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Heterogeneity of islet pathology in two infants with recent onset diabetes mellitus

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Abstract The mechanisms by which the beta cells of pancreatic islets are destroyed in insulin-dependent diabetes mellitus (IDDM) are poorly understood. In this report the pancreatic histo- and immunopathology of two children, both HLA-DR 3/4, DQ 2/8 positive and who both died from cerebral oedema within a day of clinical diagnosis of IDDM, were investigated. Patient 1, a 14-month-old girl, had a 4-week history of polydipsia and polyuria. Patient 2, a 3-year-old boy, had 2 days of illness. Both patients had a similarly severe loss of insulin cells but differed markedly as to the extent of lymphocytic islet infiltration (insulitis). Apart from insulitis, marked islet macrophage infiltration was demonstrated in both patients with the HAM-56 monoclonal antibody. Neither patient showed aberrant expression of HLA class II antigens on insulin-immunoreactive cells, but allele-specific HLA-DQ8 expression was evident on endothelial cells. Glutamic acid decarboxylase immunoreactivity was detected in both insulin- and glucagon-immunoreactive cells. It is concluded that the heterogeneity of islet pathology, especially insulitis, may reflect different dynamics and extent rather than different pathomechanisms of immune destruction of islets in IDDM.

Key words Recent-onset insulin dependent diabetes mellitus · Insulitis · Islet cell macrophages · Glutamic acid decarboxylase · Islet destruction

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Introduction

Insulin-dependent diabetes mellitus (IDDM) is clinically evident after a major loss of pancreatic islet beta cells. The natural history of the disease prior to the onset of symptoms is poorly understood, but several autoimmune phenomena have been documented (for review see [13, 44]. The prodrome may be long since islet cell (ICA), insulin (IAA), or glutamic acid decarboxylase (GAD65Ab) autoantibodies have been reported as long as 8-12 years before the clinical onset [3, 4, 6, 23, 42, 52, 55, 59]. A prodrome would obviously be shorter in infants and young children, but the prevalence of ICA, IAA or GAD65Ab at diagnosis of IDDM in children is not different from that in teenagers [11, 35]. Young children, however, tend to have higher frequency and levels of IAA than older children [1, 2, 36]. The clinical onset of IDDM in infants and young children is also associated with lower plasma and urine levels of C-peptide [60]. The „pre-onset" destruction of the islet beta cells could therefore proceed more rapidly in infants and young children.

Insulitis is often [8, 14, 15, 17, 18, 21, 22, 31, 33, 46, 49, 51, 64], but not always [12, 26] seen at the clinical onset (Table 1). However, since the number of multiple-case studies on newly diagnosed children is small, there

Table 1 Literature review of reports on insulitis in insulin-dependent diabetes mellitus. Italicized entries not included on Fig. 5.

Age at onset[a]	Duration[a]	Insulitis (positive cases/total cases)		
		All cases	<16 years	Reference
10 years	*„Acute"*	*1/1*	*1/1*	*54*
46.5y (9–73y)	2y (1 days–22y)	7/59	0/4	10
25.7y (20–51y)	1y (1d–3y)	1/6	0/0	29
45y (4.5–80y)	*Not given*	*8/183*	*5/12*	29
6.5y, 18y	2 weeks, 6y	1/2	1/1	14
36.75y (11–63y)	4.75y (26d–30y)	3/26	1/3	62
9y (2–15y)	1.6y (17d–29y)	1/9	1/9	61
6y, 11y	6w, 6d	2/2	2/2	56
18y	10m	1/1	0/0	43
14y (11w–28y)	*13d (2d–8w)*	*3/18*	*Not given*	41
13.7y (11m–30y)	15.5d (1–180d)	15/22	14/18	21
12y (2–29y)	20y (2–37y)	0/32	0/24	21
9m, 33m	4d, 7d	2/2	2/2	57
17y (6–21y)	3.5w (1–78w)	0/13	0/5	12
5y	20d	1/1[b]	1/1	22
14.7y (1–31y)	3q (1d–8w)	6/11	5/6	31
10y	3d	1/1[b]	1/1	64
14m	5d	1/1[b]	1/1	46
7m	3m	1/1[c]	1/1	51
4y (2–12y)	4w (1–12w)	8/9	8/9	14
6y (5–7y)	4y(2–6y)	0/2	0/2	14
12y	1m	1/1	1/1	8
8.5y (1.5–18.5y)	2w (1w–9m)	47/47	47/47	15
10.5y (DOB–18y)	3y (13m–9y)	3/8	2/17	15
8.4y (1.5–21.4y)	3w (2d–3m)	21/23	16/18	16
7.5y (1–14y)	3y (1y–9y)	3/12	3/12	16
17.5y (7–20y)	1y (1–15y)	4/4	2/2	18
31y (24–49y)	3.1m (2–4m)	0/7[d]	0/0	26
12y (1–18y)	*<3m*	*12/12*	*11/11*	19
31y (17–53y)	*(0–7m)*	*12/18§*	*0/0*	30

[a]For total cases >2, figure shown is the median (range in parentheses)
[b]Additional viral pathology observed
[c]Additional endocrine autoimmunities
[d]Biopsies obtained at laparoscopy

is no understanding of the possible relationship between the degree of insulitis and clinical onset. A systematic study of islet pathology at the onset of IDDM is obviously difficult to undertake, and therefore all except a few [26, 30] studies of islet pathology in IDDM (see Table 1) have been conducted in autopsy cases. The few autopsy pancreases studied recently were obtained from children, because children less than 4 years of age at onset of IDDM are at increased risk of fatal cerebral oedema [53].

In this report, we describe two children, both heterozygous HLA-DR 3/4, DQ 2/8 positive: a 14-month-old girl (patient 1) and a 3-year-old boy (patient 2), both of whom died with cerebral oedema within a day of clinical diagnosis of IDDM. The two children were different in that patient 1 had a 4-week history of polydipsia and polyuria, whereas patient 2 had only 2 days of illness. We demonstrate that both patients had a similarly severe loss of insulin beta cells; however, while both had marked islet macrophage infiltrations, significant insulitis was only detected in patient 1. The question is addressed whether the difference in islet pathology between the two infants reflects different pathomechanisms or different dynamics of immunoreactivity.

Patients, materials and methods

Patient 1

A 14-month-old girl was admitted to the hospital unconscious and with irregular breathing. Her parents and brother (3 years older) were healthy, but both IDDM and non-insulin dependent diabetes mellitus (NIDDM) were present among second- and third-degree relatives. The girl had been well until 4 weeks earlier when increased thirst and polyuria developed. The day before admission she had a fever of approximately 38° C, her breathing was abnormal and she was lethargic. She improved spontaneously during that day, but was unconscious the next morning and brought to a primary health care center where ketonuria 3+ and glucosuria 3+ were found. On arrival at the hospital in the afternoon, blood glucose was 30 mmol/l (540 mg/100 ml), pH 7.11, PCO_2 1.6 and white blood cells 36 700. She was treated with 12.5 ml/kg per hour acetated Ringer's the first 4 h and 25 mmol sodium bicarbonate was given to counteract the acidosis. Regular insulin at 0.1 U/kg per hour was given intravenously. After 4 h the blood glucose value dropped below 10 mmol/l and the acetated Ringer's was changed to 50 mg/ml glucose at an infusion rate of 7.5 ml/kg per hour for another 4 h and 4 ml/kg per hour thereafter. During the night both her metabolic and clinical status improved. The next morning, however, she had convulsions and 2 h later she developed bradycardia and irregular breathing. She was intubated, hyperventilated and given beta-methasone to decrease the cerebral oedema. She had no pupillary reflex and was pronounced brain dead in the afternoon following two aortocervical angiographies. Bacterial cultures taken from blood, urine and cerebrospinal fluid were all negative. The patient's kidneys were taken for transplantation and the pancreas but no serum was made available for investigation. The HLA type determined at the University of Lund, Lund, Sweden was DR3/4; DQ2/8.

Patient 2

A 3-year-old boy was brought to a physician's office following 1 day's signs of some dehydration and of lethargy subsequent to a day of stomach upset experienced when travelling with his parents. The family history was negative for autoimmune diseases including IDDM; the mother (30 years old) had developed diet-treated gestational diabetes in the 8th month and a maternal aunt has NIDDM. At the physician's office, ketonuria was 4+, Chemstrip blood glucose was 45 mmol/l (810 mg/100 ml) and the boy was administered 3 U regular insulin subcutaneously and given 30 ml/kg lactated Ringer's prior to his arrival at the hospital. Admission laboratory venous blood values were pH 7.11, PCO_2 14 and white blood cells 36 200. Since his mental status was deteriorating, a CT scan was performed immediately, revealing cerebral oedema. The patient was intubated, hyperventilated, given mannitol, fluids were restricted and he was taken to the operating room for placement of an intracranial pressure monitor and a ventriculoscope. On return to the intensive care unit, intracranial pressure subsided to normal and his metabolic status improved. However, subsequent to this blood pressure dropped and he did not respond to dopamine and fluid boluses. He was pronounced brain dead following negative apnoea tests and no flow on a cerebral perfusion study. The heart, liver and kidneys were removed for transplantation while the pancreas and serum were made available for investigation. The serum was positive for both ICA and IAA determined using standardized methods [7, 34, 45] but negative for GAD65Ab in immunoprecipitation assays with either dog [5] or recombinant human islet GAD65 [24]. The ICA reaction was also tested at multiple dilutions on the pancreas of the patient. However, there were no differences in end-point titres whether the patient's pancreas or the pancreas for standard ICA determination was used. The HLA type determined at the Puget Sound Blood Bank, Seattle, Wash. was DR3/4; DQ2/8.

Non-diabetic patients

Pancreatic tissue was also obtained from nine age- and sex-matched non-diabetic children. In Brussels three control specimens were obtained from children who had died after congenital heart disease (4 years old), carbon monoxide poisoning (4 years old) or a traffic accident (1.5 years old) and in Seattle six specimens were obtained (courtesy of Dr. Joseph R. Siebert) after congenital heart disease (8 months old), acute myelocytic/lymphocytic leukaemia (1 year old), asplenia complex (3.5 years old), congenital heart disease (3.8 years old), acute lymphoblastic leukaemia (5 years old) or pulmonary hypertension (10 years old).

Histology, immunocytochemistry and in situ hybridization

The pancreases were fixed in formalin (patient 1 and controls) or Bouin-Hollande solution (patient 2) and embedded in paraffin. Immunocytochemical studies were performed using the standard avidin-biotin immunoperoxidase technique, with the antibodies listed in Table 2. In situ hybridization of insulin was carried out as described previously [58]. To determine the proportion (i.e. volume density) of islet tissue in relation to the total pancreatic parenchyma, the point counting method [25, 33] was used in the two patients and in the three Brussels controls. The analysis was performed at 100× magnification with a 100-point graticule on sections stained by haematoxylin and eosin (H&E), which permitted point counting of islets whether infiltrated by mononuclear cells or not. Only the exocrine parenchyma was used as a reference volume, whereas connective tissue, vessels and nerves were excluded. Immunostained insulin and glucagon cells and islet macrophages in percent of islet tissue were thereafter determined at 400× magnification. At least 80 fields were evaluated to cover a profile area of about 2 mm^2.

Results

In patient 1, histological examination of samples from different regions of the pancreas revealed the presence of a marked mononuclear cell infiltration (insulitis) in and around many islets (Fig. 1A, B). The islets which lacked insulitis (not shown) were composed of small endocrine cells and had irregular outlines. Normal-appearing islets were very rare. In patient 2, only two islets out of 200 examined showed a discrete mononuclear cell infiltration at the islet periphery (Fig. 1C). Most islets were composed of small endocrine cells often difficult to distinguish from the surrounding acinar cells. The remaining islets were readily recognizable and contained some slightly hypertrophic islet cells staining for insulin. The distribution of the latter islets was uneven in the pancreas (Fig. 1D). Mononuclear cell infiltrations were not detected in the control pancreases. This analysis revealed that insulitis was severe and involved most islets in the younger child (patient 1), while affecting only single islets in the older child (patient 2). Lymphocytic infiltration in patient 1 was confirmed by positive immunostaining with both T- and B-lymphocyte-specific antisera, as well as antiserum against the common leucocyte antigen. It was next tested whether the difference in intensity of insulitis was correlated to a difference in the presence of insulin-, proinsulin- or glucagon-immunoreactive cells.

Immunostaining showed cells positive for insulin and glucagon in both patients. In addition, the islets contained somatostatin and pancreatic polypeptide cells (data not shown). In patient 1, islets had insulin-positive cells (Fig. 1B); however, the architecture of the islets was often disrupted and both insulin (Fig. 2A) and glucagon-immunoreactive cells were detected in strands broken up by inflammatory cells. Patient 2 showed clusters of islets with a normal number of insulin cells (Fig. 1D); most islets, however, were devoid of insulin-immunoreactive cells but stained for glucagon as well as somatostatin and pancreatic polypeptide (data not shown for the latter two hormones). Islet structures are still detectable due to weakly stained insulin cells (Fig. 1B, D). The islets with insulin-immunoreactive cells were also found positive by a proinsulin antibody (Fig. 2B) as well as by in situ hybridization for proinsulin mRNA (Fig. 2C) or islet amyloid polypeptide (IAPP) (not shown). The number of cells which hybridized to proinsulin mRNA did not exceed the number of insulin-immunoreactive cells. Proinsulin expression at the RNA and the protein level was reduced when compared with results in controls.

Morphometric evaluation of the proportion of insulin- and glucagon-immunostained cells in the islets of four sections from each individual revealed that patient 1 had 8% insulin- and 27% glucagon-positive cells, while patient 2 had 13% and 28%, respectively, compared with a mean of 54% and 16% respectively in the three controls (Table 3). The remaining proportion of the islet area was occupied by somatostatin, pancreatic polypeptide and vascular cells. In both diabetic pancreases a considerable

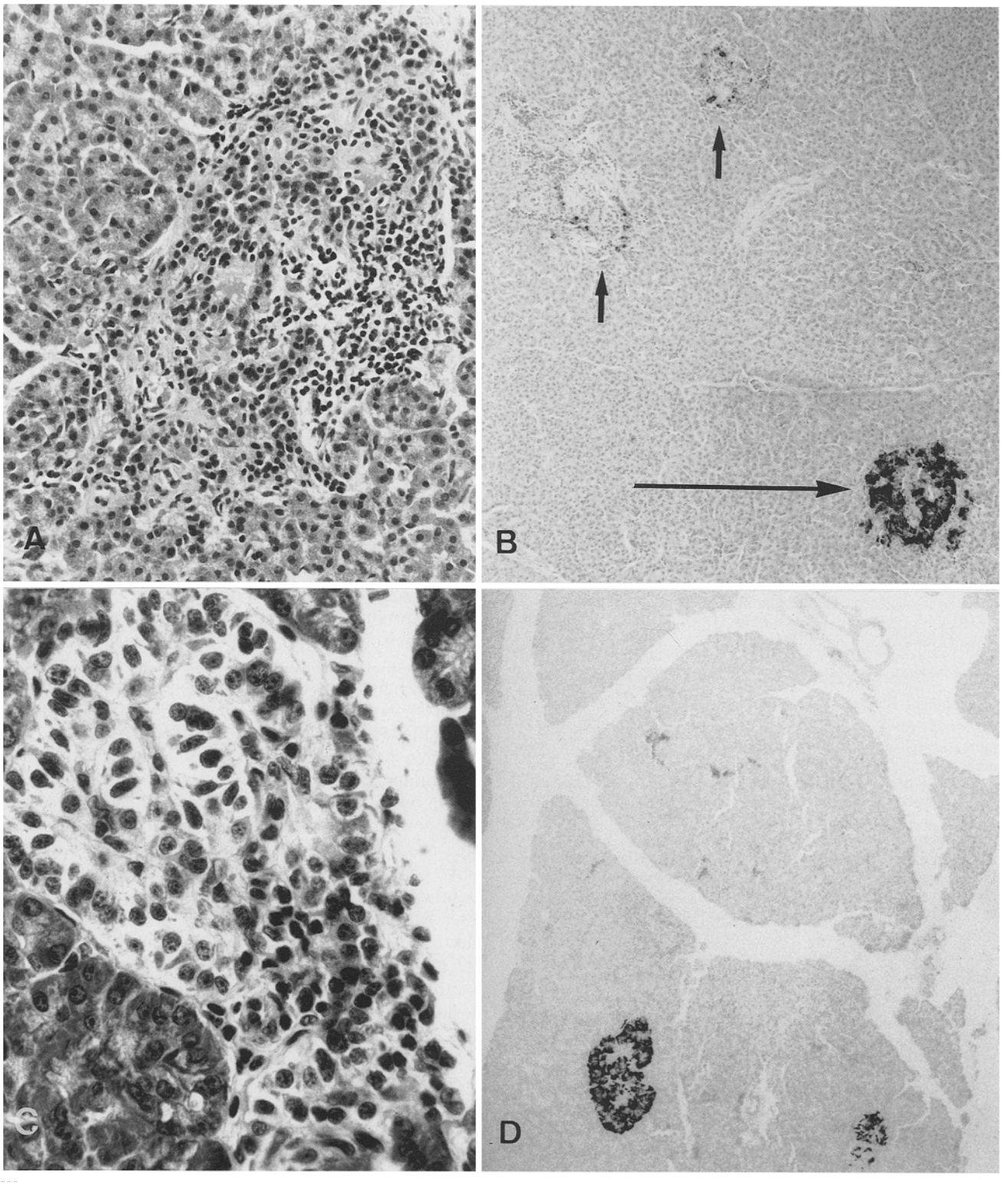

Fig. 1A–D Patient 1. **A** Islet showing severe mononuclear cell infiltration (insulitis). H&E, ×120. **B** Immunostaining for insulin showing a normal-appearing islet with many insulin cells *(long arrow)* and two other islets almost devoid of insulin cells but with insulitis *(short arrows)*. ×60. **C** Patient 2. One of the two islets detected showing minimal (peri)-insulitis. ×250. **D** Immunostaining for insulin reveals the uneven distribution of the few insulin-positive islets in the pancreas. ×40

portion of the islet area is represented by mononuclear cells and a marked expansion of the capillary and pericapillary space (Fig. 1A, C).

Our rabbit peptide antiserum (7647) developed against amino acid positions 570-585 of GAD65 [39] but also representing the C-terminal end of GAD67 [9] stained islet cells in the pancreas of patient 2 (Fig. 3, left column),

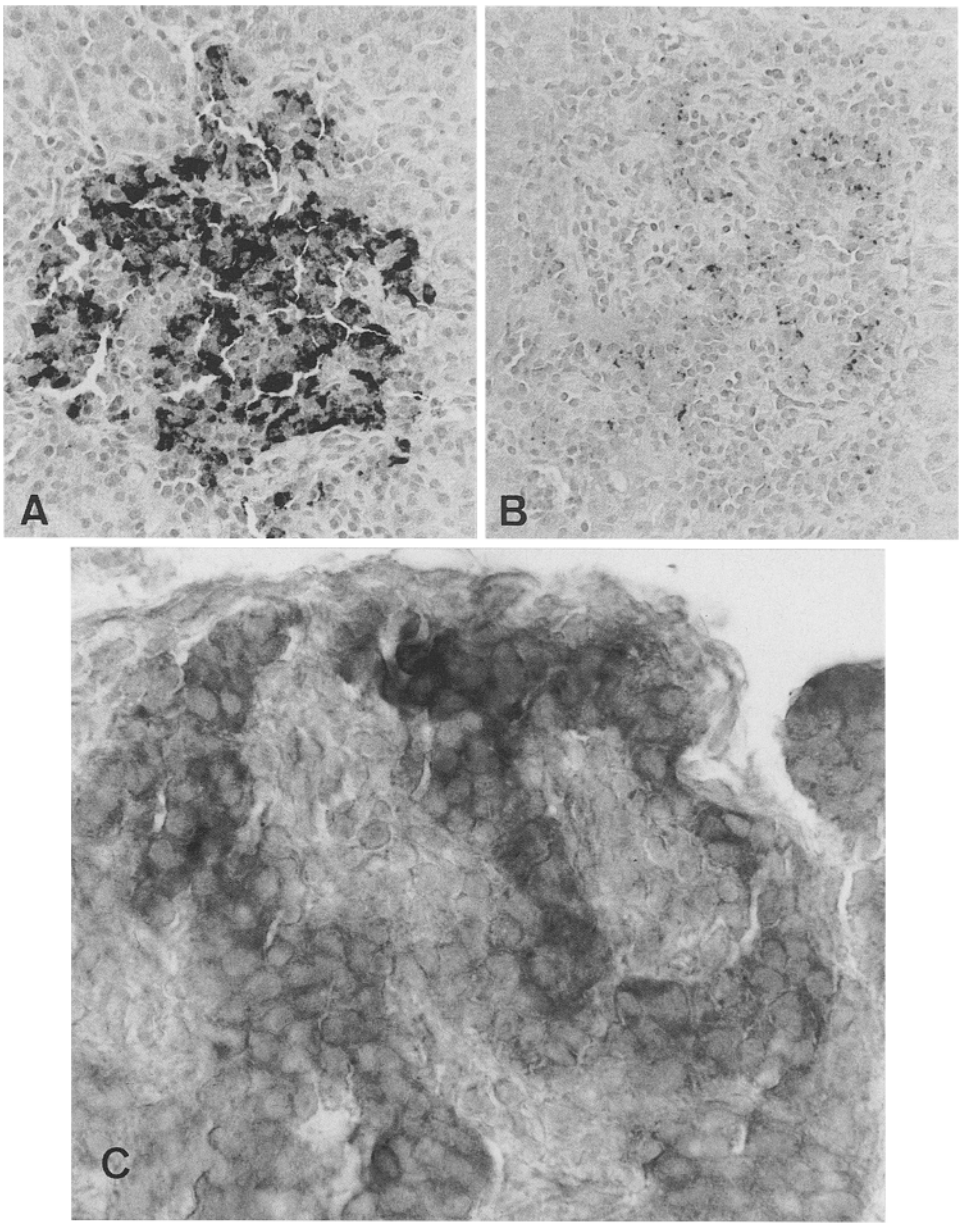

Fig. 2A Islet from patient 1 showing severe insulitis and reduced numbers of beta cells staining for insulin. ×120. **B** The same islet in an adjacent section shows a reduced proinsulin staining. ×120. **C** Another islet from patient 1 reveals a positive hybridization signal against proinsulin mRNA. ×250

as well as in the control children (Fig. 3, right column). When insulin (Fig. 3, panels A and D) and glucagon (panels B and E) immunostaining was compared with that of GAD (panels C and F), it was observed that GAD immunoreactivity is independent of insulin positivity and was also present in glucagon cells. The intensity of insulin immunoreactivity (Fig. 3A) did not correlate with that of GAD immunoreactivity in adjacent sections (Fig. 3C).

In a subsequent analysis we examined whether macrophages contributed to the mononuclear islet infiltrate. Staining with monoclonal antibody HAM-56, which detects macrophages (Table 2), showed the presence of positive cells within the islets of both patients (Fig. 4). The number of HAM-56-positive cells detected per islet varied, but was most dramatic in patient 2. In Fig. 4, immunostaining with HAM-56 is shown in panel A and the corresponding staining with normal mouse serum on an adjacent section in panel B. The morphometric evaluation of HAM-56-immunostained sections showed that patient 1 had 4% and patient 2 had 12% HAM-56-positive cells in the islets. In the control pancreases only single HAM-56 positive cells were found (Table 3).

The presence of macrophages in islets which were either devoid of or contained a significant number of insulin-positive cells suggested that HAM-56-positive cells might be involved in the destructive process. The presence of macrophages in the islets also suggests a marked potential for antigen presentation. Since both children were HLA-DR 3/4-DQ 2/8-positive, we tested thereafter whether these molecules were present on islet cells.

The pancreatic tissue was found to contain scattered HLA-DR-immunoreactive cells (<10%) which appeared

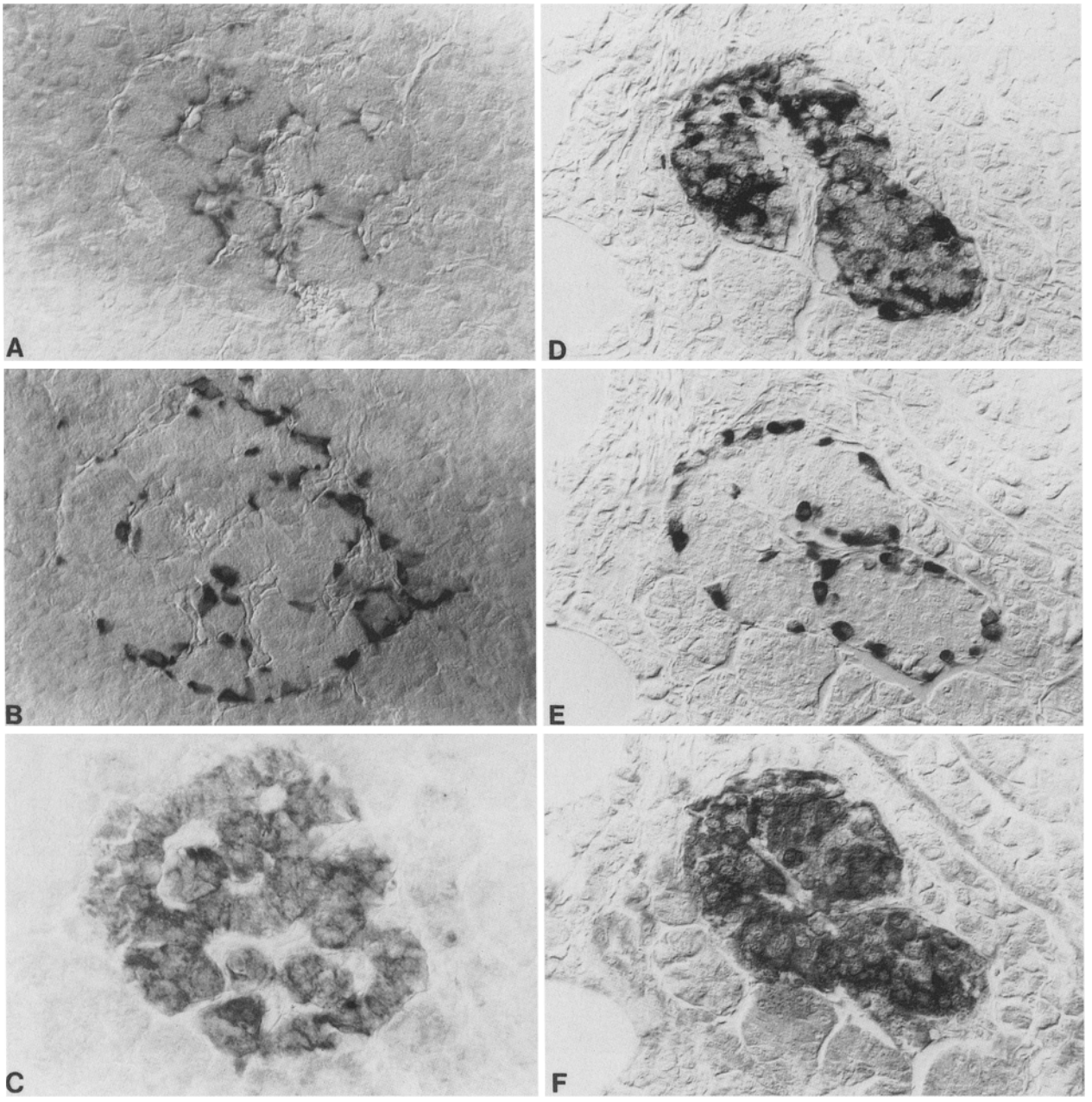

Fig. 3A–F Islets from patient 2 *(left column)* and from a control pancreas *(right column)*. Immunoreactive insulin (**A, D**), glucagon (**B, E**) and glutamic acid decarboxylase (GAD) (**C, F**). GAD immunoreactivity is independent of insulin positivity and appears also to be present in glucagon immunoreactive cells. ×400

to be macrophages or endothelial cells. No apparent endocrine cells were found to express HLA-DR. The factor-VIII-positive endothelial cells of larger vessels failed to express HLA-DR. Staining with a monoclonal antibody specific for HLA-DQ8 [48] (Fig. 5A) showed staining of endothelial cells and macrophages in sections from both children, confirming their tissue typing. The HLA-DQ8 staining was unaffected by an excess of the HLA-DQ7 synthetic peptide (Fig. 5A), but was completely abolished by the immunizing peptide of DQ8 (Fig. 5B). Aberrant expression of HLA-DQ on beta cells was not detected in either child.

Discussion

The two patients were HLA DR 3/4-DQ 2/8 heterozygous, a common HLA type in IDDM, and both died from cerebral oedema following attempts to correct their ketoacidosis and hyperglycaemia. Both patients showed a similarly severe loss of beta cells and reduced proinsulin staining of the few remaining beta cells. In addition, insulitis, the key finding in patients who die at the clinical onset of IDDM (Table 1), was present in both. However, although both patients suffered from the same disease with the same rapid and fatal outcome, patient 1 had

Table 2 Antibodies used for immunocytochemical analysis.

Antigen	Antiserum	Source
Insulin	GP–1	Å. Lernmark
Proinsulin		O. Madsen, Copenhagen, Denmark
Glucagon		Novo Nordisk A/S, Bagsvaerd, Denmark
Macrophage-specific antigen	HAM-56	A. Gown
CD4		Dako, Copenhagen, Denmark
CD8		Dako
Leucocyte antigen	LCA	Dako
HLA-DQ8		J. Petersen
HLA-DR		A. Foulis, Edinburgh, Scotland
IAPP 11–37		O. Madsen
Factor VIII		Amersham, England
Glutamic acid decarboxylase, 570–585	R7647	Å. Lernmark

Table 3 Morphometric analysis of the pancreas in newly diagnosed insulin-dependent diabetic children. Volume density of islet tissue is shown as percentage of pancreatic parenchyma. Islet beta and alpha cells, as well as macrophages, are shown as a percentage of islet tissue

Tissue or cell type	Patients		Controls		
	1	2	1	2	3
Islet tissue	2	2	4	4	3
Beta cells	8	13	53	55	55
Alpha cells	27	28	18	15	20
Islet macrophages	4	1.2	neg.	neg.	neg.

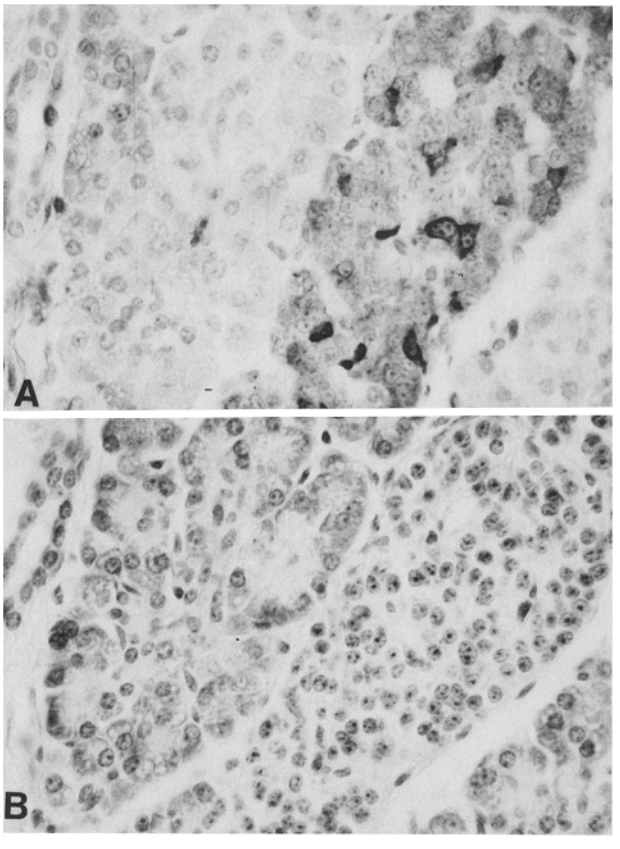

Fig. 4A Islet from patient 2 showing immunocytochemical staining with the macrophage marker HAM-56. **B** The same islet in an adjacent section shows the control staining with normal mouse serum. ×350

prominent insulitis affecting most islets in the tissue specimens analysed, while patient 2 had minimal insulitis detected in only two islets out of about 200 examined. This raises the question whether the extent and quality of the immune reaction against the beta cells differs from one patient to another.

Analysis of the literature reveals that insulitis is rarely found after the age of 17 (Table 1) and is more often reported in infants younger than 5 years (Fig. 6). Moreover, severe insulitis has previously been observed in young children [29, 58, 61]. It is therefore conceivable that very young diabetic infants are particularly prone to severe insulitis because a diabetic manifestation during the first 10-20 months of life necessarily implies a major destruction of beta cells within a relatively short period of time. Our patient 1 would fall into this category. If, however, IDDM appears later in life, insulitis may be mild, because in these patients even a slowly progressing immune reaction has enough time to eradicate sufficient beta cells (approximately 80%, [33]) for manifestation of diabetes. These pancreases, as seen in patient 2, will then reveal predominantly insulin-negative islets and occasional insulitis.

The immune process in the two patients might differ not only in extent but also in quality. Here, several findings are of interest, although none of them uncovered clear differences. The first is that the insulitis infiltrate was composed of both B- and T-lymphocytes, confirming previous observations [8, 28]. The second finding is that immunostaining with the human macrophage-specific antibody HAM-56 revealed an infiltration of macrophages. HAM-56-positive cells were not only found in the islets affected by insulitis but also in those without insulitis, where they were barely detected in H&E-stained slides. The significance of this observation is obvious from our inability to find HAM-56-positive cells in the islets of control children. Our documentation of macrophages in human islets at the time of clinical onset of diabetes confirms a recent report [19] of macrophages in IDDM patients of less than 3 months' duration selected on the basis that insulitis was present. Monocytes/macrophages were also found in an 8-year-old girl with new-onset IDDM [28] along with signs of endothelial cell activation. To date it is not known whether the presence of macrophages may be sufficient to cause beta cell loss but

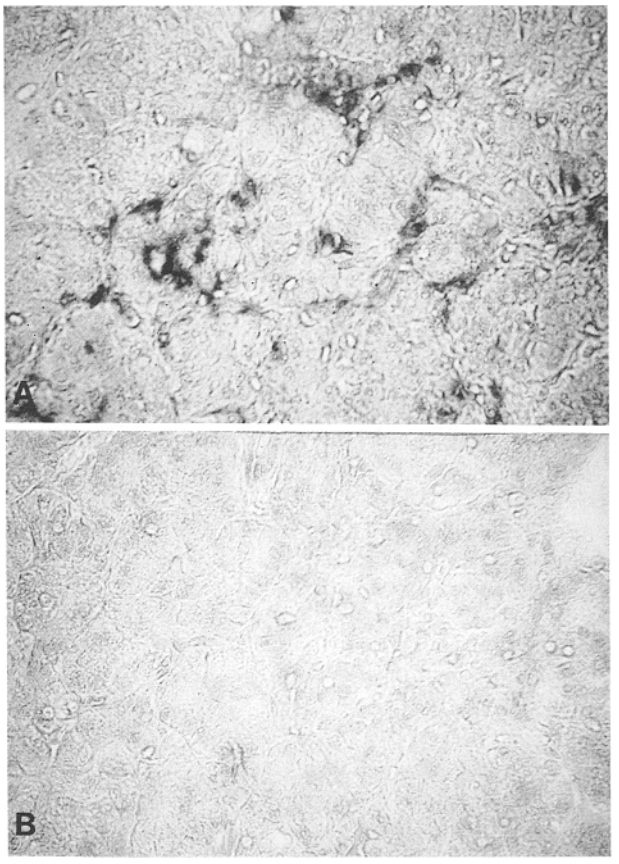

Fig. 5A, B Islet from patient 2 showing HLA-DQ8-positive cells mostly of endothelial origin but possibly also macrophages. Islets in adjacent sections are shown stained with the HLA-DQ8 specific monoclonal antibody incubated with an excess of the HLA-DQ7 sequence specific peptide (**A**) and in an excess of the HLA-DQ8 sequence specific peptide (**B**) which completely blocked the staining reaction. ×350

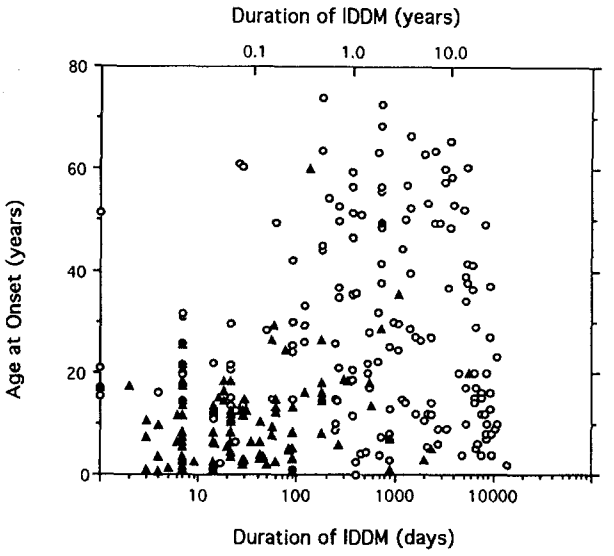

Fig. 6 Relationship of age at onset and diabetes duration to human insulitis. Age in years at onset of insulin-dependent diabetes mellitus (*IDDM*), plotted against duration of IDDM in days (semi-log scale, *bottom*), and years (semi-log scale, *top*) for 302 patients from the literature examined for the presence of insulitis. Patients include 172 that were negative for insulitis (○), 130 that were positive (▲) (see Table 1)

the fact that these cells are beta cell-cytotoxic in vitro [49] could indicate that they may also be involved in beta cell destruction in vivo.

A third finding is that neither of the patients showed aberrant expression of HLA-DR or HLA-DQ class II molecules on beta cells. These data support observations in the spontaneously diabetic BB rat [40, 50] that the MHC class II expression previously described [8] may be explained by macrophages containing beta cell debris. It is of interest in this regard that GAD-immunoreactive cells were present in all islets irrespective of the insulin staining. In man, GAD65 is present predominantly in beta cells but low levels are also detected in alpha cells [32, 47].

Finally, it has been suggested that the loss of beta cells has reached 80% when overt diabetes occurs [33]. In our patients we were not able to determine the absolute beta cell volume, but the small percentage of beta cells detected in the islets supports the assumption that diabetes only manifests after major loss of beta cells. The functional capacity of the remaining beta cells is difficult to assess, but judged by the very low proinsulin staining and the low in situ hybridization signal for proinsulin mRNA, it seems that the biosynthetic activity of these cells is severely impaired. Whether this is due to the general metabolic situation or the action of cytokines such as interleukin-1, tumour necrosis factor, or interferon gamma, alone or in combination, released by the immune cells (see [49] for review], is unclear.

In summary, our observations support the hypothesis that the extent of the immune reaction is usually much greater in IDDM patients who are only several months old than in patients who present later in life. We could not find any major difference in the features of the immune process, but in agreement with evidence from animals studies [50], and biopsies in new-onset IDDM patients [26, 30], our findings in these two patients fail to support the hypothesis [8] that aberrant expression of class II antigens on beta cells is significant with regard to the pathogenesis. Instead they suggest that, similar to autoimmune diabetes in NOD mice [37] and BB rats [27, 38], macrophage infiltration of the islets is also closely associated with the development of IDDM in man. In the animals, bone-marrow-derived macrophages are detected first, followed by CD8- and CD4-positive T-cells as well as B-lymphocytes.

The islet pathology heterogeneity in our two patients emphasizes the need for further pancreatic investigations in children. Our study suggests that it should be possible to investigate quantitatively both archive (Table 1) and new cases with antisera such as those we have used to localize (1) the target cell, (2) its autoantigens insulin and GAD65,(3) immune effector cells including macrophages, and (4) HLA typing for DQ8 by immunocytochemical staining. The latter technique has, to our

knowledge, not been reported previously. The causation of IDDM is still unclear and it remains to be determined by which immune processes the beta cells are destroyed.

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