinical onset of diabetes, are good markers of the autoimmune process.[1,2]

Several lines of evidence have suggested that autoimmunity is not the only cause of beta-cell destruction. We and others have described young patients who presented with the abrupt onset of symptoms of hyperglycemia and who were prone to the development of ketoacidosis, as is characteristic of patients with type 1 diabetes, but who did not have insulitis on either biopsy[3-5] or autopsy.[6,7] Furthermore, at least 10 percent of patients with newly diagnosed type 1 diabetes do not have any diabetes-related antibodies.[8,9]

The American Diabetes Association and the World Health Organization have proposed that type 1 diabetes be subdivided into autoimmune (immune-mediated) diabetes (type 1A) and idiopathic diabetes with beta-cell destruction (type 1B).[10,11] However, the specific characteristics of the idiopathic subtype are largely unknown. In a previous study,[5] we found that the presence of glutamic acid decarboxylase antibodies, but not islet-cell antibodies, was closely correlated with direct evidence of insulitis in patients with type 1 diabetes. In this report, we describe the results of detailed clinical and histologic studies of patients with idiopathic type 1 diabetes.

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*Other members of the Osaka IDDM Study Group are listed in the Appendix.

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METHODS

Patients

We studied 56 consecutive patients with type 1 diabetes who came to our hospitals within one year after receiving the diagnosis, during the period from 1994 to 1998. All 56 patients met the criteria of the American Diabetes Association for type 1 diabetes — that is, pancreatic beta-cell destruction as the primary cause of the disorder and a tendency toward ketoacidosis[10,11] — as determined by at least two physicians independently. Patients who had a period of remission that lasted for six months or more after the diagnosis had been made were excluded.[12] None of the enrolled patients consumed moderate or large amounts of alcohol. The study was approved by the ethics committee of Osaka University Medical Hospital, and written informed consent was obtained from each patient.

Clinical Characteristics and Serum Glutamic Acid Decarboxylase Antibodies

At the time of the onset of overt diabetes, all patients were hospitalized. Their clinical characteristics were recorded, and plasma glucose, serum electrolytes, arterial pH, glycosylated hemoglobin, and serum total or pancreatic amylase and elastase I were measured within two days after the initial diagnosis, and an ultrasonographic study of the pancreas was performed. Patients with arterial pH values lower than 7.35 and serum bicarbonate concentrations lower than 18 mmol per liter received the diagnosis of metabolic acidosis. Urinary C-peptide excretion was measured daily for at least three days, and the mean value was calculated. Subsequently, all patients underwent clinical examinations and measurements of glycosylated hemoglobin at monthly intervals.

The patients were divided into two groups according to the presence or absence of glutamic acid decarboxylase antibodies in serum samples obtained within three months after the initial diagnosis of diabetes, with the use of a radioimmunoassay kit (Rip-GAD, Hoechst Japan, Tokyo, or GAD-Ab Cosmic, Cosmic, Tokyo). A value greater than 5 units per milliliter (with the first kit) or 1.5 units per milliliter (with the second) was considered positive. The specificity and sensitivity of the first kit were 100 percent and 89.5 percent in the Second International GADAb Workshop, respectively,[13] and the specificity and sensitivity of the second kit were both 100 percent in the Second and Third GAD Proficiency Test Results Evaluations (University of Florida, Gainesville).

Diabetes-Related and Thyroid Antibodies

Serum antibodies were measured within three months after the initial diagnosis of diabetes. Islet-cell antibodies were measured by an indirect immunofluorescence method (with an abnormal value defined as more than 5 Juvenile Diabetes Foundation units),[4] IA-2 antibodies with an immunoprecipitation assay kit (with an abnormal value defined as more than 0.75 unit per milliliter) (Cosmic),[14] and insulin antibodies with a liquid-phase radioimmunoassay (with an abnormal value defined as higher than the 99th percentile for 140 normal subjects).[15,16] Thyroid antimicrosomal antibodies were measured by a hemagglutination assay (with an abnormal value defined as greater than 1:100).[17]

HLA Typing and Mitochondrial-DNA Analysis

Genomic and mitochondrial DNA were extracted from peripheral-blood leukocytes. The HLA class II antigen haplotype and the presence or absence of a guanine-for-adenine substitution at position 3243 in mitochondrial DNA were determined.[5,18] No mitochondrial mutations were detected.

Pancreatic Studies

Pancreatic biopsies were performed in six patients within five months after the initial diagnosis of diabetes, as reported previously.[3-5] The biopsy specimens were examined after staining with hematoxylin and eosin and by indirect immunohistochemical methods. To detect insulitis, a double immunofluorescence method was used with monoclonal antihuman CD3+ T-lymphocyte antibody, B-lymphocyte antibody, or macrophage antibody and anti-insulin or anti-glucagon antibodies. We also examined the expression of major histocompatibility complex (MHC) class I antigens in islets with the use of antihuman HLA-A, B, and C antibodies. The sources of these antibodies have been reported previously.[4]

To examine the relation between lymphocytes or macrophages and pancreatic exocrine tissue, we stained the cells with peroxidase substrate solution containing diaminobenzidine tetrachloride–nickel chloride (Zymed Laboratories, South San Francisco, Calif.). The sections were then incubated with antihuman alpha-amylase antibody (Sigma Chemical, St. Louis), and the pancreatic exocrine

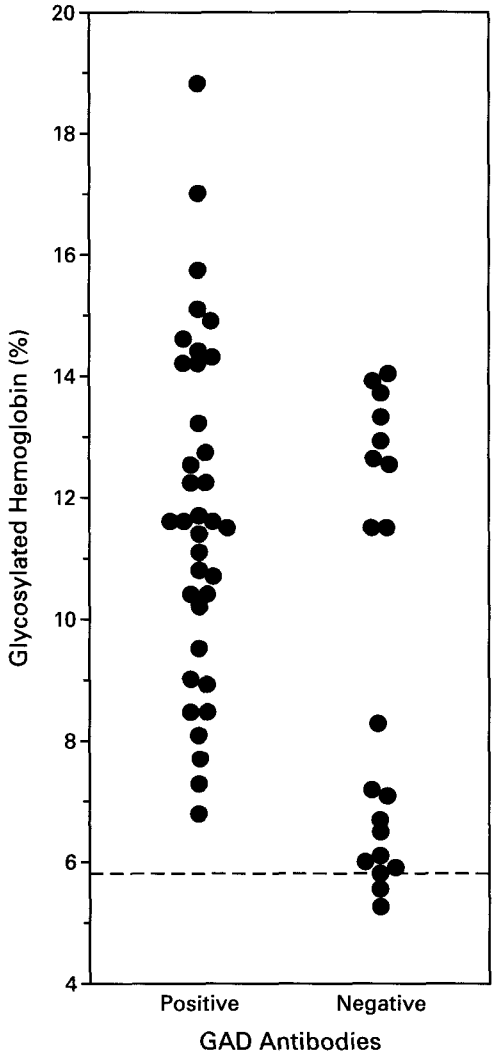

Figure 1. Glycosylated Hemoglobin Values at the Time of the Diagnosis of Diabetes in 56 Patients, According to Whether the Test for Glutamic Acid Decarboxylase (GAD) Antibodies Was Positive or Negative.

The values for glycosylated hemoglobin in the patients with positive antibody tests are scattered, whereas the values in the patients with negative antibody tests are clearly divided into two groups: those below 8.5 percent and those above 11.5 percent. The broken line indicates the upper limit of the normal range for glycosylated hemoglobin.

TABLE 1. CHARACTERISTICS OF 56 PATIENTS WITH TYPE 1 DIABETES, ACCORDING TO WHETHER THE TEST FOR GLUTAMIC ACID DECARBOXYLASE ANTIBODIES (GAD) WAS POSITIVE OR NEGATIVE.*

CHARACTERISTIC	GAD-POSITIVE (N=36)	GAD-NEGATIVE, HIGH HbA_{1c} (N=9)	GAD-NEGATIVE, LOW HbA_{1c} (N=11)	P VALUE	
				GAD-NEGATIVE, LOW HbA_{1c} VS. GAD-POSITIVE	GAD-NEGATIVE, LOW HbA_{1c} VS. GAD-NEGATIVE, HIGH HbA_{1c}
HbA_{1c} (%)	11.7±2.8	12.9±1.0	6.4±0.9	<0.001	<0.001
Age (yr)					
Mean	32	27	38		0.05
Range	14–75	14–52	25–57		
Male sex (no.)	14	5	6		
Body-mass index†	19.1±2.6	20.0±2.9	21.0±3.8		
First-degree relative with diabetes (no.)‡	7	1	4		
Duration of hyperglycemic symptoms before diagnosis (days)	52.4±54.1	45.9±36.2	4.0±1.7	0.005	0.001
Abdominal pain (no.)	0	0	1		
Abnormal findings on pancreatic ultrasonography (no.)	0	0	0		
Plasma glucose (mg/dl)	398±198	439±179	773±250	<0.001	0.004
Urinary C peptide (μg/day)	21.0±11.2	19.7±10.3	3.2±1.9	<0.001	<0.001
Arterial pH	7.36±0.07	7.34±0.11	7.09±0.22	0.001	0.03
Serum bicarbonate (mmol/liter)	20.6±6.2	19.5±7.3	9.8±6.8	0.004	0.03
Serum amylase§	0.39±0.16	0.63±0.74	4.24±4.27	<0.001	0.02
Serum elastase I§	0.45±0.15	0.14±0.02	3.47±2.15	0.006	
Insulin dose during first yr (U/kg of body weight)	0.43±0.21	0.34±0.24	0.61±0.14	0.02	0.01

*Data were obtained at the time of diagnosis. Plus–minus values are means ±SD. To convert the values for glucose to millimoles per liter, multiply by 0.056. To convert the values for C peptide to millimoles per day, multiply by 0.33. HbA_{1c} denotes glycosylated hemoglobin.

†The body-mass index was calculated as the weight in kilograms divided by the square of the height in meters.

‡All affected relatives had type 2 diabetes except that one relative of each of two patients in the antibody-positive group had type 1 diabetes.

§Values for amylase and elastase I are expressed as multiples of the upper limit of the normal range.

cells were stained with 3-amino-9-ethylcarbazole (Dakopatts, Glostrup, Denmark).

Statistical Analysis

Statistical analysis was performed with Student's t-test or Fisher's exact probability test, as appropriate.

RESULTS

Serum glutamic acid decarboxylase antibodies were detected in 36 patients (64 percent) and were not detected in 20 patients (36 percent). The patients without glutamic acid decarboxylase antibodies were divided into two subgroups according to the initial glycosylated hemoglobin value: those with values of less than 8.5 percent, and those with values of more than 11.5 percent (Fig. 1).

The clinical characteristics of the patients with positive tests for glutamic acid decarboxylase antibodies and those of the two groups of patients with negative tests are shown in Table 1. The mean duration of hyperglycemic symptoms in the patients with negative tests for glutamic acid decarboxylase antibodies and low glycosylated hemoglobin values was only 4.0 days. This group had a significantly higher mean glucose concentration, despite lower glycosylated hemoglobin values, and a significantly lower mean value for urinary C-peptide excretion than did the other two groups. All the patients with negative antibody tests and low glycosylated hemoglobin values had diabetic ketoacidosis, as compared with 20 percent of the patients with positive antibody tests and 40 percent of the patients with negative tests and high glycosylated hemoglobin values. Serum pancreatic enzyme concentrations were high in all the patients who had negative antibody tests and low glycosylated hemoglobin values but not in the other two groups (Table 1), and the values fell to the normal range in 3 to 38 days (median, 17).

All patients received multiple insulin injections, with a higher mean dose during the first year in the group of patients with negative antibody tests and low glycosylated hemoglobin values than in the other two groups (Table 1). Diabetes-related antibodies were not

TABLE 2. RESULTS OF OTHER ANTIBODY TESTS AT THE TIME OF DIAGNOSIS, ACCORDING TO WHETHER THE TEST FOR GLUTAMIC ACID DECARBOXYLASE ANTIBODIES (GAD) WAS POSITIVE OR NEGATIVE.*

TEST	GAD-POSITIVE (N=36)	GAD-NEGATIVE, HIGH HbA_{1c} (N=9)	GAD-NEGATIVE, LOW HbA_{1c} (N=11)
	no. with positive test/no. tested		
Islet-cell antibodies	15/22	3/7	0/11†
IA-2 antibodies	16/19	3/7	0/11†
Insulin antibodies	8/22	1/4	0/10‡
Thyroid microsomal antibodies	12/30	0/7	0/10§

*HbA_{1c} denotes glycosylated hemoglobin.

†$P<0.001$ for the comparison with the patients who had positive tests for glutamic acid decarboxylase antibodies, and $P=0.04$ for the comparison with the patients who had negative tests for glutamic acid decarboxylase antibodies and high HbA_{1c} values.

‡$P=0.04$ for the comparison with the patients who had positive tests for glutamic acid decarboxylase antibodies.

§$P=0.02$ for the comparison with the patients who had positive tests for glutamic acid decarboxylase antibodies.

detected in serum samples from any of the patients with negative tests for glutamic acid decarboxylase antibodies and low glycosylated hemoglobin values (Table 2).

The characteristics of the 11 patients with negative tests for glutamic acid decarboxylase antibodies and low glycosylated hemoglobin values are shown in Table 3. The HLA class II haplotype was determined in 10 of the patients. The haplotypes most often associated with type 1 diabetes in Japanese patients,[19] HLA-DRB1*0405,DQA1*0303,DQB1*0401 and HLA-DRB1*0901,DQA1*0302,DQB1*0303, were present in five and three patients, respectively. Two haplotypes associated with resistance to type 1 diabetes, HLA-DRB1*1501,DQA1*0102,DQB1*0602 and HLA-DRB1*1502,DQA1*0103,DQB1*0601, were found in two patients and one patient, respectively.

Pancreatic biopsies were performed in one patient with a negative test for glutamic acid decarboxylase antibodies and a low glycosylated hemoglobin value (Patient 2 in Table 3) and in two other patients with similar findings (described previously[5]) who had been hospitalized before we started this study. In all three patients, no islet-cell antibodies were detected, hyperglycemic symptoms lasted for fewer than six days,

TABLE 3. CLINICAL CHARACTERISTICS OF THE 11 PATIENTS WITH NEGATIVE TESTS FOR GLUTAMIC ACID DECARBOXYLASE ANTIBODIES AND LOW GLYCOSYLATED HEMOGLOBIN (HbA_{1c}) VALUES AT THE ONSET OF DIABETES.*

PATIENT NO.	AGE	SEX	BMI†	DURATION	SERUM GLUCOSE	HbA_{1c}	URINARY C PEPTIDE	pH	SERUM AMYLASE§	SERUM ELASTASE I§	HLA-DRB1, DQA1, DQB1 HAPLOTYPE
	yr			days‡	mg/dl	%	μg/day				
1	33	F	15.8	7	854	8.3	3.6	7.23	2.88	ND	0405,0303,0401/ 0803,0103,0601
2	40	M	17.6	6	643	6.7	1.1	7.29	0.32	1.28	0101,0101,0501/ 0405,0303,0401
3	57	M	29.8	2	811	6.5	6.5	7.22	6.96	1.64	ND
4	48	F	18.1	3	591	5.3	2.1	ND	1.16	3.38	0901,0302,0303/ 1502,0103,0601
5	25	F	21.0	3	1272	5.8	3.0	6.83	9.38	2.75	0901,0302,0303
6	29	M	19.7	5	555	6.0	0.6	ND	1.00	4.73	0405,0303,0401
7	35	F	19.1	4	993	5.6	5.7	6.81	12.21	7.05	1301,0103,0603/ 1501,0102,0602
8	36	F	22.1	3	726	7.2	1.3	6.86	1.13	ND	0802,0401,0302/ 1501,0102,0602
9	35	M	19.9	3	1041	6.1	<3.6	7.10	3.07	ND	0101,0101,0501/ 0405,0303,0401
10	34	M	22.7	6	447	5.9	3.0	7.33	ND	ND	0405,0303,0401/ 1405,0303,0303
30	41	M	24.4	2	569	7.1	4.7	ND	ND	ND	0901,0302,0303

*To convert the values for glucose to millimoles per liter, multiply by 0.056. To convert the values for C peptide to nanomoles per day, multiply by 0.33. ND denotes not determined.

†The body-mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters.

‡Duration refers to the period of hyperglycemic symptoms before the diagnosis of diabetes.

§Values are expressed as multiples of the upper limit of the normal range.

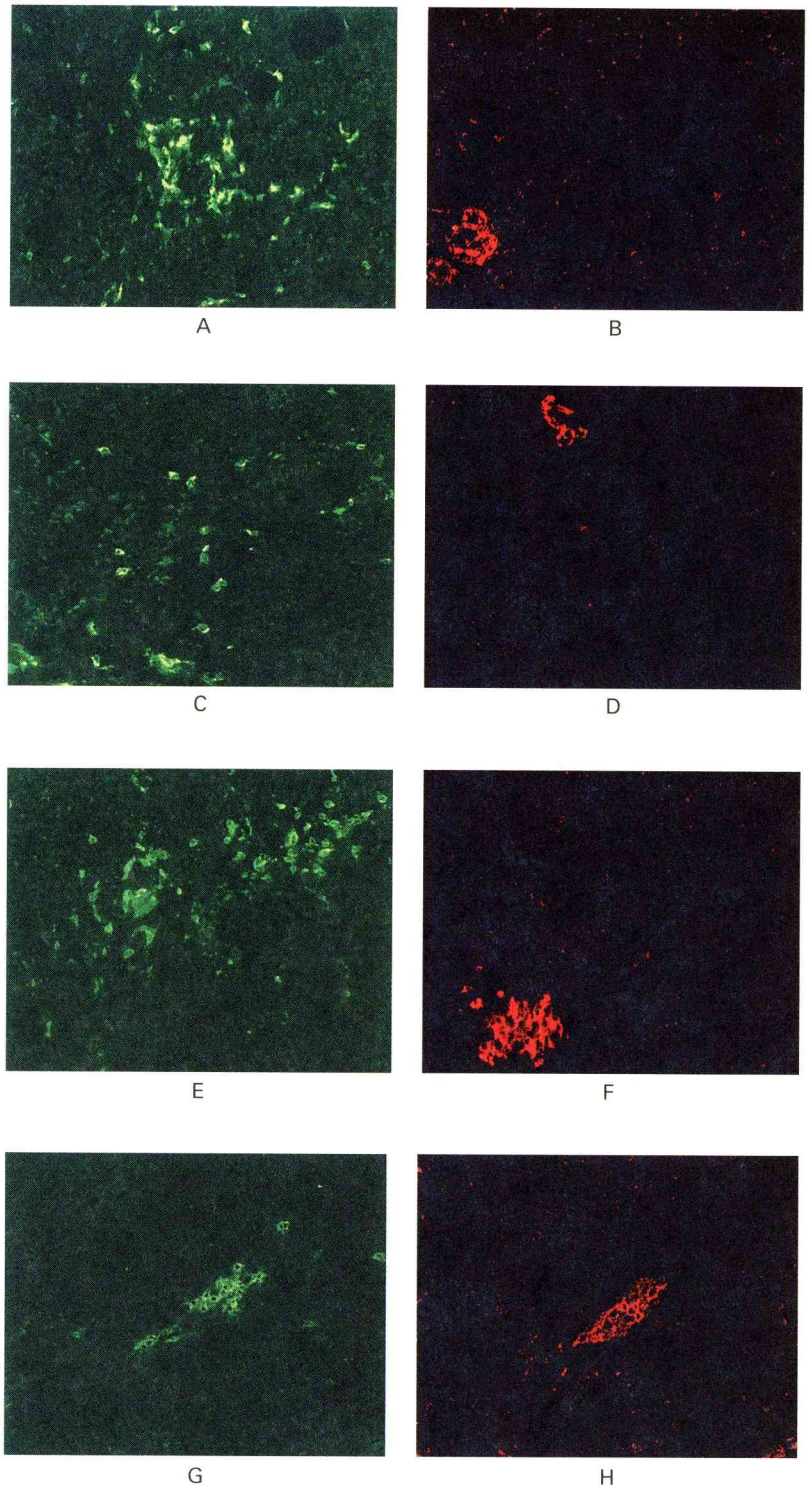

Figure 2. Photomicrographs of Double-Stained Pancreatic-Biopsy Specimens Showing Diffuse Infiltration of T Lymphocytes in the Exocrine Pancreas (×140).

Each pair of panels shows CD3+ T lymphocytes (green) and pancreatic alpha cells (red) in a biopsy specimen from one patient. Panels A and B, C and D, and E and F show specimens from three patients with negative tests for glutamic acid decarboxylase and islet-cell antibodies, and Panels G and H show specimens from one patient with positive tests for glutamic acid decarboxylase and islet-cell antibodies.

and at least one serum pancreatic enzyme value was elevated initially. As a control, pancreatic studies were also performed in three patients with positive tests for glutamic acid decarboxylase antibodies or islet-cell antibodies and glycosylated hemoglobin values of 8.5 to 15.1 percent.

Light-microscopical examination of sections of pancreas stained with hematoxylin and eosin revealed small, atrophic, distorted islet cells in all six patients. However, none of the patients had edema, necrosis, hemorrhage, suppuration, cyst formation, fibrosis, or apparent atrophy of the exocrine pancreas. Immunohistochemical examination revealed a markedly reduced beta-cell mass in all six patients, as would be expected in patients with type 1 diabetes. The sections from all three patients with negative tests for glutamic acid decarboxylase antibodies had T-lymphocyte–predominant infiltration of the exocrine pancreas but no insulitis (Fig. 2 and 3). On the other hand, insulitis but no cellular infiltration of the exocrine pancreas was seen in the sections from all three patients with positive antibody tests (Fig. 3). Hyperexpression of MHC class I molecules, another immunologic abnormality in islets, was seen only in the sections from the patients with insulitis.

DISCUSSION

Among 56 consecutive Japanese patients who had type 1 diabetes, we identified 11 with a subtype of diabetes that differed from autoimmune diabetes in three respects. First, no autoimmune features were detected. The patients did not have diabetes-related serum antibodies, such as islet-cell, glutamic acid decarboxylase, IA-2, or insulin antibodies. Pancreatic biopsies revealed neither insulitis nor hyperexpression of MHC class I molecules in islets.

Second, the onset of overt diabetes was rapid, and diabetic ketoacidosis occurred soon after the onset of hyperglycemic symptoms. The mean duration of hyperglycemic symptoms before the diagnosis was only four days. The short duration of hyperglycemia was reflected by the patients' nearly normal glycosylated hemoglobin values. Insulin-secretory capacity, estimated on the basis of urinary C-peptide excretion, was low, and the metabolic derangement at the onset was severe.

Third, the patients had markedly elevated serum pancreatic enzyme concentrations, a finding in accordance with the lymphocytic infiltration of the exocrine pancreas seen in the biopsy specimens. In contrast, the other patients had normal serum pancreatic enzyme concentrations and insulitis but did not have lymphocytic infiltrates in the exocrine pancreas. The edema, necrosis, hemorrhage, suppuration, cyst formation, and fibrosis that characterize classic acute or chronic pancreatitis were not present in our patients.[20] In addition, none of the patients with negative tests for glutamic acid decarboxylase antibodies and low gly-

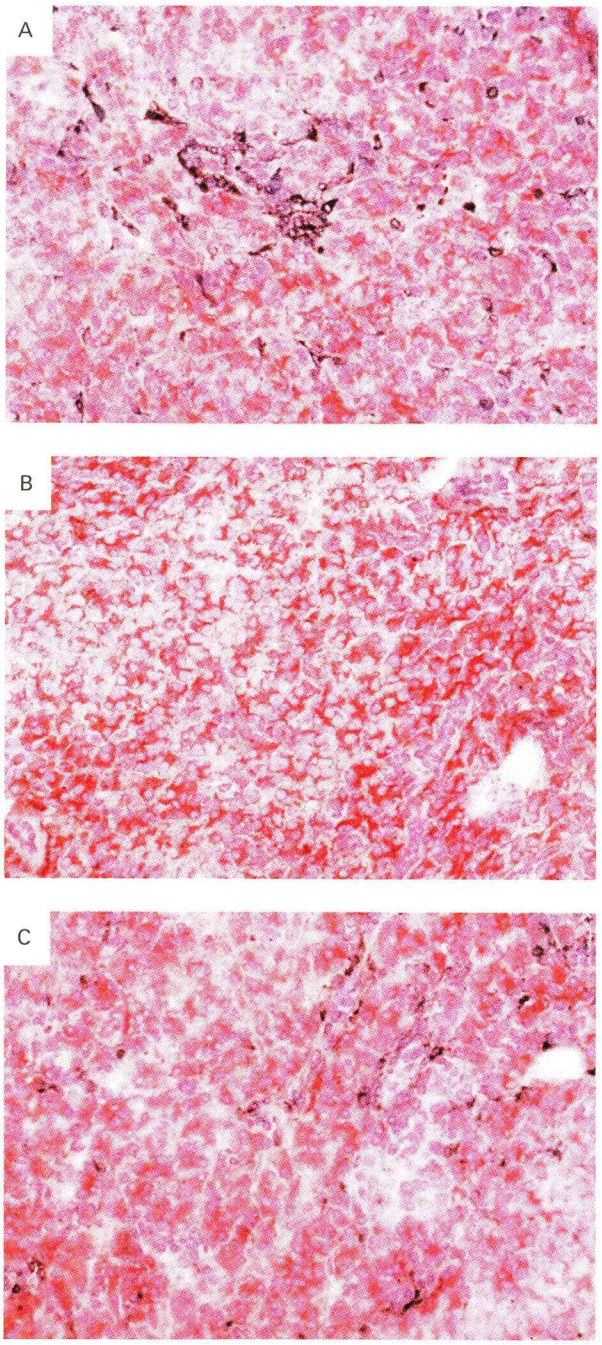

Figure 3. T-Lymphocyte–Predominant Infiltration of the Exocrine Pancreas in a Patient with a Positive Test for Glutamic Acid Decarboxylase Antibodies (×200).

T lymphocytes (Panel A), B lymphocytes (Panel B), and macrophages (Panel C) were stained with peroxidase substrate solution containing diaminobenzidine tetrahydrochloride–nickel chloride (black), and exocrine pancreas was stained with antihuman alpha-amylase antibody followed by 3-amino-9-ethylcarbazole (pink). Counterstaining was performed with hematoxylin.

cosylated hemoglobin values drank moderate or large amounts of alcohol, only 1 had abdominal pain, and all 11 had normal findings on ultrasonography of the pancreas. Therefore, these 11 patients had a type of diabetes other than that caused by classic pancreatitis.[10,11]

On the basis of these findings, we believe that diabetes characterized by the absence of glutamic acid decarboxylase antibodies and low glycosylated hemoglobin values should be classified as nonautoimmune, fulminant type 1 diabetes, a subtype of idiopathic (type 1B) diabetes. Some similar cases have been reported previously.[21,22]

The precise mechanism of beta-cell destruction in patients with this subtype of diabetes is not known. A viral cause is suggested by the abrupt onset of diabetes, the presence of lymphocytic infiltrates in the exocrine pancreas, and the affinity of several viruses for exocrine pancreatic tissue.[23,24] In preliminary studies, however, none of our patients had high titers of antiviral antibodies (data not shown).

Further studies with younger patients and other ethnic groups may provide a better understanding of this subtype of diabetes. All our patients were adults, and the clinical features of type 1 diabetes differ to some extent in children and adults.[25] Diabetes-related antibodies are more often detected in white patients than in Japanese patients,[26] suggesting that nonautoimmune, fulminant type 1 diabetes may be rare in whites and that this subtype may therefore have been overlooked in studies of autoimmune diabetes in whites.

In conclusion, nonautoimmune, fulminant type 1 diabetes mellitus in Japanese adults is a novel subtype of type 1 diabetes characterized by the absence of both insulitis and diabetes-related antibodies, an abrupt onset, and high serum pancreatic enzyme concentrations.

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APPENDIX

Other members of the Osaka IDDM Study Group included M. Namba, H. Nakajima, K. Yamamoto, H. Iwahashi, K. Yamagata, M. Moriwaki, T. Nanmo, S. Kawata, and S. Tamura (Osaka Univeristy); N. Itoh and T. Matsuyama (Toyonaka Municipal Hospital); I. Mineo and C. Nakagawa (Otemae Hospital); Y. Yamada (Sumitomo Hospital); H. Itoh (Ikeda Municipal Hospital); M. Kawachi (Izumi-otsu Municipal Hospital); H. Toyoshima (Mino Municipal Hospital); N. Watanabe, M. Hashimoto, and T. Kinoshita (Nishinomiya Prefectural Hospital); and H. Asakawa (Itami Municipal Hospital).

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参考論文

Diabetologia

Short communication

Immunological abnormalities in islets at diagnosis paralleled further deterioration of glycaemic control in patients with recent-onset Type I (insulin-dependent) diabetes mellitus

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Abstract

Aims/hypothesis. To determine whether the clinical heterogeneity observed in the development of Type I (insulin-dependent) diabetes mellitus correlates with immunohistochemical differences observed at diagnosis.
Methods. Patients ($n = 17$) with recent-onset diabetes clinically considered to be insulin dependent (Type I), underwent pancreatic biopsy for immunohistological analysis. These patients were divided into two groups based on the presence or absence of islet immunological abnormalities (insulitis or hyperexpression of MHC class I antigens or both). The patients were also HLA typed and tested for islet cell antibodies and antibodies to glutamic acid decarboxylase (GAD-Ab). All patients were followed monthly for 2 years and their fasting plasma glucose, haemoglobin A_{1C} and daily insulin doses were recorded. The clinical course of patients with islet immunological abnormalities was compared with that of patients without those abnormalities.
Results. Patients with and without islet immunological abnormalities did not differ with regard to HLA type or islet cell antibodies. Antibodies to glutamic acid decarboxylase correlated with the presence of insulitis and MHC class I hyperexpression. These local immunological abnormalities were also associated with higher haemoglobin A_{1C} values ($p < 0.05$) and a trend towards greater insulin requirements. Further, patients with the islet abnormalities had higher fasting plasma glucose concentrations 2 years after the biopsy than at the time of the biopsy ($p < 0.05$).
Conclusion/interpretation. The heterogeneous clinical course observed following diagnosis in patients with Type I diabetes correlates with islet immunological abnormalities. Insulitis and hyperexpression of MHC class I correlate with deteriorating glycaemic control. [Diabetologia (1999) 42: 574–578]

Keywords Type I diabetes, insulitis, ICA, GAD, biopsy, immunohistochemistry, HLA typing.

Type I (insulin-dependent) diabetes mellitus results from the selective destruction of pancreatic beta cells and is considered to be a cell-mediated autoimmune disease [1]. To understand the pathogenesis of the disease better we studied pancreatic biopsy specimens from recent-onset Type I diabetic patients. These studies showed hyperexpression of MHC class I antigens and that the predominant islet-infiltrating cells to be $CD8^+$ T cells and macrophages [2–3]. Others have examined autopsy specimens from Type I diabetic patients and have reported similar immunohistological findings [4–6].

We previously reported the surprising observation that only about half of the biopsies from patients with recent-onset Type I diabetes display the charac-

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Abbreviations: AP, Autoimmune-proven; AUP, autoimmune-unproven; ICA, islet cell antibodies; GAD-Ab, antibodies to glutamic acid decarboxylase; JDFU, Juvenile Diabetes Foundation Units.

Table 1. Patient's profiles and clinical and experimental data

Patient No.	Age	Sex	Insulitis	MHC class I	ICA (JDFU)	GADAb (U/ml)	HLA						
							A	B	C	DR	DQ	DRB1	DQB1
1	29	F	–	H	160	329	2/24	13/54	w2/w3	4,9	3,4	0405/0901	03032/0401
2	26	M	+	H	160	52	24/26	40/w62	w3.1/w4	9,9	3,3	0901/0901	03032/03032
3	49	F	+	H	40	21	2/24	35/54	1/–	4,4	3,4	0405/0407	0302/0401
4	25	M	–	H	80	322	2/11	48/54	1/8	1,8	3,3	NE	NE
5	24	M	+	H	< 5	< 4	2/11	39/w62	w4/w7	13,15	6,6	1302/1501	0602/0604
6	26	M	+	H	< 5	< 4	24/w33	44/w54	w1/–	4,13	4,6	0405/1302	0401/0604
7	24	F	+	H	5	92	2/24	w54/60	w1/w3	4,4	4,4	0405/0405	0401/0401
8	22	F	+	H	640	29	2/24	w54/w61	w1/–	4,9	3,4	0405/0901	0401/03032
9	34	M	+	N	320	168	2/24	7/w61	w7/–	9,9	3,3	0901/0901	03032/03032
10	43	M	+	H	< 5	< 4	2/24	44/w59	w1/–	4,13	4,6	0405/1302	0401/0604
11	25	M	+	N	640	8	24/–	7/–	w7/–	1,1	5,5	0101/0101	0501/0501
12	25	F	–	N	40	9	24/–	54/–	1/–	4,6	3,3	NE	NE
13	24	M	–	N	< 5	< 4	2/24	54/–	1/–	4,4	4,4	0405/0405	0401/0401
14	18	M	–	N	5	< 4	11/24	54/62	1/4	4,4	NE	NE	NE
15	40	F	–	N	5	< 4	31/w33	44/w56	w4/–	9,13	3,6	0901/1302	03032/0604
16	17	M	–	N	< 5	< 4	11/24	44/w61	NE	9,13	3,6	0901/1302	03032/0604
17	20	M	–	N	< 5	< 4	24/–	7/w54	w1/w7	1,4	4,5	0101/0405	0501/0401

H, hyperexpression in islets; N, normal expression in islets; NE, not examined

teristic immunohistological findings of insulitis or MHC class I hyperexpression or both [2–3]. The patients whose biopsies did not show either insulitis or MHC class I hyperexpression otherwise manifested all the typical Type I diabetic clinical characteristics. Similar patients have been reported among Caucasians [5, 7]. They were diagnosed using clinical criteria as having typical Type I diabetes but minimal or no insulitis was observed in their pancreas specimens at autopsy. These findings suggest that Type I diabetes is an immunohistologically heterogeneous disease with perhaps a more multi-factorial pathophysiology than is currently appreciated.

Recently, the expert committee on the diagnosis and classification of diabetes mellitus proposed that Type I diabetes resulted from mainly "immune-mediated" beta-cell destruction, except for a few "idiopathic" cases [8]. Nevertheless, detailed immunohistological features have not been correlated with temporal changes in clinical status. We therefore studied the immunohistological features present in pancreatic biopsy specimens from patients with recent-onset Type I diabetes and followed the clinical status of these patients for 2 or more years after the onset of the disease.

Subjects and methods

Patients. Patients (n = 17) with recent-onset diabetes clinically consistent with the early Type I disease were recruited for this study. Accordingly, all patients were lean and presented with some degree of ketosis. They were therefore started immediately on insulin therapy. Only patients with an initial C-peptide excretion of 5 μg/day were included in the study because we wished to study the effect of immunohistological abnormalities on the beta-cell function still present when diabetes first became clinically manifest. Written informed consent was obtained from all the study patients before the pancreatic biopsy was carried out as previously reported [2–3]. The 17 patients were then divided into two groups based on the immunohistological analysis of their biopsy specimen. Those patients (n = 11, 1–11 on Table 1) who displayed insulitis or the hyperexpression of MHC class I antigens or both were designated the autoimmune-proven (AP) group. The biopsies from six patients (12–17 on Table 1) displayed no immunological abnormalities and this group was designated the autoimmune-unproven (AUP) group. The time from diagnosis to biopsy was similar for the two groups; 3.3 months (range 1–7 months) for the AP and 3.5 months (range 0–7 months) for the AUP group.

HLA typing and clinical characteristics at diagnosis. At the time of the diagnosis we documented: age, sex, body mass index (BMI), HbA_{1C}, urinary C-peptide secretion, presence or absence of a family history of diabetes and presence or absence of probable infection preceding diabetes onset. In addition, we determined HLA class I and class II subtypes.

Islet autoantibodies. At the time of the biopsy, we also assayed each patient's serum for islet cell antibodies (ICA) and GAD-Ab using an indirect immunofluorescent method and a radioimmunoassay, respectively [9]. Values greater than 5 Juvenile Diabetes Foundation Units (JDFU) were considered positive for the ICA and greater than 5 U/ml were considered positive for the GAD-Ab. The fourth and fifth ICA proficiency tests done under the auspices of the Immunology and Diabetes Workshops determined that our laboratory had a 100% sensitivity and specificity.

Long-term clinical features. All patients were admitted to hospital for their pancreatic biopsy and were subsequently followed in our diabetes outpatient clinic. All patients were treated with multiple daily insulin injections and self-monitored their blood glucose aiming to maintain normoglycaemia. Each patient's daily insulin doses were monitored and documented once a month. Each patient visited the laboratory once a month for a fasting plasma sugar and HbA_{1C} determination.

Statistical analysis. Statistical analysis was by Chi-squared test, Mann-Whitney U test or Student's t-test where appropriate.

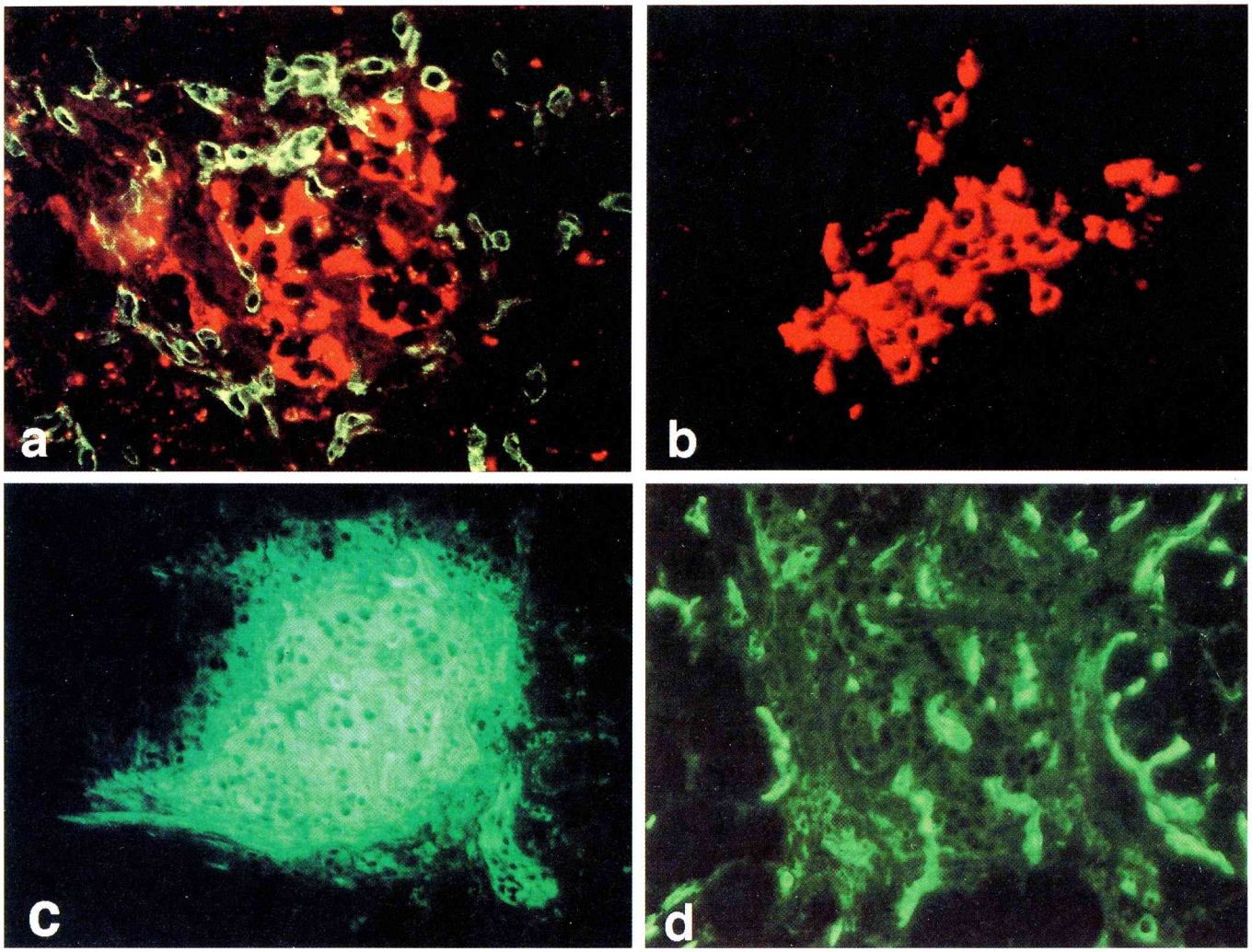
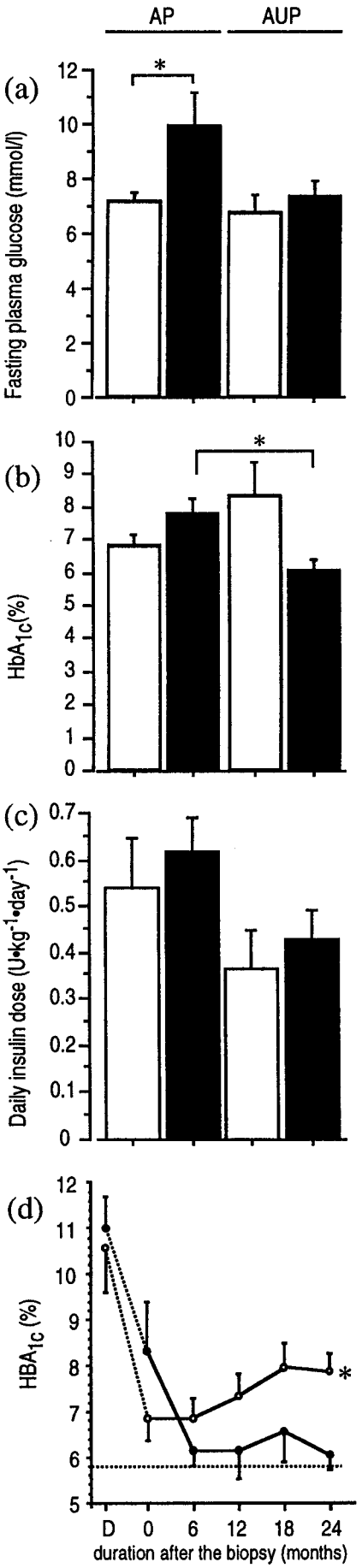


Fig. 1. a-d Photomicrographs of biopsies showing **a** insulitis (patient 10) compared with **b** an un-infiltrated islet (patient 16) and **c** an islet with increased MHC class I expression (patient 10) compared with **d** one with the more typical faint expression (patient 16). Glucagon-positive islet cells are shown in red and $CD3^+$ T cells are shown in green

Results

Immunological and immunohistological analysis. All immunological and immunohistological data for the 17 patients is shown in Table 1. In the six AUP patients, six *DQB1* alleles are considered to be diabetes susceptible and two to be neutral. In the 11 AP patients, 14 *DQB1* alleles are considered to be diabetes susceptible, 5 neutral and 1 resistant for Type I diabetes [10]. Although the sample is small, we observed no significant difference between the AP and AUP patients in HLA class I and class II antigens and the genotype of HLA-DRB1 or DQB1 antigens. Typical photomicrographs showing the appearance of an inflamed islet (insulitis) compared with an un-infiltrated islet together with an islet with increased MHC class I expression as opposed to one with the more typical faint expression are represented in Fig. 1. All 17 patients were found to harbour residual beta cells. We examined, on average (± SD), 104 (± 98) islets in AP patients and 60 (± 45) islets in the AUP patients. In those biopsy specimens showing insulitis and using an islet counting method we have reported previously [3], we found 20.9 ± 22.6 % of the islets to be affected islets (means ± SD). Immunological abnormalities in islets were correlated with the existence of GAD-Ab ($p < 0.05$, chi-squared analysis).

Clinical characteristics at diabetes onset. At the time of diabetes diagnosis, there were no significant differences between AP and AUP patients in (all data expressed as mean ± SD): age (29.7 ± 8.7, 24.0 ± 8.5), sex (men/women = 7/4, 4/2), BMI (18.8 ± 1.9, 17.2 ± 2.0), HbA_{1C} values (11.0 ± 2.4, 11.4 ± 1.4) and urinary C-peptide secretion (22.7 μg/day ± 11.6, 32.3 μg/day ± 7.9). None of the patients had a relative with Type I diabetes but 6 of the 11 patients in the AP group and 5 of the 6 patients in AUP group had relatives with Type II (non-insulin-dependent) diabetes



mellitus. Signs or symptoms suggesting antecedent infection were observed in 4 of the 11 AP patients and 4 of the 6 AUP patients. Lastly, at diagnosis there was no significant difference between AP and AUP patients in fasting plasma glucose, HbA_{1C} or daily insulin dose (Fig. 2).

Clinical characteristics on follow-up. Although fasting plasma glucose concentrations in AUP patients did not change over the 2-year observation period after pancreatic biopsy, these concentrations increased in the AP patients ($p < 0.05$) (Fig. 2). The HbA_{1C} values were also higher in the AP than in the AUP group at the end of this period ($p < 0.05$, Fig. 2), another indication of a gradual deterioration in control in the AP patients. Finally, we observed better glycaemic control in the AUP group despite their daily insulin requirements being lower than those of AP patients.

Discussion

We report that immunohistological analysis of pancreatic biopsy specimens correlates with the heterogeneous clinical course of Type I diabetes after initial diagnosis. Insulitis or MHC class I hyperexpression or both was observed in 11 of the 17 patients and their glycaemic control deteriorated during the post-diagnosis 2-year follow-up period. On the other hand, 6 of 17 patients did not show the immunohistological changes at diagnosis and their glycaemic control was better despite relatively smaller daily insulin doses during the 2-year post-diagnosis follow-up. The improved glycaemic control with small insulin doses observed in the AUP group almost certainly reflects residual beta-cell function [11–12]. After the onset of Type I diabetes, the patients in which islet immunological abnormalities had been observed probably gradually lost their remaining beta-cells (insulin-secreting capacity) resulting in the deteriorating glycaemic control which we observed. Our results are supported by a previous report which found similar heterogeneity in the clinical course of patients with Type I diabetes depending on their ICA titre [13].

Fig. 2. a-d Chronic glycaemic control is predicted by the presence or absence of in situ immunohistological abnormalities. Fasting plasma glucose concentrations (**a**), HbA_{1C} (**b**) and daily insulin dose (**c**) are shown as means ± SEM (* $p < 0.05$). Open bars indicate data at the time of biopsy and closed bars 2 years after the biopsy. (**d**) shows the HbA_{1C} measured every 6 months after the pancreatic biopsy (means ± SEM). ○, mean HbA_{1C} from the AP group and ●, the values from the AUP group. – – –, the HbA_{1C} upper limit of normal. (*$p < 0.05$ vs AUP 2 years after the biopsy). D, at the time of initial diagnosis of diabetes. The 3 AP and 2 AUP patients are not included in (**d**) at the time of initial diagnosis of diabetes

All the patients were clinically diagnosed with classical Type I diabetes based on the presenting features, e.g. all patients were lean and prone to ketosis. The Type I diagnosis was then supported by the histological finding of beta-cell depletion, an observation we have reported previously [14]. Notably, the beta-cells of the patients without in situ immunohistological abnormalities were decreased to the same extent as those of the patients with such abnormalities. In addition, we found no significant differences between the two groups in: age, sex, BMI, HbA_{1C}, urinary C-peptide secretion, signs of antecedent infection, HLA type or disease duration before insulin therapy. Similarly, in data not shown, we found no difference between the AP and AUP groups either in: peripheral lymphocyte subsets ($CD3^+$ cells, $CD4^+$ cells, $CD8^+$ cells, natural killer cells, interleukin-2 receptor positive cells and $HLA\text{-}DR^+$ cells) or the titre of viral antibodies to mumps, measles, rubella, cytomegalovirus, Epstein-Barr, and coxackie B4 virus. Despite these similarities, the clinical course after disease onset was different between the two groups.

The presence of at least two clinically distinct groups with recent-onset Type I diabetes suggests the existence of at least two different mechanisms leading to beta-cell death. In AP patients, beta-cells appear to be damaged via a cell-mediated autoimmune pathway before and even after the onset of overt diabetes. Our data suggest that $CD8^+$-cytotoxic T cells have an important pathogenic role. This is based on two observations. Firstly, the $CD8^+$ cells predominate in the infiltrate and secondly we have consistently observed the hyperexpression of MHC class I in the islet cells [2–3]. Our data does not allow us to comment on the pathogenesis of beta-cell destruction in the AUP patients, but we found no evidence for an autoimmune mechanism. We surmise that a genetic disorder or direct viral attack on the pancreas contributed to the decreased beta-cell mass we observed. Others, for example, have reported patients with a clinical diagnosis of Type I diabetes to have an associated mitochondrial DNA mutation and severe loss of beta-cell volume [15]. Another group reported a Type I diabetic patient who was ICA-negative, this apparently having been caused by a cytomegalovirus infection [16].

We conclude from this study that the heterogeneous clinical course following the onset of Type I diabetes is associated with islet immunological abnormalities seen at diagnosis. The presence of either insulitis or MHC class I hyperexpression is associated with clear clinical evidence of ongoing beta-cell loss.

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Islet-Infiltrating T Lymphocytes in Insulin-dependent Diabetic Patients Express CD80 (B7-1) and CD86 (B7-2)

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Insulin-dependent diabetes mellitus (IDDM) results mainly from T cell mediated pancreatic β cell destruction. To fully activate antigen specific T cells, current evidence suggests that two signals are required. One signal is delivered via the antigen specific T cell receptor (TCR) when engaged by major histocompatibility complex presented antigen (MHC:Ag), the other via the T cell's CD28 when engaged by CD80/86. Recent studies have demonstrated that transgenic mice expressing CD80 on their pancreatic β cells are susceptible to autoimmune β cell destruction. To further explore whether CD80/86 expression plays a role in IDDM pathogenesis, we analysed pancreatic biopsy specimens from 16 recent-onset IDDM patients (13 men and 3 women; age 29.7±8.8 years) for CD80/86 expression. While no biopsy revealed any islet cell specific CD80 or CD86 expression, biopsies from six of the nine patients with insulitis revealed both CD80 and CD86 expression on the islet infiltrating cells. Triple immunofluorescent staining for CD80/86, CD3, and glucagon revealed that the CD80/86-positive cells were also CD3-positive. Of the CD3-positive cells, 19.4% expressed CD80 and 21.7% expressed CD86. CD80 and CD86-positive cells were similarly distributed throughout the inflamed islets. These data suggest that CD28 engagement with CD80/86 may play a pathogenic role in the β cell destruction underlying IDDM.

Key words: IDDM, co-stimulatory factor, insulins, pancreas biopsy, immunohistochemistry

Introduction

Insulin-dependent diabetes mellitus (IDDM) results mainly from autoimmune pancreatic β cell destruction [1, 2]. Several groups have reported that the pancreatic islets in patients with recent-onset IDDM are infiltrated with mononuclear cells, a lesion called insulitis [3–7]. Detailed immunohistochemical analysis of pancreatic biopsy specimens from such patients has revealed that the islet-infiltrating mononuclear cells are predominantly $CD8^+$ T lymphocytes and macrophages [6]. Further, electron microscopic studies have provided evidence that these islet infiltrating T cells are actively and directly engaged in β cell destruction [8]. These findings suggest that the islet infiltrating T lymphocytes are activated against β cell-specific antigens. Yet current evidence suggests that at least two signals are required for antigen-specific T lymphocytes to be fully activated [9, 10]. One signal is delivered via the antigen-specific T-cell receptor (TCR) when engaged by major histocompatibility complex presented antigen (MHC:Ag). The other signal, called the costimulatory signal, is delivered via the T cell's CD28 when engaged by CD80 (B7/B7-1) or CD86 (B70/B7-2). While CD80 and CD86 expression is thought to be limited to professional antigen presenting cells and lymphocytes in normal individuals [9, 10], transgenic mouse studies have demonstrated that β cell CD80 expression can activate β cell-specific T lymphocytes resulting in the development of autoimmune diabetes [11, 12]. For example, when C57BL/6 transgenic mice expressing CD80 on their β cells are bred into the non-obese-diabetic (NOD) mouse strain, and offspring of the first backcross generation (F2) are selected for $H\text{-}2^{g7}$ and for the presence or the absence of the CD80 transgene, mice homozygous for $H\text{-}2^{g7}$ and also expressing β cell CD80 developed diabetes much sooner than their CD80-negative littermates [11]. In another study 'double transgenic' mice expressing both a viral glycoprotein (GP) on their pancreatic β cells and a transgene-encoded TCR specific for that GP on their T lymphocytes did not spontaneously develop insulitis

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Table 1. *Characteristics of IDDM patients*

Patient	Age	Sex	Disease duration (months)	Urinary CPR (μg/day)	ICA (JDF units)	GAD Ab (U/ml)	HLA DR phenotype
1	33	M	4	6–15	160	<4	4, 8
2	24	M	3	8–27	<5	<4	4
3	25	M	2	6–23	80	322	4, 9
4	24	M	2	7–14	<5	<4	2, 6
5	40	F	0	10–27	5	<4	6, 9
6	26	M	1	5–19	<5	<4	4, 6
7	24	F	6	14–36	5	92	4
8	17	M	3	18–47	<5	<4	6, 9
9	42	M	5	1–3	<5	<4	4, 9
10	22	F	4	4–15	640	29	4, 9
11	34	M	7	5–19	320	168	9
12	31	M	13	4–13	5	<4	4, 6
13	43	M	2	6–9	<5	<4	4, 6
14	25	M	3	10–51	640	8	1
15	20	M	10	11–31	<5	<4	1, 4
16	47	M	2	ND*	<5	<4	ND*

*ND: not determined.

or diabetes. However, when these 'double transgenic' mice were bred with mice expressing CD80 on their β cells to create 'triple transgenic' mice, i.e. GP and CD80 on their pancreatic β cells, and GP-specific TCRs on their T lymphocytes, all the mice developed insulitis and overt diabetes [12]. Less is known about CD86, the other ligand for CD28 [13, 14], and the role that ligand might play in autoimmune disease pathogenesis. CD86 is known to provide similar costimulatory signals for T lymphocyte proliferation, cytokine production and the generation of cytotoxic T lymphocytes *in vitro* [15].

We therefore asked whether CD80 or CD86 (CD80/86) was expressed in the pancreas of IDDM patients. While we did not find CD80 or CD86 expressing islet cells, we did observe $CD80^+$ and $CD86^+$ islet-infiltrating mononuclear cells in the insulitis lesions and determined which type of cells expressed CD80/86.

Materials and Methods

Subjects

Sixteen patients with recent-onset IDDM, 13 men and 3 women with a mean (±SD) age of 29.7 (±8.8) years were included in this study. Their detailed profiles are provided in Table 1. All patients fulfilled National Diabetes Data Group criteria for IDDM [16], and all except two (patients 3 and 5) presented with the typical abrupt disease onset with ketosis. In the two patients with somewhat atypical onset the disease progressed more slowly to the insulin dependent state [17]. In these two cases, IDDM onset was assumed when insulin was required to prevent ketosis. Islet cell antibodies (ICA) were detected in eight patients by an indirect immunofluorescence (IFL) assay [18]. Serum antibodies to glutamic acid decarboxylase (GAD Ab) were detected in five patients with a radioimmunoassay kit (Hoechst Japan, Tokyo, Japan) [19]. Data from some of the subjects (patients Nos 1–11) have been included in previous publications which included detailed histological findings [2, 6].

Pancreas biopsy

After obtaining written informed consent from each patient, we performed a pancreatic biopsy. This study was approved by the Ethics Committee of Osaka University Medical School. A detailed description of the biopsy procedure has been published [2, 6]. Briefly, a laparoscopic biopsy from the body of the pancreas was performed and about 20 mg of tissue was obtained. The tissue was immediately frozen and serial 5-μm-thick sections were prepared with a cryostat. Approximately 300 consecutive sections were prepared from each biopsy sample and analysed immunohistochemically.

Immunohistochemistry

An indirect immunofluorescence (IFL) method was employed to detect CD80 and CD86 expression in the pancreatic sections. These sections were air dried for 30 min, prefixed in cold acetone for 10 min, and washed 3 times at 5-min intervals in PBS, pH 7.4, and incubated overnight at 4°C with one of the following monoclonal mouse antibodies: anti-human B7-1 (EW3, 4B2; Repligen, Cambridge, MA), anti-human B7-2 (HA3, IF9; Repligen), anti-human CD3 (UCHT-1; Dakopatts, Glostrup, Denmark), and anti-human macrophage (CD68) (EBM11; Dakopatts). After washing in PBS, the sections were incubated for 60 min at room temperature with biotinylated horse anti-mouse immunoglobulins (Vector Laboratories, Burlingame, CA) and then for an additional 30 min with FITC-

conjugated avidin (Vector Laboratories). To examine the relation between CD80/86 positive cells or CD3/CD68 positive cells and the islet, the above mentioned procedures were followed by an incubation with guinea pig anti-insulin (Dakopatts) or rabbit anti-glucagon (Dr S. Iwasa, Takeda Chemical, Osaka, Japan) antibody with the corresponding secondary antibody, rhodamine-conjugated anti-guinea pig (Zymed Laboratories, Inc, South San Francisco, CA) or Texas red-conjugated anti-rabbit (Amersham International, Amersham, UK) immunoglobulins. The sections were washed in PBS, mounted with Perma Flour Aqueous Mounting Medium (Immunon, Pittsburgh, PA), and observed using a fluorescence microscope with epi-illumination (Olympus, Tokyo, Japan).

A triple immunohistochemical method was employed to examine the relation among CD80/86 positive cells, CD3/CD68 positive cells and the islet cells. After incubation with anti-CD80 or CD86 antibody, the sections were incubated for 60 min with phycoerythrin (PE)-conjugated rat anti-mouse immunoglobulin kappa chain (Becton Dickinson, San Jose, CA). As the second step, the sections were preincubated with normal mouse serum (Dakopatts) for 10 min, and then incubated with biotinylated anti-human CD3 (UCHT-1; Dakopatts) followed by an additional 30 min with FITC-conjugated avidin (Vector Laboratories) to detect CD80/86 positive T lymphocytes or incubated with HRP-labelled anti-human CD68 (PG-M1; Dakopatts) followed by an additional 2 min in peroxidase substrate solution containing DAB 3,3'-diaminobenzidine)/nickel chloride (Zymed Labs) to detect CD80/86 positive macrophages. As the third step, the sections were incubated with anti-glucagon (Dakopatts) and AMCA (7-amino-4-methylcoumarin-3-acetic acid)-conjugated goat anti-rabbit immunoglobulins (Dakopatts) to indicate the islet area for CD3 and CD80/86 staining. The sections were washed in PBS after each incubation except after application of normal mouse serum. As for CD68 and CD80/86 staining, the triple immunostaining method with glucagon did not work well in our preliminary study, hence only double immunostaining method was done to determine the relation between CD80/86 positive cells and macrophages.

Results

CD80 and CD86 expression in the pancreas

We defined an islet as insulitis-positive when two or more mononuclear cells were present in an islet area, since in some instances only one mononuclear cell was seen [6]. Using this criterion, only 9 of 16 patients in this study were insulitis-positive (Table 2). In six of the nine insulitis-positive patients, we found that islet infiltrating cells stained for CD80 and/or CD86 (Figure 1). $CD80^+$ and $CD86^+$ cells were similarly distributed throughout the inflamed islets in all six patients. We did not observe islet cell staining for CD80/86 in any patient. None of the seven insulitis-negative patients displayed CD80 or CD86 expression in the pancreas.

Table 2. *Immunohistochemical analysis of insulitis and CD80/86 expression in the IDDM pancreas*

Patient	Insulitis	CD80 expression*	CD86 expression*
1	+	+	+
2	−	−	−
3	−	−	−
4	+	+	+
5	−	−	−
6	+	+	+
7	+	−	−
8	−	−	−
9	−	−	−
10	+	+	+
11	+	+	+
12	+	−	−
13	+	+	+
14	+	−	−
15	−	−	−
16	−	−	−

*: Expression on islet-infiltrating cells.

Phenotypic analysis of CD80 and CD86 positive cells

We have previously reported that the predominant islet-infiltrating cells in patients with recent-onset IDDM are T lymphocytes and macrophages [6]. To determine which cells express CD80 or CD86, we tried to detect CD80 or CD86 positive cells with anti-CD3 or anti-macrophage antibodies. A single section triple immunostained for CD86, CD3, and glucagon (Figure 2) shows that an islet-infiltrating $CD3^+$ T lymphocyte is also positive for CD86. In other sections, some $CD80^+$ were also $CD3^+$ T lymphocytes. While we could not determine whether some $CD3^+$ T lymphocytes express both CD80 and CD86, the distribution of CD80 and CD86 expressing cells was similar. This was the case in all six patients with CD80/86 positive cells.

In five of the six patients with $CD80^+$ and $CD86^+$ islet-infiltrating cells, we determined the proportion of $CD3^+$ T lymphocytes that stained for CD80 or CD86. In the biopsies from these five patients, we could find anywhere from 3 to 34 islets, of which approximately half were identified as insulitis positive. For each individual patient, the proportion of $CD3^+$ islet infiltrating cells that also stained for CD80 ranged from 12.5% to 33.3%, while the proportion of $CD3^+$ islet infiltrating cells that also stained for CD86 ranged from 16.7% to 38.5%. When the $CD3^+$ cells observed in the five biopsies were considered collectively, 14 cells out of 74 $CD3^+$ T lymphocytes were positive for CD80, giving the total prevalence of 19.4%. On other sections from these five patients, 39 cells out of 184 $CD3^+$ T lymphocytes were also $CD86^+$, giving a prevalence of 21.7%.

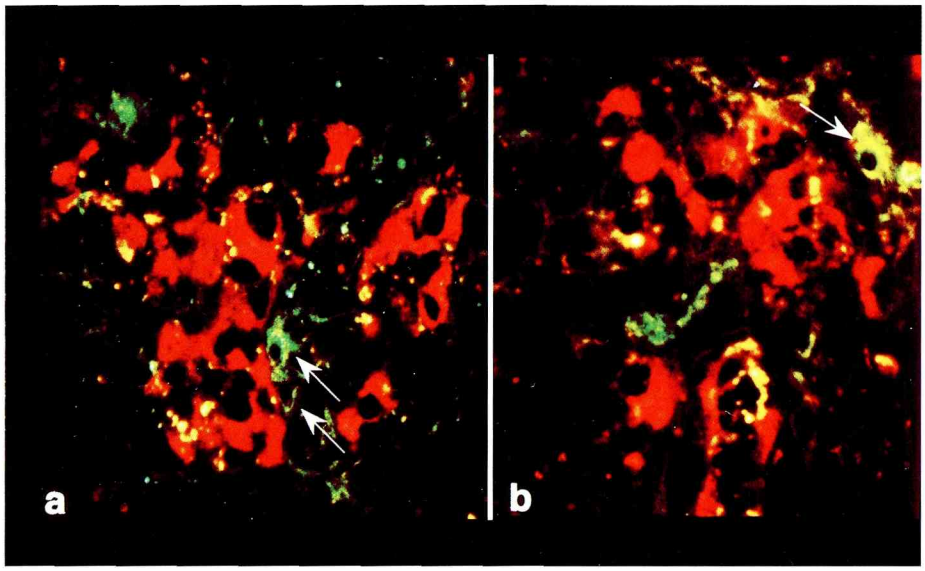

Figure 1. The expression of CD80 and CD86 in double-exposed photographs of a pancreatic biopsy specimen (patient 13). The red colour shows glucagon-positive cells indicating an islet area, and green colour (arrow) shows $CD80^+$ (a) and $CD86^+$ (b) islet-infiltrating cells. Islet-infiltrating cells are shown to stain for both CD80 and CD86 expression.

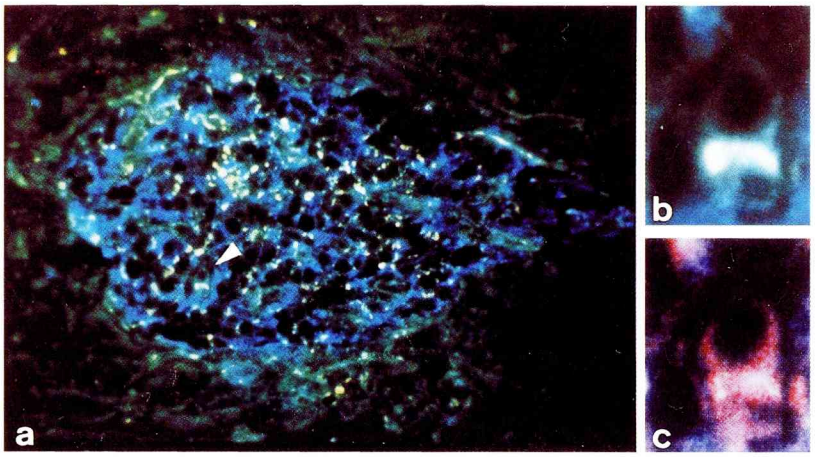

Figure 2. Triple immunofluorescent staining for CD86, CD3, and glucagon in a single section (patient 11). (a) Glucagon-positive cells are shown in blue, and $CD3^+$ cells are shown in green in this double-exposed photograph. (b) High magnification of a $CD3^+$ islet-infiltrating cell (green), indicated by an arrowhead in (a). (c) The same $CD3^+$ cell that is shown in (b) is also positive for CD86 shown in red.

A few $CD80^+$ and $CD86^+$ macrophages were also detected by the double immunostaining method. We were unable to determine whether these CD80/86 positive macrophages infiltrated to islets, however, because the triple staining did not work using the PG-M1 (macrophage-specific) antibody.

CD80/86 expression and clinical characteristics

CD80 and/or CD86 expression was observed only in the patients with insulitis. Clinical characteristics including patient age, sex, disease duration, urinary C-peptide excretion, ICA titre, GAD Ab titre and HLA phenotype did not correlate with CD80/86 positivity.

Discussion

Bottazzo *et al.* [3] first reported that the majority of islet-infiltrating cells were T lymphocytes expressing both HLA-DR and interleukin 2 receptor. Somoza *et al.* [7] corroborated these findings, reporting that 20% of islet-infiltrating cells expressed DR antigen. After carefully examining serial sections of the pancreatic biopsy tissue described in this report, we have also confirmed that islet-infiltrating cells are DR^+ (Itoh N., Hanafusa T., Imagawa A., *et al.*, unpublished observation). These findings suggest that islet-infiltrating T lymphocytes are activated and moreover, that these activated T lymphocytes may serve as antigen presenting cells and thereby stimulate naive T

lymphocytes that traffic to the islet. However, naive T lymphocytes must be costimulated via a CD28–CD80/86 interaction to be fully activated [20]. We are now the first to report that in patients with recent-onset IDDM, islet-infiltrating cells also express CD80 and CD86. Further, that these islet-infiltrating $CD80^+$ and $CD86^+$ cells are predominantly if not exclusively T lymphocytes. These data suggest that islet-infiltrating T lymphocytes may costimulate autologous naive T lymphocytes and thereby amplify the immune destructive process leading to β cell death and IDDM.

We have proposed that the co-expression of HLA-DR and CD80/86 on β cells could trigger an autoimmune cascade by directly activating T lymphocytes, i.e. the antigen specific signal provided by the MHC:Ag:TCR interaction and the costimulatory signal generated by the CD80/86:CD28 interaction [9, 12]. We have previously reported that the biopsy specimen from one patient (patient 1 in this study) revealed β cell HLA-DR staining [2]. In the group now reported, the biopsy from patient 13 also revealed islet cell HLA-DR antigen expression (Itoh N., Hanafusa T., Imagawa A., *et al.*, unpublished observation). At least by the methods employed in the present study, however, we could not detect any islet cell CD80 or CD86 expression. Nevertheless, Yagi *et al.* have reported electron microscopic immunohistochemical data showing that NOD mouse islet cells do express intercellular adhesion molecule (ICAM)-1, but that this expression was not apparent by light microscopic study [21]. Therefore, it is possible that immunoelectron microscopic study or other sensitive methods such as in situ hybridization or in situ polymerase chain reaction (PCR) would be useful to investigate further whether islet cells may express CD80 or CD86 in patients with insulitis.

In this study we found that 19.4% of islet-infiltrating T lymphocytes co-expressed CD80, and that 21.7% of islet-infiltrating T lymphocytes co-expressed CD86. The prevalence of CD80 positive cells was similar to that observed in a study of patients with rheumatoid arthritis (RA) in which 30% of T lymphocytes infiltrating the rheumatoid synovium were positive for CD80 [22]. Infiltrating lymphocytes in both RA and IDDM express HLA-DR and the IL-2 receptor [23]. These results suggest a similar pathogenesis underlying RA and IDDM, two intensively studied organ-specific autoimmune diseases histologically notable for their marked tissue-specific lymphocytic infiltration.

We observed a few $CD80^+$ and $CD86^+$ pancreatic macrophages by the double immunostaining method, but we could not determine whether these macrophages were infiltrating the islets. In other studies we have used clone EBM11 to demonstrate that significant numbers of macrophages can be identified in the insulitis lesions. For the present study, however, we attempted to use clone PG-M1 to stain macrophages. This antibody has the advantage of direct HRP-labelling but the disadvantage of poor sensitivity. Thus, our inability to detect macrophages in the insulitis lesions may be secondary to an insufficient sensitivity of the macrophage-specific antibody employed for the double staining procedure.

Recently, CD80 and CD86 have been reported to differ in their ability to potentiate the differentiation of Th0 T lymphocytes into either Th1 or Th2 phenotypes [24, 25]. CD80 and CD86 molecules might have different functions *in vivo* though they show similar actions *in vitro* [15]. We found no difference between CD80 and CD86 expression in the insulitis lesions of these patients with recent onset IDDM.

In conclusion, our present results demonstrate that in patients with recent-onset IDDM, islet-infiltrating T lymphocytes express CD80 and CD86. This suggests that these CD80/86 positive T lymphocytes may play a role in the autoimmune reaction against β cells resulting in IDDM.

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