Pancreatic Pathology in Type 1 (Insulin-dependent) Diabetes Mellitus

By

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DECLARATION

All the studies presented in this thesis were instigated and developed by me. The contributions of others as regards materials and technical assistance have been acknowledged.

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SUMMARY

While the serum and leucocytes of diabetics have been studied exhaustively in attempts to elucidate the pathogenesis of type 1 (insulin-dependent) diabetes, the pancreas, the stage on which the drama of B cell death is played, has been largely ignored.

In an attempt to partially correct this a 25 year computerised survey of deaths in the United Kingdom among diabetics under the age of 30 years was performed. Suitable pancreatic material was available in 155 cases. The disease was known to have been present for less than a year in 74. Insulitis (a chronic inflammatory cell infiltrate affecting islets) was present in 59 of these 74 cases (80%). Insulitis affected 20% of islets containing insulin but only 1% of islets which were insulin deficient. This observation has provided the most substantial direct evidence that insulitis represents an immunologically mediated destruction of B cells. The fact that insulitis could be found up to 6 years after presentation showed that destruction of B cells may take place over a prolonged period of time. Several unusual cases of diabetes were found which highlighted the heterogeneity of the disease. In the most interesting of these there seemed to have been massive simultaneous necrosis of B cells, suggesting an acute viral cytopathic effect.

Two tangential studies were prompted by the observation that acini around insulin containing islets in recent-onset diabetes appeared to be atrophic. In the first of these the anatomy of the blood supply to normal

human islets and islets from a patient with type 1 diabetes was studied. This showed that islets were supplied by arterioles and drained by multiple capillaries. In the second study a new animal model of a "partially diabetic" pancreas was produced. In rabbits, a vascular clamp was placed across the junction of body and tail of pancreas, thus occluding the circulation to the tail. Alloxan (200mg/kg) was injected intravenously and four minutes later dextrose (0.5g/Kg) was given by the same route. After a further 2 minutes the clamp was removed. Those animals which survived the first post operative week had an almost total lack of B cells, but a normal population of A,D and PP cells, in the head and body of the pancreas. The islets in the tail of pancreas appeared entirely normal. This model appears suitable for the investigation of the effects of locally produced insulin on pancreatic exocrine function.

T helper lymphocytes only recognise the antigen to which they are directed if it is 'presented' to them by a cell expressing the same class II major histocompatibility complex (MHC) antigen as themselves. Since insulin secreting B cells do not normally express class II MHC antigens on their surface, any cell specific surface antigens they possess may never have been presented, and therefore immunological tolerance to them may not exist. If B cells were induced to express class II MHC aberrantly this might allow presentation of such cell specific surface antigens to potentially autoreactive T helper lymphocytes and autoimmunity to them may be initiated.

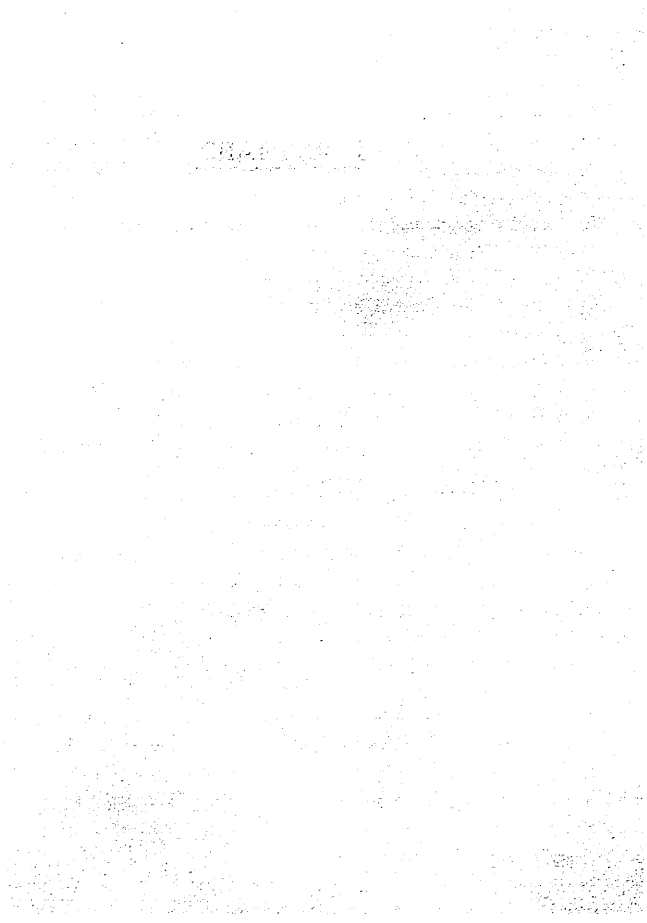
The presence of class II MHC on B cells was studied

initially on 14 diabetic pancreases using the monoclonal antibody TAL-1B5. Two pancreases had no B cells and no class II MHC expression was seen on endocrine cells in these cases. The remaining 12 pancreases had residual B cells and endocrine cells expressing class II MHC were found in all 12 of them. Such cells were found exclusively in insulin containing islets and double stains for hormones and class II MHC confirmed that only the B cells expressed this antigen complex. No such expression was seen in pancreases from patients with type 2 diabetes, cystic fibrosis or chronic pancreatitis. The majority of islets in which B cells were seen to express class II MHC had no evidence of insulitis, suggesting that within an individual islet aberrant expression on B cells may precede the inflammatory infiltrate.

A second study was done to confirm these observations using 2 other antisera directed against class II MHC. In addition, a simultaneous study of class I MHC expression was done. Thirty-five pancreases from patients with type 1 diabetes were studied. Aberrant class II MHC expression, confined to B cells, was seen in 21 out of 23 cases where the disease had been present for less than a year. It was also present up to 9 years after clinical presentation of the disease. Ninety-two per cent of insulin containing islets had striking hyperexpression of class I MHC which affected all the endocrine cells in these islets. All islets in which aberrant class II MHC expression on B cells or insulitis were present exhibited hyperexpression of class I MHC, but this latter phenomenon was the only abnormality seen in the majority of insulin containing

islets. Thus, within an individual islet, class I MHC hyperexpression, affecting all the endocrine cells, appeared to precede aberrant class II MHC expression on B cells and insulitis. These abnormalities of MHC expression in islets were unique to type 1 diabetes, being absent in pancreases from patients with type 2 diabetes, cystic fibrosis, chronic pancreatitis, Coxsackie B viral infections and graft-versus-host disease.

Some possible explanations for these findings in type 1 diabetes are discussed and indications are given as to how they may be investigated in the future.



CHAPTER 1

CHAPTER 1

General Introduction

1. The aetiology and pathogenesis of type 1 (insulin dependent diabetes

Diabetes mellitus is not a disease. It is a syndrome of altered metabolism resulting in, among other things, hyperglycaemia. There are a number of different diseases, of varying aetiology and pathogenesis, which can result in a similar final common pathway. This thesis concerns that group of diseases which have become known as type 1 diabetes. Other names applied have been insulin-dependent diabetes or juvenile diabetes. In contrast to type 2 diabetes (also known as non-insulin-dependent or maturity onset diabetes) type 1 diabetes presents most commonly under the age of 25 years, is liable to be complicated by ketoacidosis as well as hyperglycaemia, and requires insulin therapy for metabolic control. Type 1 diabetes is ten times less common than type 2 diabetes and affects approximately 1 in 400 of the population in the U.K.

Much of the historical literature on diabetes is confounded by a lack of appreciation that more than one disease was involved. It is obviously a matter of opinion as to which studies have contributed most to our understanding of the pathogenesis of type 1 diabetes, but few would argue that the study of Gepts (1965), on the pancreases of 22 young people who had died of recent-onset diabetes, was a landmark of the modern era. Several facts became firmly estabished as a result of this work. Firstly, at presentation, there was a profound reduction in the number of insulin secreting B cells. This had not been

observed by Maclean & Ogilvie (1959) in their study of 18 similar patients. Gepts noted that the residual B cells showed marked hypersecretory activity which would allow the serum insulin level to be maintained at only marginally reduced levels at clinical presentation. The other principal finding was the presence of insulitis in 15 of the 22 cases. By insulitis was meant the presence of a predominantly chronic inflammatory cell infiltrate, specifically affecting the islets of Langerhans. This lesion had been first recognised in diabetes at the beginning of the century and, although it was mentioned in other studies, it was regarded as irrelevant until LeCompte (1958) described it in 4 out of 4 cases of recent-onset diabetes. He suggested that it was more common than had been thought, and Gepts confirmed this 7 years later during his fruitful visit to LeCompte's department in Boston. Both LeCompte and Gepts thought that insulitis could represent damage to B cells. They suggested that it may be the result of a viral infection, or that it could represent an immunological abnormality. To this day these remain the principal pathological processes presumed to be involved in type 1 diabetes.

Evidence for autoimmunity in type 1 diabetes

The finding that patients with type 1 diabetes had evidence of cell mediated immunity to a crude islet antigen, as demonstrated in a leucocyte migration inhibition assay (Nerup et al., 1971), prompted a search for serological evidence of autoimmunity in the disease. Initially, using indirect immunofluorescence on fresh

frozen human pancreas, islet cell cytoplasmic autoantibodies were described in patients with type 1 diabetes who also had evidence of other autoimmune diseases. (Bottazzo, Florin-Christensen & Doniach, 1974; MacCuish et al., 1974). This was followed by the finding of such antibodies in 51% of newly diagnosed type 1 diabetics who did not have other autoimmune diseases (Lendrum, Walker & Gamble, 1975). These antibodies, which reacted with glucagon and somatostatin secreting cells as well as B cells, were of IgG class, and a proportion fixed complement. In later series, islet cell cytoplasmic antibodies were found in up to 85% of recent-onset type 1 diabetics (Lendrum et al., 1976). More recently, islet cell surface antibodies have been described, and some of these were shown to lyse B cells in preference to other pancreatic endocrine cells in the presence of complement (Dobersen & Scharff, 1982). Finally, antibodies to insulin have been found, not only in newly diagnosed type 1 diabetics, but also in prediabetics (Srikanta et al., 1986).

While the finding of islet cell cytoplasmic antibodies up to 3 years before clinical presentation of type 1 diabetes provided evidence that the disease may have a long prediabetic period (Gorsuch et al., 1981), most of the autoantibodies disappear from the serum within a few years of the onset of type 1 diabetes. This may coincide with the loss of residual B cells from the pancreas (Mustonen et al., 1984).

It is generally accepted that whilst islet cell antibodies may reflect tissue damage, the destruction of B cells is primarily due to cytotoxic T lymphocytes in the

islet inflammatory infiltrate (Bottazzo et al., 1985). This was dramatically demonstrated in 4 diabetics, who had had diabetes for at least 17 years, and who were given pancreatic organ grafts from their normal identical twins. No significant immunosuppression was given and while the grafts initially functioned satisfactorily, all the patients became rapidly diabetic again. Insulitis was present on biopsy at this time, but islet cell antibodies only reappeared in one patient (Sibley et al., 1985).

Evidence for a viral aetiology in type 1 diabetes

In a frequently quoted case report of an autopsy on a 10 year old boy who died at presentation of type 1 diabetes, a Coxsackie B4 virus was cultured from the pancreas. (Yoon et al., 1979). The isolated virus was injected intraperitoneally into mice and 50% of the animals became diabetic, with evidence of insulitis. A similar virus was demonstrated in the pancreas of a 5 year old child who died of type 1 diabetes and myocarditis 3 weeks after cardiac surgery (Gladisch, Hofmann & Waldherr, 1976). Several pieces of evidence suggest that these cases may be exceptional. Virus was also demonstrated in the brain of the child reported by Yoon et al and myocarditis is an unusual finding at autopsy in type 1 diabetes. Gepts has had an opportunity to review the pancreatic pathology in these two cases and he considered them atypical (Gepts & LeCompte 1985). He found more B cells than is usual with a greater prominence of insulitis. Also, islets lacking in B cells, although present, were less conspicuous than in "classical" cases. Be that as it may, these cases prompted

serological surveys of newly diagnosed type 1 diabetics and there have been confirmed reports of the presence of Coxsackie-B-virus specific IgM responses, indicating recent or continuing infection, in approximately 30% of cases, compared to 5% in controls (King et al. 1983; Banatvala et al. 1985).

A second line of evidence suggests that rubella virus may rarely be implicated in the aetiology of type 1 diabetes. In a study of 44 patients with congenital rubella, diabetes or latent diabetes, was found in 9 (20%) (Forrest, Menser & Burgess, 1971). While the high incidence of diabetes in congenital rubella has been confirmed, there is little to suggest that post-natal rubella infection contributes to the aetiology of type 1 diabetes (Banatvala et al. 1985).

The third virus which has been implicated is mumps virus. It is well known that this virus can cause acute pancreatitis and severe mumps infection has been associated with the transient appearance of islet cell cytoplasmic antibodies (Mueller-Eckhardt et al. 1984). However, there is no more than circumstantial evidence to link this virus with type 1 diabetes, and Banatvala et al (1985) found no evidence of persistent mumps infection in their study of 122 newly diagnosed diabetic children.

Hereditary Influences

It has long been recognised that type 1 diabetes has a hereditary component to its aetiology. Initially, this was thought to be linked to the major histocompatibility complex (MHC) class I genes. Later, it was shown, that in

common with many other organ specific autoimmune diseases, the link was primarily with class II MHC genes (HLA-DR in man). Ninety-eight per cent of type 1 diabetics in one UK study possessed either DR3 (relative risk = 5), or DR4 (relative risk = 6.8) or both antigens (relative risk = 14.3). Half the patients studied were DR3, DR4 heterozygotes (Wolf, Spencer & Cudworth, 1983). By contrast DR2 had a protective role (relative risk 0.1). Any proposed pathogenesis for type 1 diabetes should attempt to explain this striking association. This subject will be covered in more detail in chapter 4 of the thesis.

2. Normal micro-anatomy of the endocrine pancreas

The pancreas develops from two separate buds of the primitive foregut. The dorsal bud eventually forms 90% of the volume of the pancreas and constitutes the tail, body and anterior part of the pancreatic head. The ventral bud forms the posterior part of the pancreatic head.

Four endocrine cell types are found in the pancreas: the insulin secreting B cell, glucagon secreting A cell, somatostatin secreting D cell and pancreatic polypeptide secreting PP cell. Malaisse-Lagae et al (1979) were the first to recognise that PP cells were not distributed evenly within the pancreas. They showed that the vast majority of these cells were found in the posterior part of the pancreatic head, in an area that corresponded to that part of the pancreas derived from the ventral bud. This area has become known as the PP rich lobe, while the remainder of the gland is known as the glucagon rich lobe. In the glucagon rich lobe of an adult 82% of endocrine cells are B cells, 13% A cells, 4% D cells and 1% PP cells. By contrast, in the PP rich lobe, 18% are B cells, less than 1% A cells, 1% D cells and 80% are PP cells (Stefan et al. 1982). Islets in the glucagon rich lobe are generally well defined, and spherical. The A cells line the vascular sinusoids and the B cells lie in small groups separated from the sinusoids by the other endocrine cells (Fig.1.1). By contrast, islets in the PP lobe are much more numerous relative to the exocrine tissue, are ill-defined and appear to merge into the surrounding exocrine tissue. They are not spherical but are totally irregular in outline (Fig.1.2).

Figure 1.1.

Normal islets from glucagon rich lobe of an adult pancreas

Distribution of B cells. Note that these lie in cords and that there is a rim of negatively staining endocrine cells at the periphery of the cords.

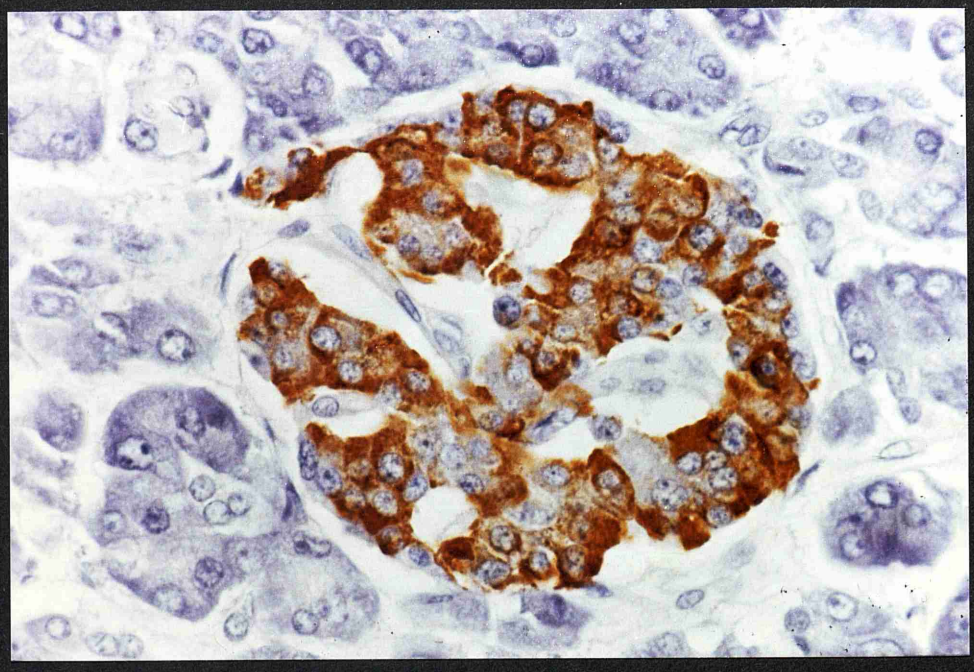
Indirect immunoperoxidase (IP) for insulin x 500.

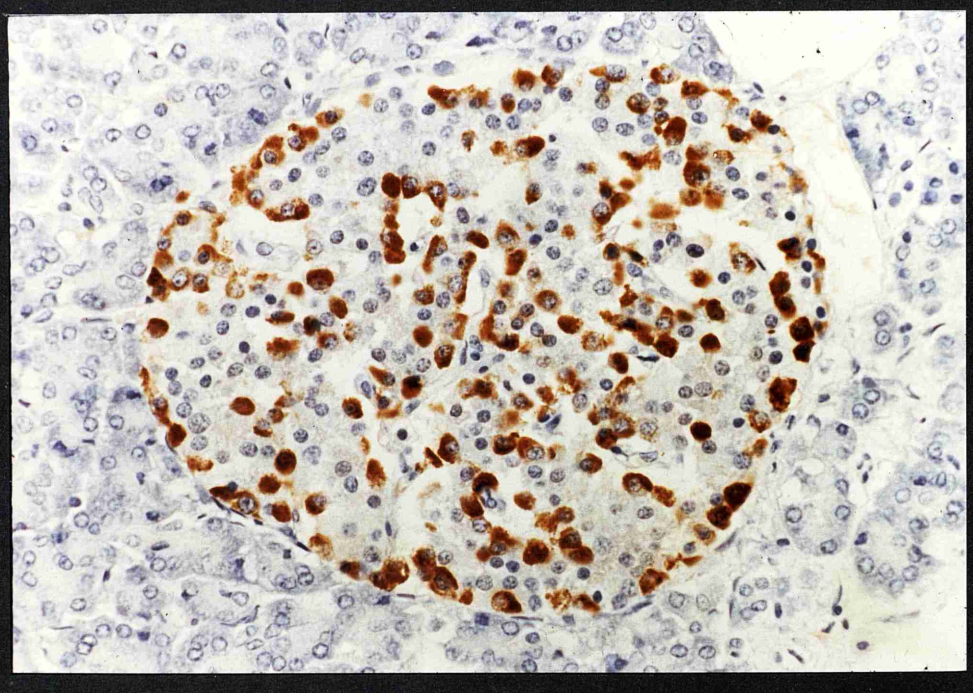
Distribution of A cells. Note that these line the vascular sinusoids and generally lie to the periphery of the cords of B cells.

IP for glucagon x 300.

Distribution of D cells. These are more randomly dispersed. Many are elongated.

IP for somatostatin x 325.





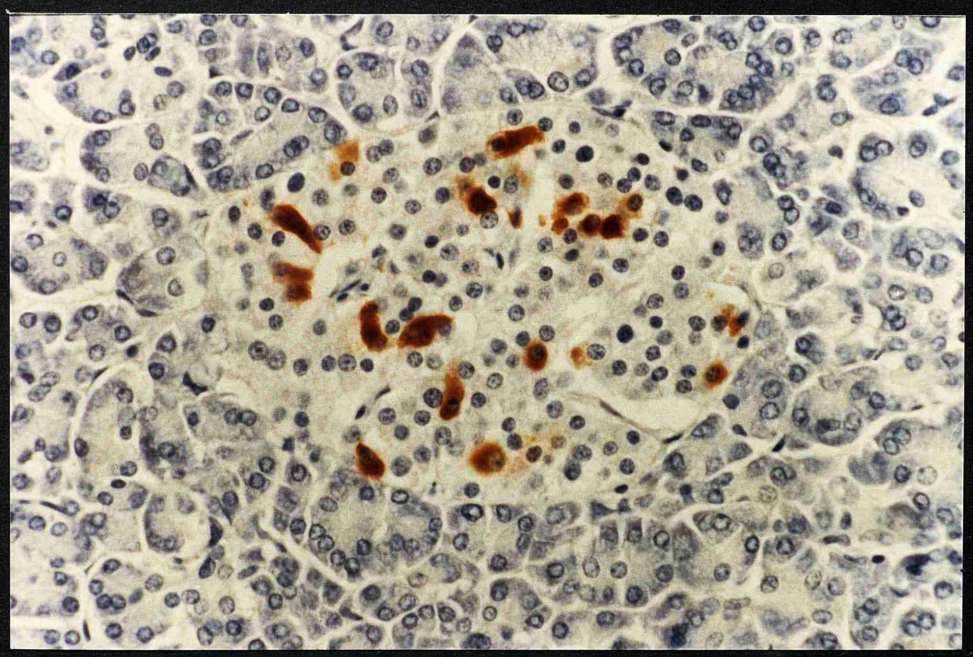


Figure 1.2

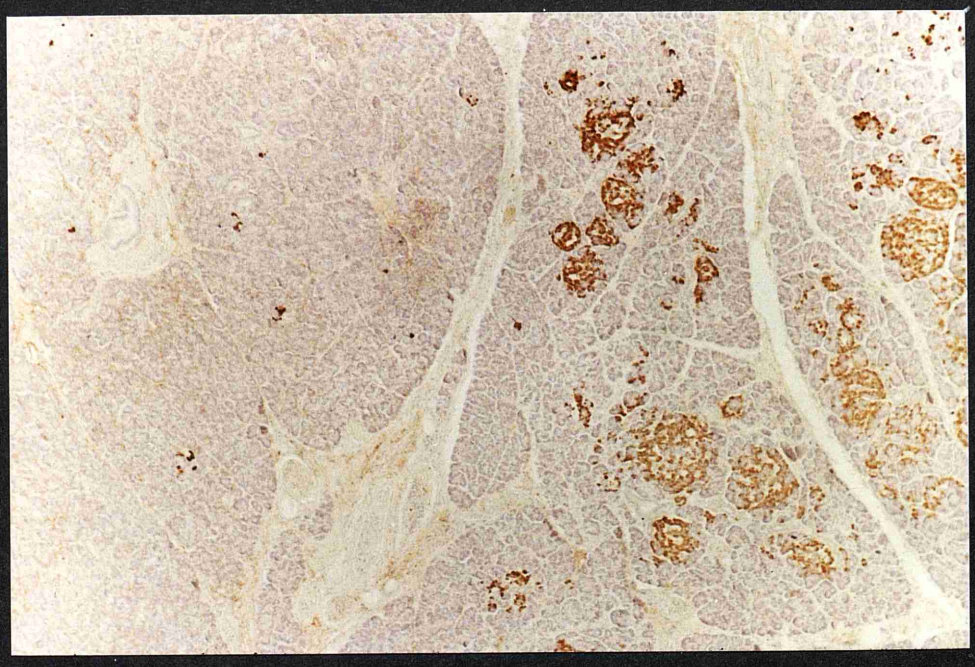
Normal islets from PP rich lobe

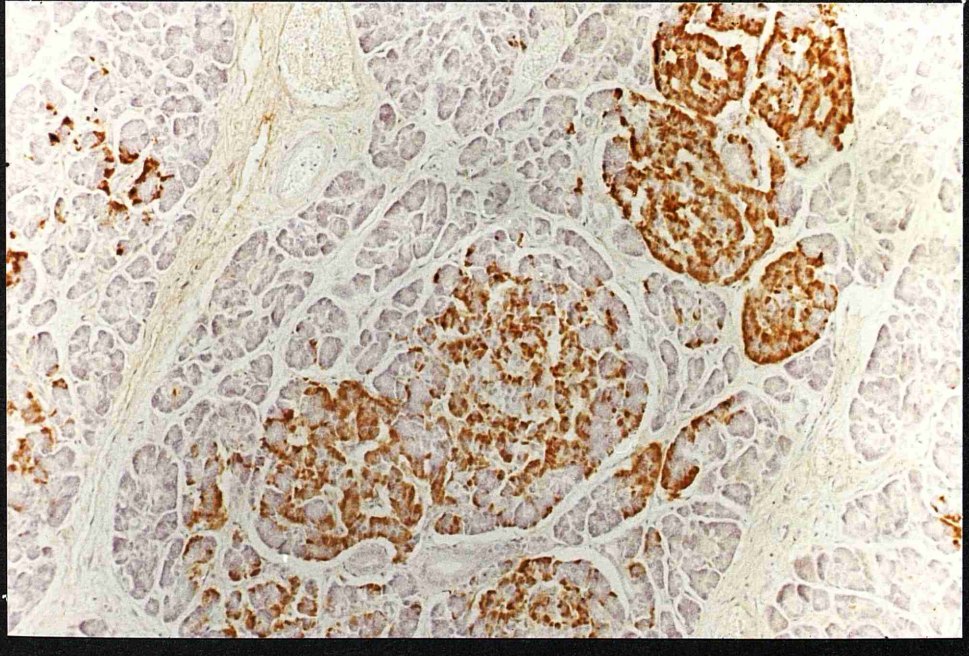
The junction between the glucagon rich lobe (left) and PP rich lobe (right) is shown. Note the density of islet tissue with respect to the exocrine tissue in the PP rich lobe.

IP for PP x 40

Islets in the PP rich lobe are not well demarcated from the exocrine tissue and are irregular in shape.

IP for PP x 110.





In neither lobe are the endocrine cells solely confined to recognisable islets. Thus they can be grouped in very small numbers or they may occur singly within acinar tissue or ducts.

Islet blood supply

INTRODUCTION

Henderson, in a Lancet hypothesis, posed the question, "Why are the islets of Langerhans?" (Henderson 1969). This article served to highlight the anatomy of the islet blood supply. It has been established that while islets are generally supplied by an arteriole they are not drained by veins. Instead the islets are drained by capillaries which ramify in the exocrine tissue around the islet (Henderson & Daniel 1979). While this arrangement holds for all laboratory animals studied, there are variations. Thus there are relatively few endocrine-exocrine connections in mice, while in the cat they are extremely well developed (Henderson & Daniel 1979). In the rabbit, the animal in which most of this work has been done, approximately 80% of the exocrine tissue receives its blood supply directly from the systemic circulation, while 20% is supplied by blood which has passed through islets (Lifson et al. 1980). This is reflected in the appearance of acini around islets. These 'peri-insular' acini tend to be larger and have more zymogen granules than 'teleinsular' acini (Fig.1.3). This is called the "halo" effect.

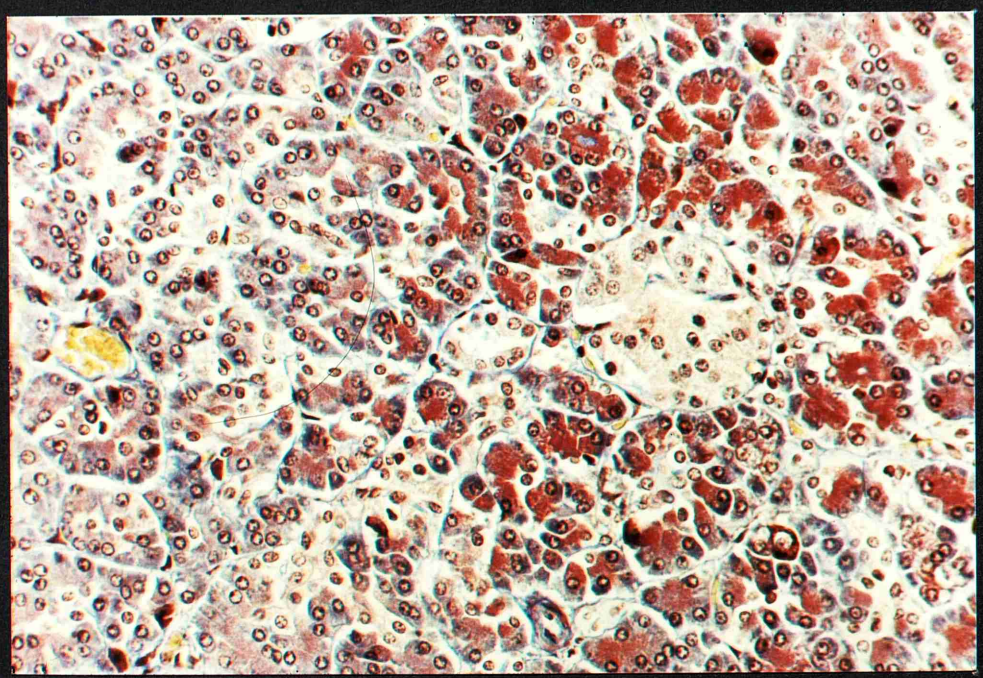
There have been only two reports on the blood supply of the islets in man but neither of these studies provided any photographic evidence for the description given (Vandamme, Van der Schueren & Bonte, 1968; Sapin & Vdovin

Figure 1.3

"Halo" around islet

The acinar cells adjacent to this islet are larger and contain more zymogen granules than acini which are more distant from the islet.

Martius Scarlet Blue x 270



1981). The clearer description is given by Vandamme et al who showed that islets were usually supplied by an afferent arteriole, which often gave a branch to the exocrine tissue before entering the islet. The arteriole divided into intra-islet sinusoids, which might anastomose with one another. The efferent vessels were very thin and left from all sides of the islet, anastomosing with the capillary bed of the surrounding acinar tissue.

Since part of the work to be described in chapter 2 depended on an accurate knowledge of the blood supply of normal islets and islets from patients with type 1 diabetes, it was decided that an attempt should be made to repeat this work.

MATERIALS AND METHODS

Over 20 pancreases were studied, but the early attempts were marred by faulty technique or excessive autolysis. However the procedure was technically successful on 4 normal pancreases and one pancreas from a 56 year old patient who had had type 1 diabetes for 30 years.

On a routine autopsy, performed less than 12 hours after death, the body was eviscerated keeping all the thoracic and abdominal organs intact. The aorta was opened and cannulae placed in the coeliac axis and superior mesenteric artery. Heavy clamps were placed across the splenic and hepatic hila to prevent circulation through the spleen and liver. Up to one hundred and twenty mls of 10% india ink in saline was injected at hand pressure into each vessel. The volume injected depended on the size of the

organs and the number of leaks encountered. Naturally, as well as injecting the pancreatic vessels, this procedure filled vessels in the entire gastrointestinal tract. The pancreas was excised and placed in buffered formalin. It was cut into 5mm thick blocks to allow adequate fixation. After this the blocks were trimmed and processed as usual to paraffin. Forty 5μ serial sections, stained by haematoxylin and eosin, were cut from representative blocks. This allowed whole islets to be studied and the nature of the vessels entering and leaving the islets determined. Sections were also cut at 25μ and 50μ. Those from the normal pancreases were stained by an indirect immunoperoxidase technique (details in chapter 2) for insulin. Sections from the diabetic pancreas were stained, using a similar technique, for glucagon.

RESULTS AND DISCUSSION

Study of the serial sections demonstrated that islets were supplied by small arterioles. No veins were ever seen leaving an islet. The thick sections allowed the supplying vessel and the small endocrine-exocrine connecting vessels to be visualised simultaneously (Fig.1.4). All the islets in the diabetic patient were insulin deficient but the circulation appeared to be the same as that found in the normal pancreases (Fig.1.5). Thus the endocrine-exocrine connections were still preserved. The importance of this hitherto unreported observation on the diabetic pancreas will become apparent when the findings in the exocrine pancreas of patients with recent-onset type 1 diabetes are described (chapter 2).

No attempt was made to assess the proportion of exocrine tissue supplied directly by arterioles, as opposed to the indirect route where arterioles first supply islets, capillaries from which then drain among the surrounding acini. The study on rabbits in which this was calculated used a technique involving the injection of graded microspheres which impacted either in arterioles or capillaries (Lifson et al. 1980). Because of the volume being used in the human study it would be extremely expensive to try to repeat this in man (about £250 per case).

Figure 1.4

Normal islet blood supply

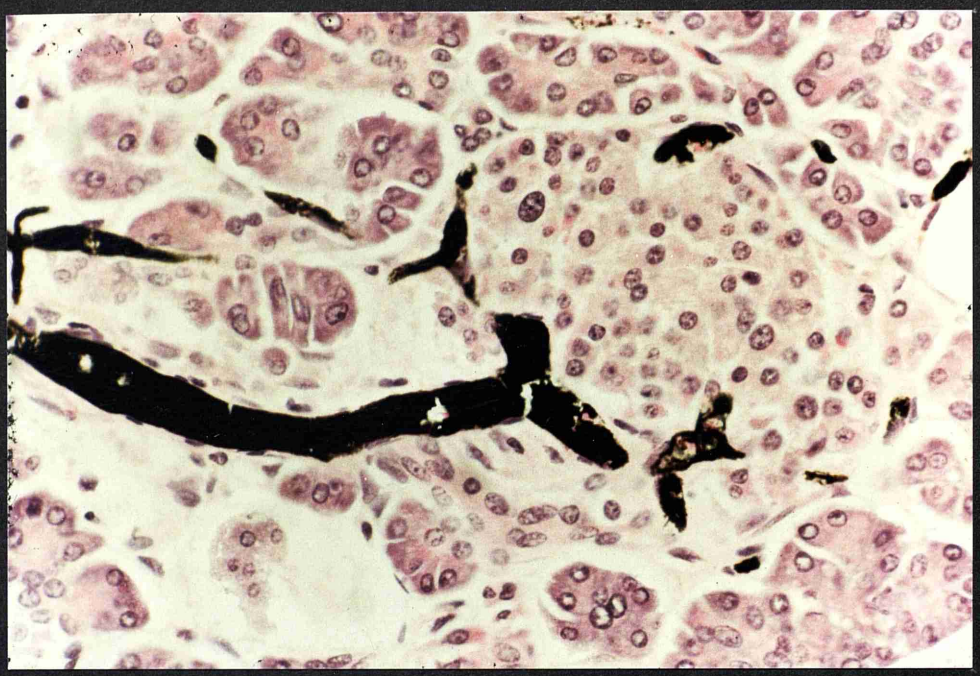
The vascular system has been injected with India ink.

This is a 5μ section which shows the supplying arteriole and demonstrates 3 endocrine-exocrine capillary connections.

Haematoxylin & Eosin (H & E) x 340.

This is a 50μ thick section. The B cells have been stained to show the position of the islet. Both the supplying arteriole and multiple endocrine-exocrine connecting capillaries can be clearly seen.

I.P. for insulin x 130.



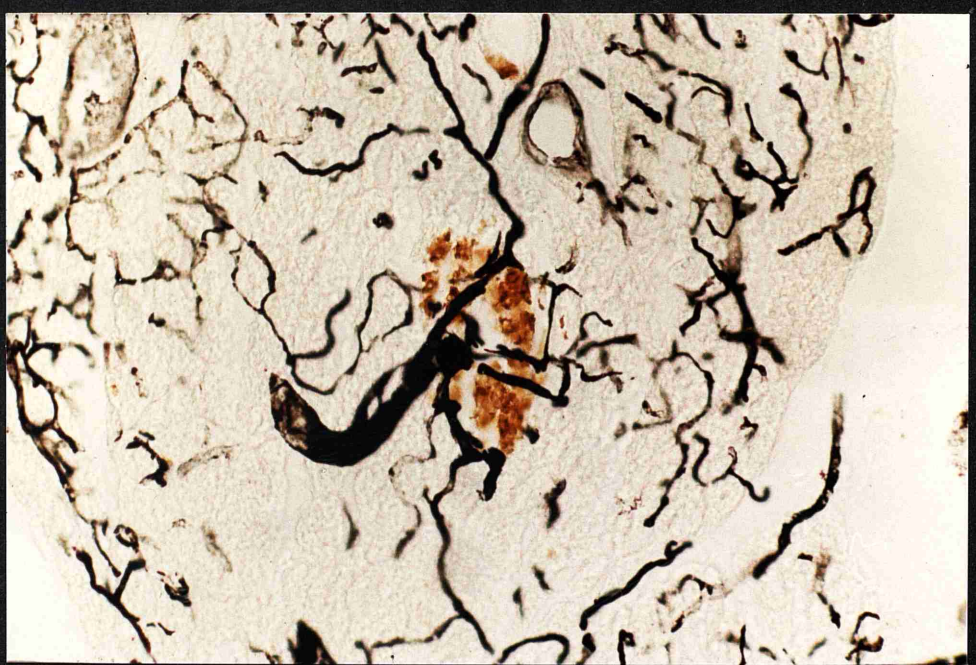


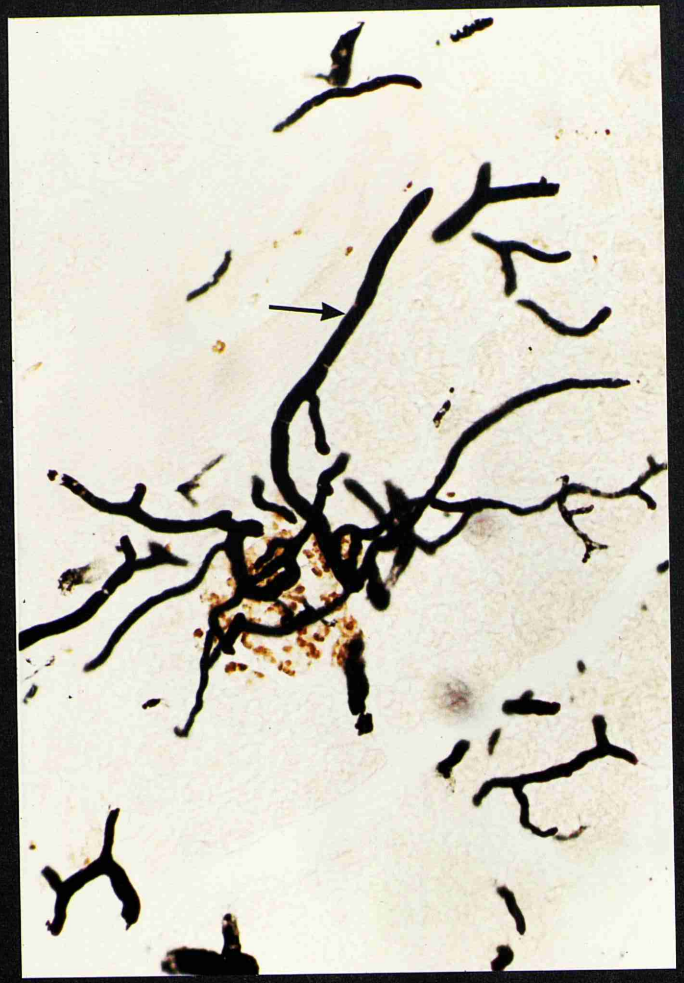
Figure 1.5

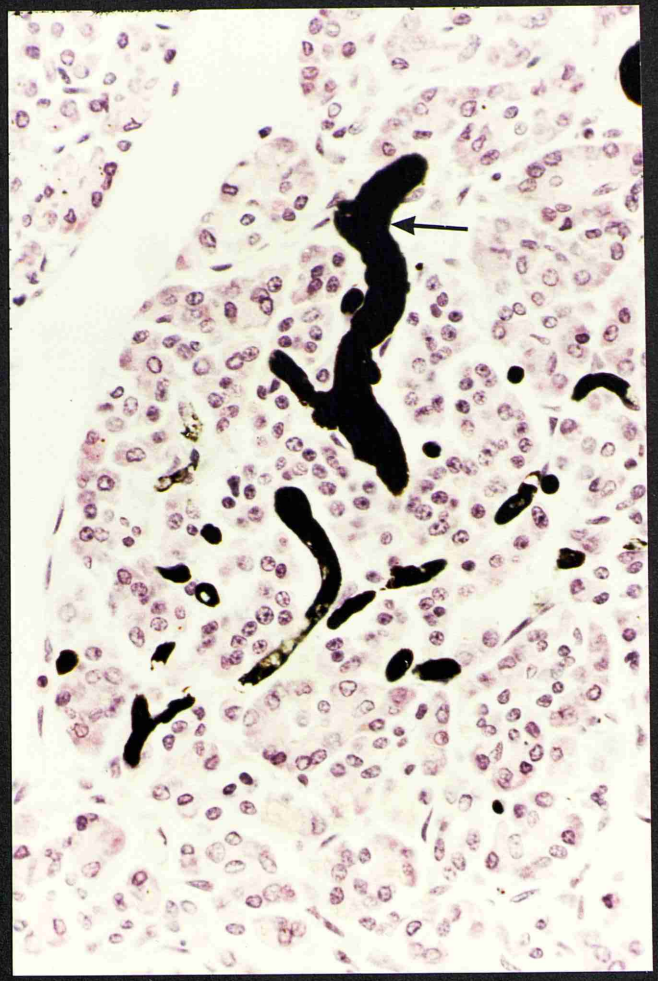
Islet blood supply in long-standing type 1 diabetes
5μ section to show the supplying arteriole (arrowed).

H & E x 340.

50μ thick section which shows preservation of endocrine exocrine connections. The supplying arteriole is arrowed.

I.P. for glucagon x 140.





CHAPTER 2

CHAPTER 2

The histopathology of the pancreas in type 1 diabetes mellitus.

INTRODUCTION

Gepts (1965) showed that insulitis was present in the pancreases of 15 out of 22 diabetics who had had the disease for less than six months. This lesion is thought to be pathognomonic of type 1 diabetes. Since Gepts did not find insulitis in patients who had had the disease for more than 6 months, any attempt to gather pancreatic material to study this process would have to concentrate on autopsies done on patients who had had the disease for a short time.

For a disease as common as type 1 diabetes there are remarkably few pathological reports of the pancreas in patients with "recent-onset" disease (duration less than 1 year). In the last 30 years, 4 series have been published in which significant numbers of patients were included. (MacLean & Ogilvie 1959, Gepts 1965, Doniach & Morgan 1973, Junker et al. 1977). The total number of patients with recent-onset disease in these studies is 64. The report of MacLean & Ogilvie (1959) is now largely irrelevant, since no attempt was made to study the endocrine cell composition of the islets, even with the techniques available at that time. This study concentrated on the size of the islets, almost to the exclusion of everything else. The study of Doniach & Morgan (1973) has been a thorn in the flesh of many reviewers. It has served to throw confusion over the prevalence of insulitis since this lesion was not observed in any of the 13 untreated diabetics these authors studied. Thus the report of of Junker et al. (1977) has

had to serve as the sole confirmation of Gepts' observations.

It has been widely assumed that insulitis represents an inflammatory infiltrate resulting in B cell destruction. If this were the case it would be expected to affect islets that contained residual B cells more commonly than islets which were devoid of B cells. Surprisingly, prior to the present studies, there was remarkably little evidence to support this contention. Thus in the study of Gepts & De Mey (1978), which was the only published study of recent-onset diabetes in which the endocrine cell content of the islets was assessed accurately by immunohisto-chemistry, insulitis was found to exclusively affect insulin containing islets in one case only. This finding could not be substantiated in the other 10 cases in which insulitis was present. Thus both the frequency of insulitis and its role in B cell destruction required proper study.

Another motivation for attempting to collect pancreases from patients who had died of type 1 diabetes was to attempt to show the presence or absence of any of the viruses which have been implicated in the aetiology of the disease. Given the fact that more than one virus could be involved, and that the most sanguine estimate for the proportion of cases with a viral aetiology is 30% (Banatvala et al, 1985), a large number of cases would have to be be collected to avoid missing any due to less common viruses.

The finding of pancreatic material from 9 patients with recent-onset diabetes who had died at the Royal

Hospital for Sick Children in Glasgow over a 30 year period (Foulis & Stewart 1984) suggested that such deaths were not as rare as had been supposed, and that a large autopsy series could be compiled. Junker et al. (1977), attempted to establish such a series by reviewing all death certificates from diabetics in Denmark over a 30 year period. It was decided to do a similar study in the United Kingdom where death certificates have been computerised for 25 years. It has been firmly established that the Scottish records have never previously been used to collect pathological material on any disease. No definite information is available on this matter for the records from England and Wales.

SUBJECTS & METHODS

The Scottish Hospital Inpatient Statistics have been computerised since 1961. These records, held by the Scottish Health Service Common Service Agency code, among other things, all hospital deaths and causes of death and indicate whether an autopsy was performed. A listing was provided for the years 1961-1983 of all deaths from diabetes mellitus in patients under 30 years of age. Death certificates in England and Wales have been computerised by the Office of Population Censuses and Surveys, London, since 1959. Copies of death certificates recorded between 1959 and 1983 were provided of diabetic patients who had been autopsied and who were under 30 years of age. A separate manual survey of the autopsy records of the Royal Hospital for Sick Children in Glasgow over a 60 year period was also done. The pathology departments of the hospitals

where these patients died were contacted. Where possible blocks of pancreas and copies of autopsy reports and case records were provided. In the light of the experience of the Scottish survey, in which few recent-onset cases were found in patients over the age of 20 years, only selected cases were sought from among the English and Welsh patients over the age of 23 years. Cases were chosen if the death certificate gave the impression that the disease was of short duration. For example entries such as "acute diabetes" or "diabetic pre-coma", were selected.

Cases in which there was advanced pancreatic autolysis were rejected. Suitable blocks of pancreas were available from 15 patients from the Royal Hospital for Sick Children in Glasgow, 35 out of 100 cases identified from the Scottish computer survey and 101 out 514 deaths from the survey done in England and Wales. In addition to these, two 1986 cases were referred by Scottish pathologists and two cases were identified from the autopsy records of the Royal Infirmary, Glasgow prior to 1961. The total number of cases was thus 155.

Subjects

The patients comprised 60 males and 95 females with an age range of 3 months to 77 years (there were only two patients over the age of 30 years). Case notes and autopsy records were not available in every case. Thus the duration of diabetes was known in 128; of these it had been present for less than a year in 74. The duration of diabetes in the remainder ranged from 1-19 years. Several patients were known clinically to have other diseases.

Three suffered from Down's syndrome, four had thyrotoxicosis, two had Addison's disease, two had muscular dystrophy, one had schizophrenia, one had pernicious anaemia, one had cerebral palsy, one was epileptic, one had chronic calcifying pancreatitis, one had a corrected tetralogy of Fallot and one had systemic lupus erythematosus.

The cause of death in patients with recent-onset diabetes was given as complications of ketoacidosis in all cases. Among the patients with prolonged duration diabetes (diabetes present for more than one year) the most common cause of death was still ketoacidosis. Other causes included lobar pneumonia (8 cases) hypoglycaemia (6 cases), tuberculosis (2 cases), chronic renal failure secondary to diabetes (2 cases), influenza pneumonitis, tetanus, renal abscess, road traffic accident and chronic renal failure secondary to systemic lupus erythematosus (1 case each).

Methods

Five micron serial sections were cut from all the formalinfixed paraffin embedded blocks of pancreas. Section one was stained by haematoxylin and eosin. Sections two to six were stained by indirect immuno-peroxidase techniques(Burns 1978) using the following primary antisera: guineapig anti-insulin (Wellcome, Dartford, England) rabbit anti-glucagon (Guildhay, Guildford, England) rabbit anti-somatostatin (RIA UK Ltd, Tyne & Wear, England), rabbit anti-pancreatic polypeptide (Metachem Diagnostics Ltd., Northampton, England), mouse monoclonal antibody PD7/26, which is directed against the

T200 leucocyte common antigen, present on all leucocytes (Warnke et al. 1983) (gift from Dr. D.Y. Mason, Oxford). This last antibody wasused as a sensitive marker for the presence of insulitis. The following bridges for the indirect techniques were used: peroxidase-conjugated rabbit anti-guinea pig, swine anti-rabbit and rabbit anti-mouse immunoglobulins (Dako, High Wycombe, England). The reactions were developed using diaminobenzidine as substrate.

Control pancreases and spleens from children who had died over a 50 year period of diseases unrelated to either organ were studied. There was no evidence that prolonged storage of paraffin embedded blocks of tissue altered the ability to stain pancreatic hormones or the T 200 leucocyte common antigen immunohistochemically.

Detailed morphometric studies of the relative numbers of islet endocrine cells were not done.

Statistical analysis

The proportion of insulin containing islets affected by insulitis in the 62 patients with known duration of diabetes and insulitis was compared to the proportion of insulin deficient islets thus affected. Statistical significance was assessed using the Chi-squared test.

RESULTS

Islet Pathology in recent-onset Type 1 diabetes

In most cases there appeared to be essentially three populations of islet. Firstly, insulin containing islets unaffected by any destructive process or inflammation; secondly, insulin containing islets in which there was an

inflammatory cell infiltrate (insulitis) and thirdly, insulin deficient islets. The proportions of these different islets varied from case to case as can be seen from Table 2.1, where the findings in 62 patients in whom the duration of disease was known and where insulitis was present are given.

An islet was defined as insulin containing after finding positive staining of endocrine cells with the anti-insulin antibody on one section. Islets were defined as free of insulitis when no lymphoid cells were seen on the six serial sections, paying particular attention to the section stained for T200 common leucocyte antigen. This proved useful in discriminating doubtful cases. Insulin containing islets unaffected by insulitis usually appeared normal. However, some had a reduced proportion of B cells and in many pancreases some were distinctly hyperplastic and contained polypoid B cells (Fig. 2.1). Insulitis particularly affected islets containing insulin (Table 2.1) and there appeared to be a spectrum of histological appearances suggesting progressive destruction of the B cells within the islet. In early insulitis (Fig. 2.2) there characteristically was infiltration by small lymphocytes at the periphery of an insulin containing islet. The number of B cells at this stage often appeared relatively normal. In the next stage there was a more diffuse inflammatory cell infiltrate within the islet, accompanied by a marked drop in the number of B cells (Fig.2.3). Occasionally islets in which no insulin containing cells could be detected were also affected (Fig. 2.4). This probably represented the end stage of the

destructive phase. The majority of inflammatory cells appeared to be small lymphocytes. Occasionally polymorphs were present but plasma cells were not seen.

An islet was defined as insulin deficient if no endocrine cells were stained with the anti-insulin antibody on one section. All the endocrine cells in these islets appeared to stain if anti-glucagon, anti-somatostatin and anti-pancreatic polypeptide sera were applied simultaneously (Fig. 2.5), indicating that there was not a population of cells containing a fourth hormone, and that totally degranulated B cells were not present. Insulin deficient islets formed the majority of islets in most recent-onset cases. While these islets were easily recognisable as being abnormal, the sinusoids of islets in the glucagon rich lobe still appeared to be lined by A cells (Fig 2.6) and the vascular connections to the exocrine tissue appeared undisturbed. No detailed morphometric analysis was done but the relative distribution of A, D & PP cells in these islets did not appear to be disturbed.

Insulitis was present in 78 out of the total of 155 cases. Among the 74 patients with known recent-onset disease it was present in 59 (80%). It can be seen from table 2.1 that in cases where insulitis was present it affected 20% of islets containing insulin but only 1% of islets which were insulin deficient. ($p < 0.001$, Chi squared test). While acknowledging that there must be considerable sampling error, there was a tendency towards a greater proportion of insulin containing islets being affected by insulitis in younger patients. Thirty-seven per cent of

insulin containing islets were affected in children of 3 years and under, while 8% were affected in patients aged 18-34 years inclusive (Table 2.1).

Table 2.2 gives the incidence of insulitis in patients known to have had diabetes for less than a year. From this it can be seen that among the very young and among older diabetics insulitis was less likely to be found.

Islet pathology in patients with prolonged duration type 1 diabetes

Fifty-four patients were known to have had diabetes for more than a year. In 38 patients all islets appeared to be insulin deficient. Insulin containing islets were found in the remaining 16 patients and four of these patients, who had been treated with insulin for $1\frac{1}{2}$ years, 2 years, 5 years and 6 years respectively, had evidence of insulitis (Table 2.3). Thus the 3 populations of islet described in the recent-onset cases - insulin containing islets without insulitis, insulin containing islets with insulitis and insulin deficient islets were all represented in patients with prolonged duration diabetes although obviously in quite different proportions.

Distribution of insulin containing islets and insulitis within the pancreas.

Samples from both PP rich lobe and glucagon rich lobe were available in 19 cases, 9 of whom had disease of recent origin. One of these patients was under a year old and had an apparently normal pancreas (see below). Normal insulin containing islets were present in the PP lobe in this patient. Among the other patients, 12 of whom had insulin containing islets in the glucagon rich lobe, B cells were

TABLE 2.1 **Insulin content of islets in relation to insulitis in 62 patients with type 1 diabetes of known duration.**

Sex	Age	Duration of symptoms	No. of ICI	No. of ICI with insulitis	No. of IDI	No. of IDI with insulitis
F	18 mths	1 wk	90	72	1239	3
F	21 mths	2 wks	19	12	100	2
F	2 yrs	1 wk	140	23	366	2
F	2 yrs	2 wks	1	1	160	5
M	2 yrs	2 wks	19	2	224	0
F	3 yrs	3 wks	153	69	46	1
F	3 yrs	6 wks	87	23	66	1
M	3 yrs	1 wk	23	5	191	1
M	3 yrs	3 mths	41	7	82	1
F	4 yrs	3 wks	133	66	121	14
M	4 yrs	3 wks	131	4	227	2
M	5 yrs	1 wk	66	10	457	6
F	5 yrs	3 mths	22	5	5	0
F	6 yrs	3 wks	41	1	63	0
F	6 yrs	1 wk	30	4	79	1
M	6 yrs	1 wk	114	64	375	8
F	6 yrs	5 wks	2	2	83	0
M	6 yrs	9 mths	8	6	221	0
M	7 yrs	1 wk	43	25	140	6
F	7 yrs	1 wk	71	10	101	1
F	8 yrs	3 wks	120	9	99	0
M	8 yrs	2 wks	3	2	65	0
F	8 yrs	1 wk	141	41	123	2
M	8 yrs	3 mths	72	4	69	0
F	8 yrs	5 yrs	12	6	540*	0
F	9 yrs	2 yrs	2	1	22	0
F	10 yrs	6 wks	5	4	176	0
M	10 yrs	3 wks	165	5	392	1
M	11 yrs	4 wks	19	2	167	1
F	11 yrs	1 wk	16	15	66	2
M	11 yrs	6 yrs	14	1	267	0
M	11 yrs	4 days	59	6	38	0
M	12 yrs	2 wks	75	2	190	0
F	12 yrs	2 mths	129	32	37	2
F	12 yrs	2 mths	50	30	55	2
F	12 yrs	1 mth	176	24	99	0
F	12 yrs	3 wks	39	11	45	3
M	13 yrs	2 wks	76	11	71	2
F	13 yrs	3 mths	15	3	79	0
F	13 yrs	1 wk	84	5	26	0
M	13 yrs	1 wk	45	5	72	0
F	13 yrs	3 mths	149	38	184	3
F	13 yrs	4 days	136	11	64	1
F	14 yrs	Few wks	20	7	156	2
F	14 yrs	1 wk	247	24	56	0
F	14 yrs	1 wk	36	7	139	1
M	15 yrs	6 mths	60	17	290	4
M	16 yrs	Few wks	149	58	583	5

TABLE 2.1 - continued

Sex	Age	Duration of symptoms	No. of ICI	No. of ICI with insulitis	No. of IDI	No. of IDI with insulitis
M	16 yrs	4 mths	134	30	267	0
M	17 yrs	1 wk	420	83	171	16
M	17 yrs	1 wk	182	79	291	0
M	17 yrs	1 wk	58	7	27	0
F	18 yrs	3 wks	157	3	5	0
F	18 yrs	1 wk	107	4	10	0
F	18 yrs	3 wks	34	15	68	3
F	19 yrs	1½ yrs	16	2	102	0
M	20 yrs	3 wks	329	35	278	1
M	22 yrs	2 wks	24	9	237	3
M	23 yrs	Few wks	140	4	42	0
M	26 yrs	Few days	45	2	22	0
M	29 yrs	Few days	142	31	230	1
F	34 yrs	2 mths	354	1	360	0
	TOTALS =		5490	1097	10626	109

% of ICI with insulitis = 20% **% of islets that contain insulin = 34%**
% of IDI with insulitis = 1% **% of islets affected by insulitis = 7.5%**

*** only 2 sections out of 28 are included. The remaining 26 contained insulin deficient islets only.**

TABLE 2.2

Presence of Insulitis in patients with recent-onset type 1 diabetes

	AGE 1	AGE 1-2	AGE 3-5	AGE 6-9	AGE 10-14	AGE 15-19	AGE 20-29	AGE 30-39
Insulitis present	_	5	8	11	19	9	6	1
Insulitis absent	2*	1*	-	-	3+	7**	2+	-

* all islets in these cases contained insulin.

\+ in one case all islets were insulin deficient.

** in two cases all islets contained insulin. In one all islets were insulin deficient.

found in the PP lobe in only 2 patients, both with disease of recent origin. In one of these patients only one insulin containing islet was present in that lobe while in the other there were a considerable number. Insulitis was present in both the PP lobe and the glucagon rich lobe in the latter patient and this proved to be the exception. Insulitis was present in the glucagon rich lobe in 7 of the other 18 patients but among them insulitis was not observed in the PP lobe.

The exocrine pancreas is divided into lobules which are separated from each other by connective tissue septa. The distribution of insulin containing islets and B cell destruction, as witnessed by insulitis, did not appear to be entirely random, but there appeared to be a marked lobular distribution in many cases. Thus, in a given case, while the majority of lobules may have contained only insulin deficient islets the insulin containing islets would be present in a small number of lobules in which few insulin deficient islets were present (Fig 2.7). Similarly, islets affected by insulitis tended to be grouped together within individual lobules. One case in particular served to highlight this phenomenon. This was an autopsy performed by the author on a child of 8 years who had had diabetes for 5 years. She was found dead in bed at home and no cause of death was established at autopsy. A total of 28 blocks of pancreas were sampled and sections from each stained for insulin. While there were over 7000 insulin deficient islets in the sections only 12 insulin containing islets were seen and these were found in 2 separate groups (Fig 2.8).

Findings in the exocrine pancreas

Focal acute pancreatitis was present in 10 cases, 8 of recent-onset. The acute inflammatory infiltrate was characteristically present within and around excretory ducts. Eosinophilic proteinaceous concretions were commonly found in the duct lumina. An exceptional case was that of a 21 year old woman who had had diabetes for 9 years. She had required pancreatic enzyme therapy for exocrine pancreatic failure. Her pancreas at autopsy showed the typical findings of chronic calcifying pancreatitis. In addition, all islets were insulin deficient.

A mild diffuse lymphocytic infiltrate was present in 12 cases (6 of recent origin) and this made assessment of the presence of insulitis impossible in these cases.

Apart from the presence of inflammation in a few cases, the most striking change in the exocrine tissue concerned the size of acini. Insulin containing islets were surrounded by normal acini while insulin deficient islets were surrounded by apparently atrophic acini (Fig 2.9). The atrophic acinar cells were smaller and subjectively contained many fewer zymogen granules than normal. This relationship between the presence or absence of B cells in an islet and the appearance of the adjacent acini was so marked that the insulin content of an islet could be predicted precisely in most cases simply by observing the surrounding exocrine tissue on a haematoxylin and eosin stained section. Even an islet showing severe insulitis was surrounded by normal exocrine tissue so long as some B cells were still present within the islet. As has

Figure 2.1

Hyperplastic insulin containing islet in recent-onset type 1 diabetes

Note the presence of polypoid endocrine cells. The adjacent serial section showed that this islet contained insulin.

H & E x 270.

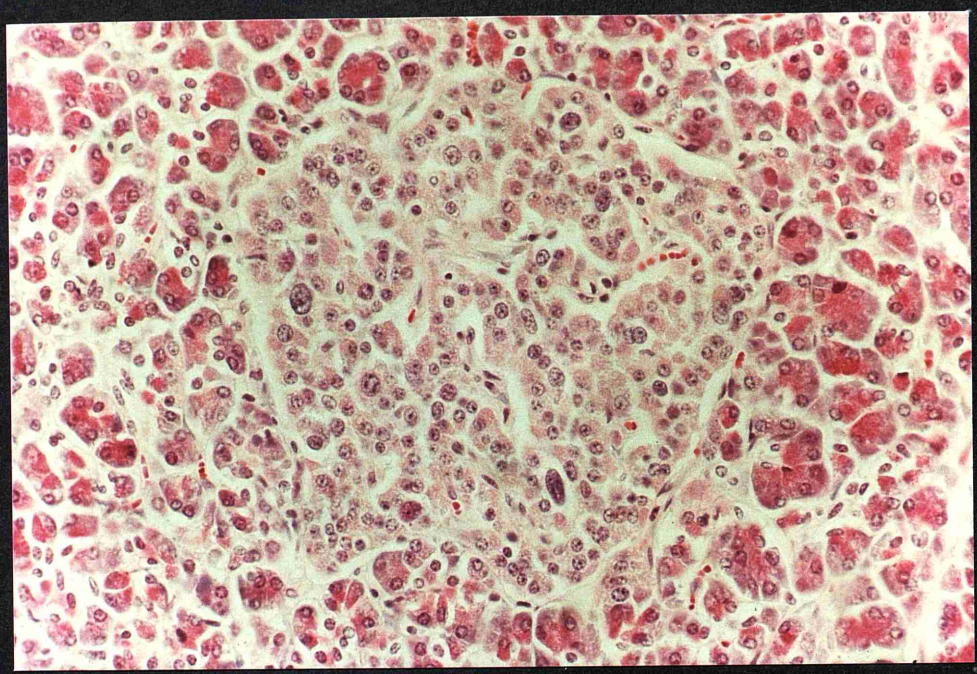


Figure 2.2

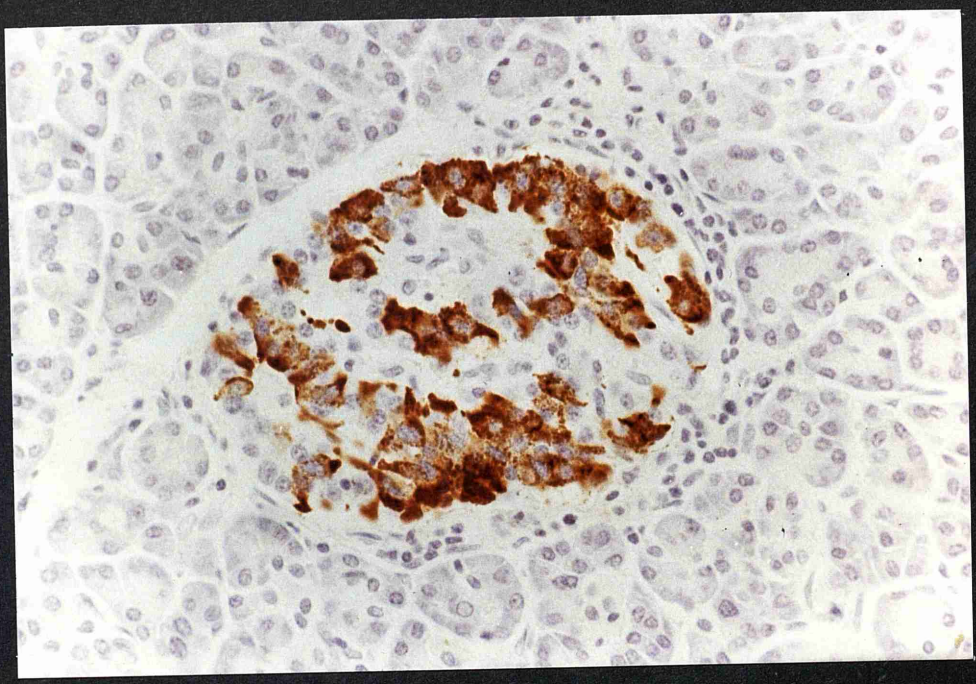
Early insulitis

There is relatively little loss of B cells.

I.P. for insulin x 350

There is a peripheral infiltrate of small lymphocytes.

I.P. for leucocyte common antigen (LCA) x 350.



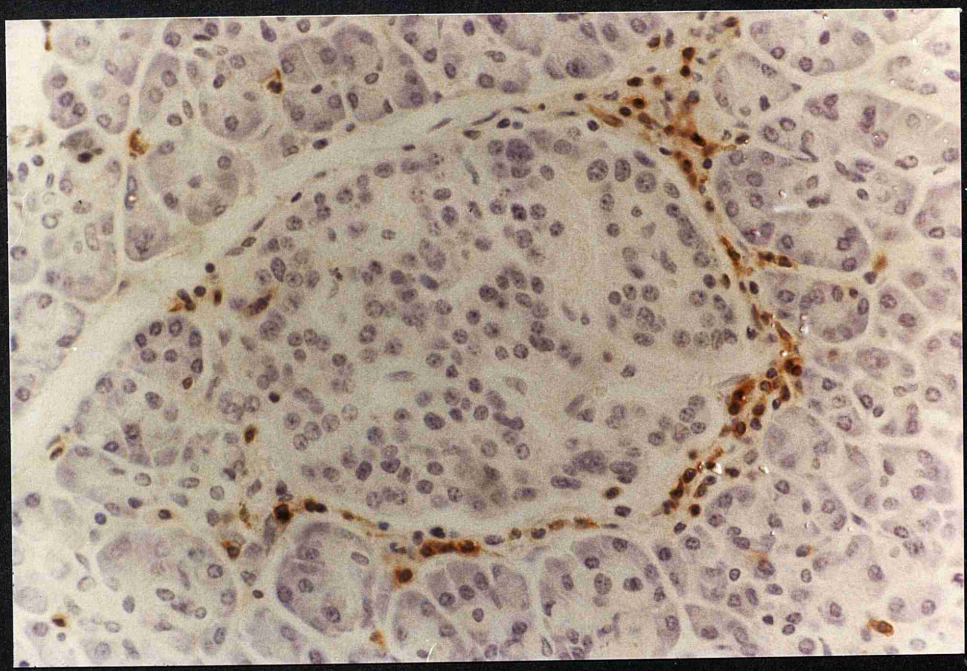


Figure 2.3

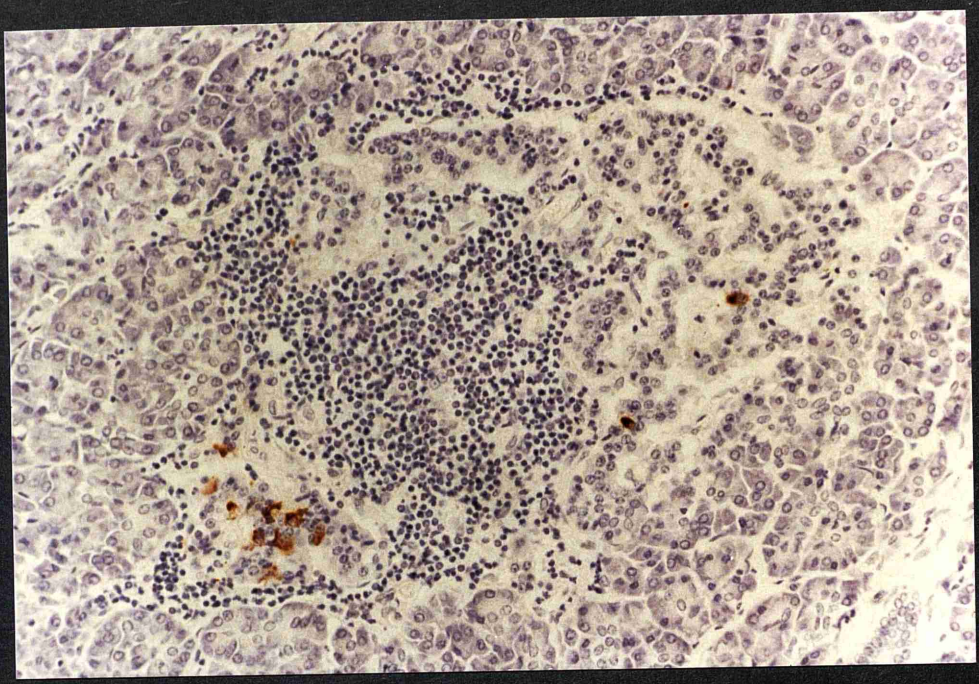
Advanced insulitis

There are very few residual B cells

I.P. for insulin x 220.

There is a diffuse lymphocytic infiltrate in the islet.

I.P. for LCA x 220.



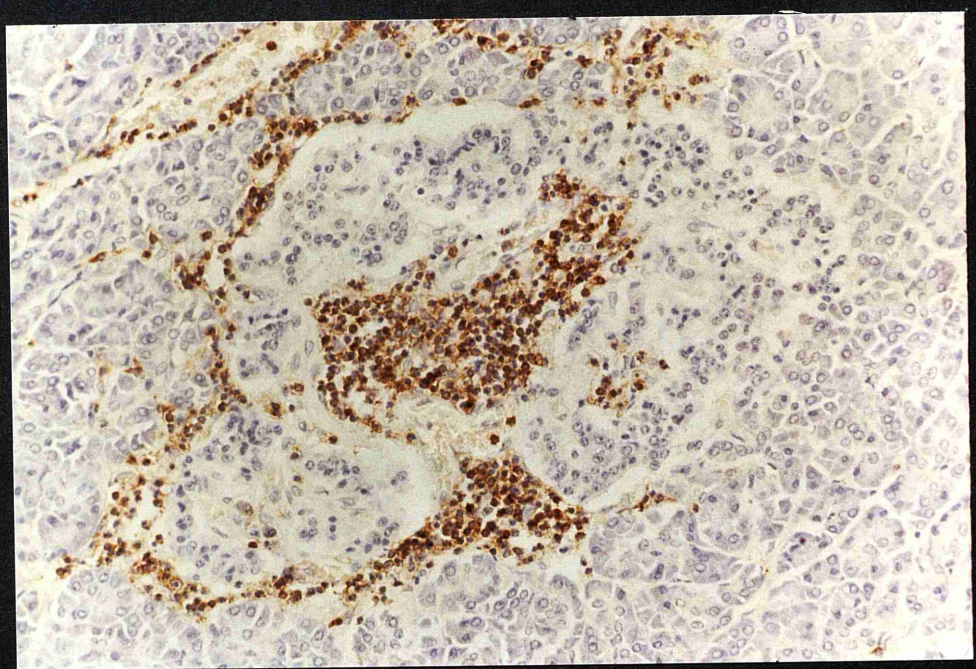


Figure 2.4

Insulin deficient islet with insulitis

An unaffected insulin containing islet is shown on the right of the picture. There is a marked lymphocytic infiltrate adjacent to the insulin deficient islet on the left.

I.P. for insulin x 100.

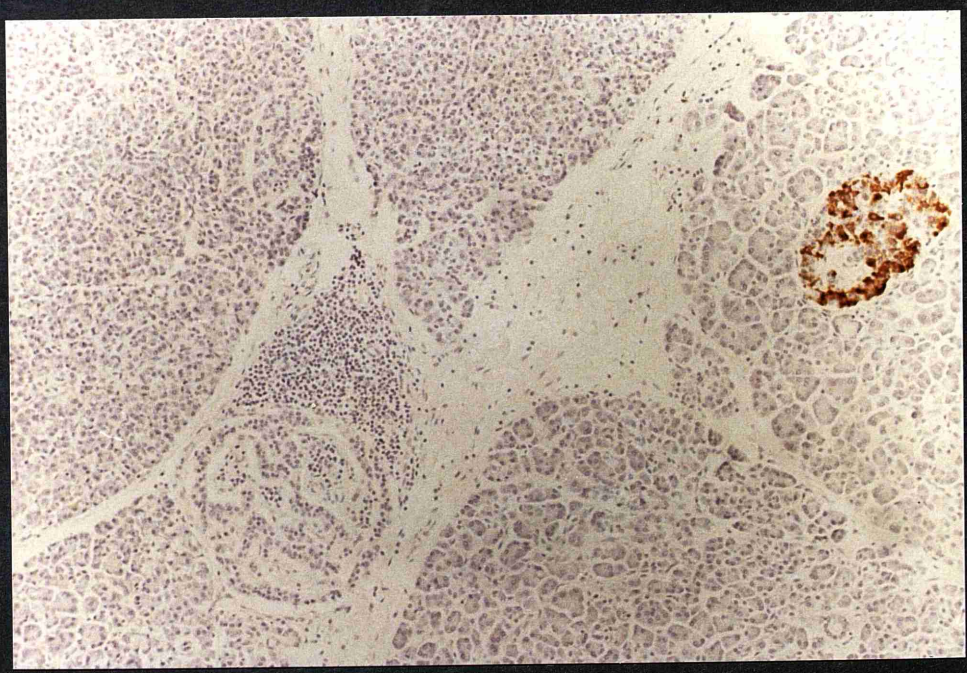


Figure 2.5

Endocrine cell content of insulin deficient islet

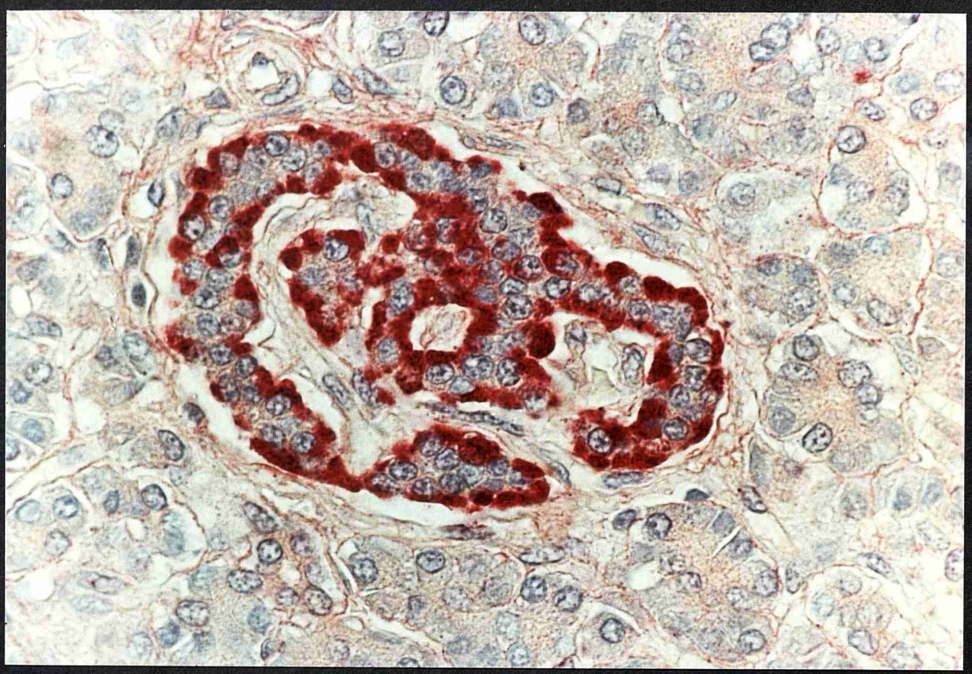
This section has been stained for glucagon, somatostatin and PP by an immunoalkaline phosphatase method. All the endocrine cells appear to have been stained. x 440.

Figure 2.6

Insulin deficient islet: distribution of A cells

The vascular sinusoids are still lined by A cells. When compared to Fig 1.1 it can be seen that the distribution of A cells has been scarcely disturbed during the process of B cell destruction.

I.P. for glucagon x 550.



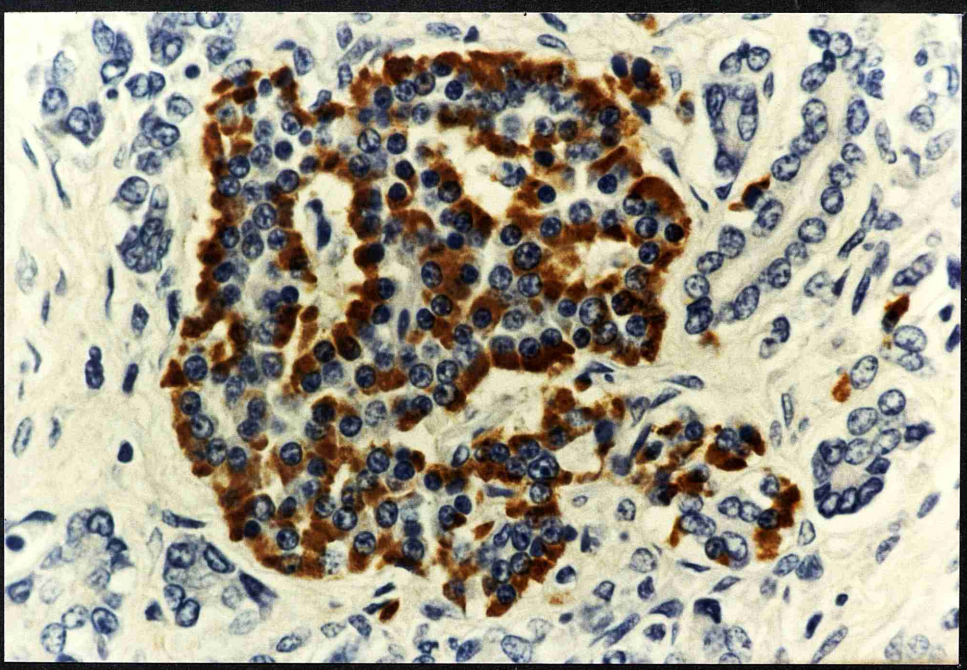


Figure 2.7

Lobular distribution of insulin containing islets in recent-onset diabetes

Note that the lobule on right has many insulin containing islets while those on the left have only insulin deficient islets.

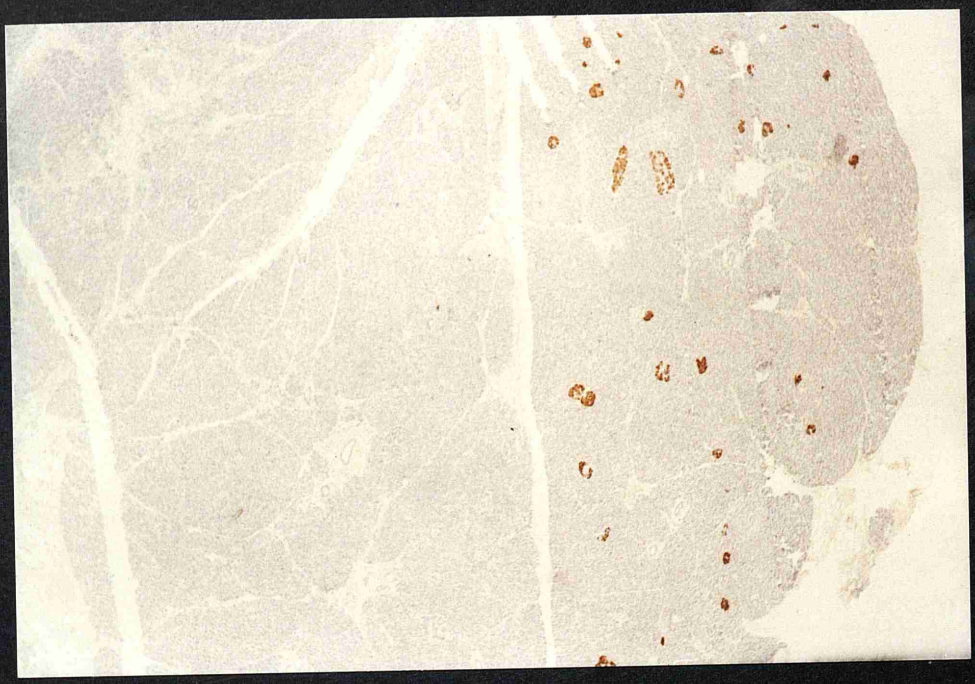
I.P. for insulin x 22.

Figure 2.8

Lobular distribution of insulin containing islets in diabetes of 5 years duration

The few residual insulin containing islets in this case are grouped together.

I.P. for insulin x 14.



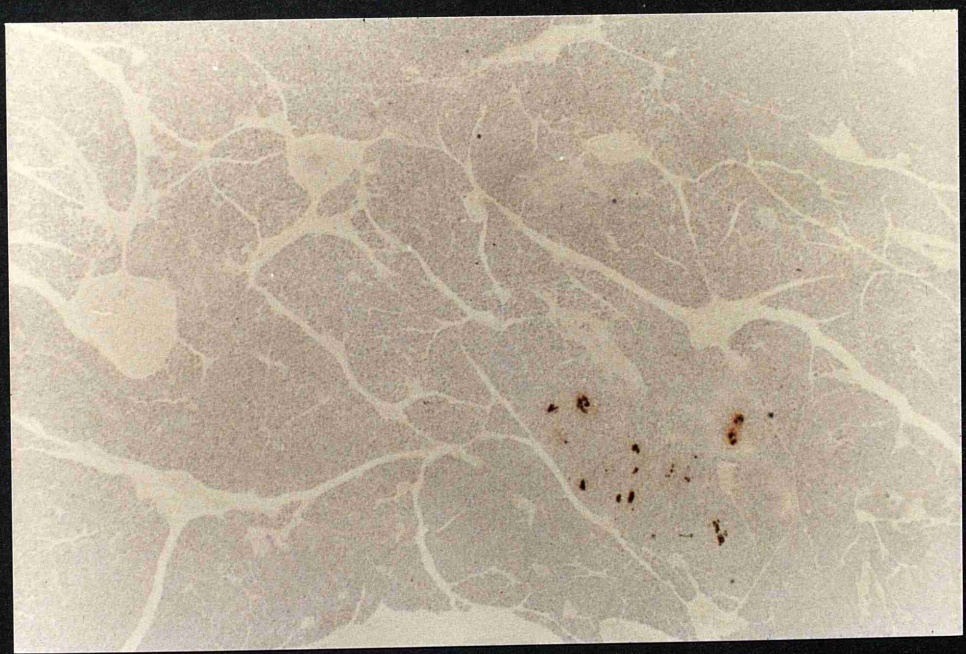


Figure 2.9

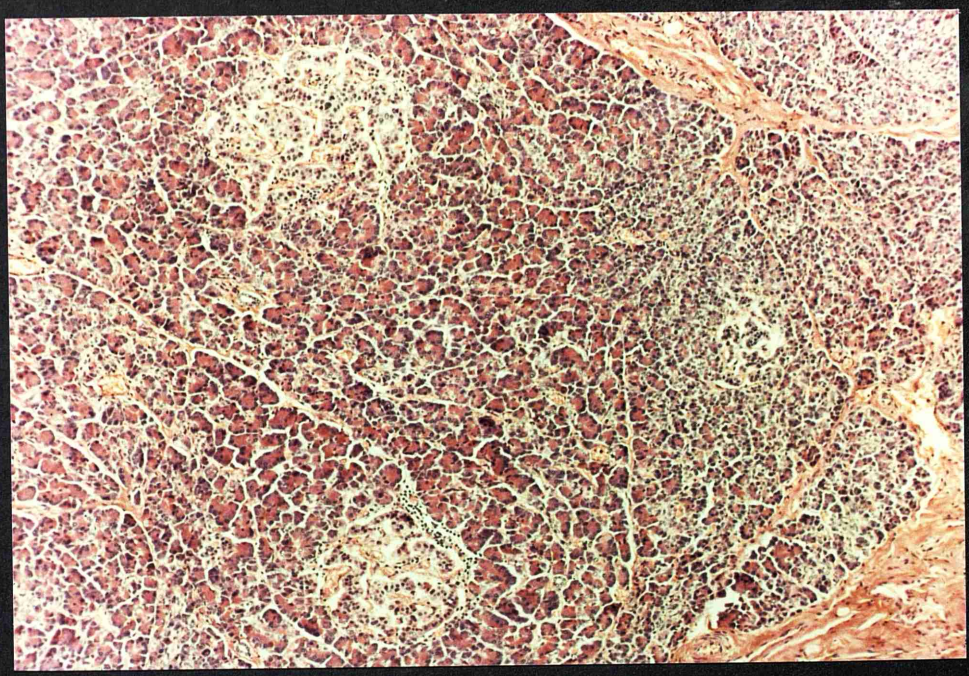
Acinar degranulation around insulin deficient islets in recent-onset diabetes

Acini surrounding the two large islets on the left are large and well granulated. Those around the small islet on the right are small and degranulated.

H & E x 80

The two islets on the left contain insulin. The one on the right is insulin deficient.

I.P. for insulin x 80.



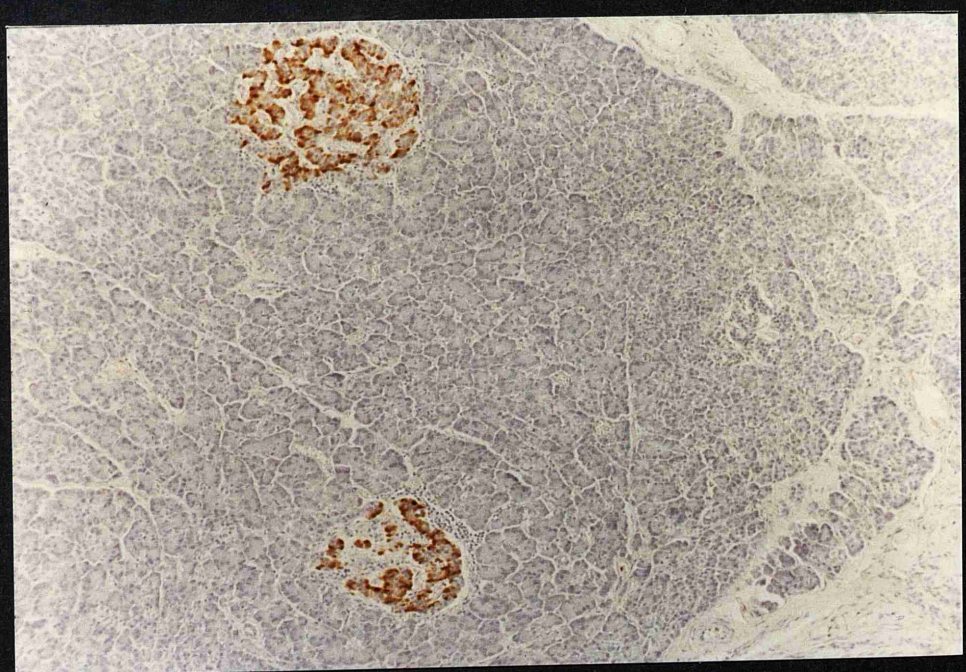


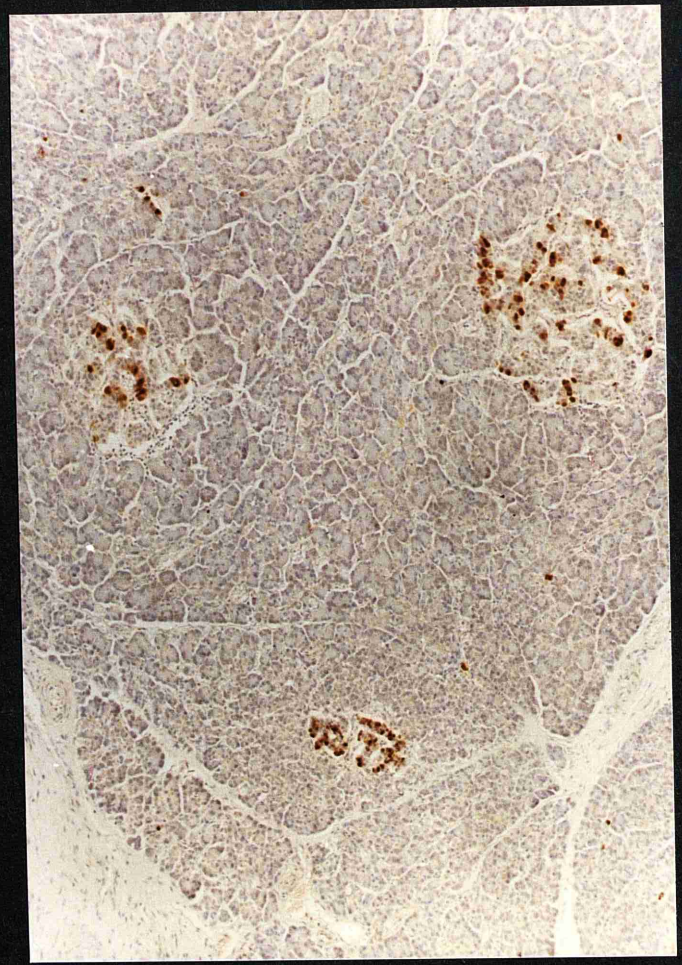
Figure 2.9 continued

All 3 islets contain A cells.

I.P. for glucagon x 80.

All 3 islets contain D cells.

I.P. for somatostatin x 80.



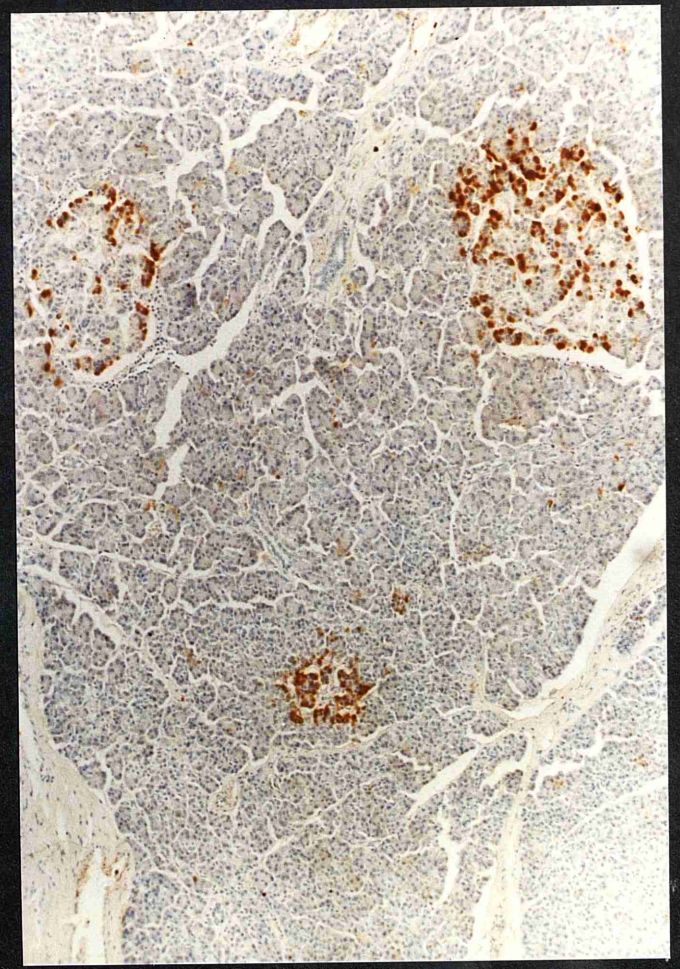


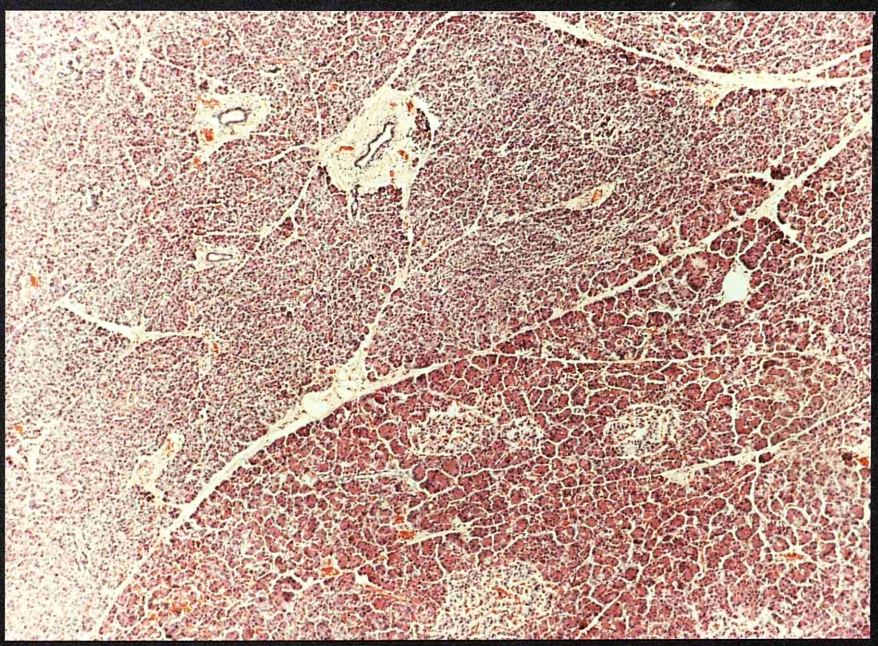
Figure 2.10

Exocrine atrophy in insulin deficient lobules

A connective tissue septum separates two pancreatic lobules. All islets in the top left lobule are insulin deficient (as revealed by a serial section stained for insulin). Those in the bottom right lobule contain insulin.

Note the acinar atrophy in the insulin deficient lobule.

H & E x 35.



been described above, there was often a marked lobular distribution of insulin containing and insulin deficient islets. Acini in lobules in which all islets contained insulin were normal while they were uniformly atrophic in lobules containing only insulin deficient islets (Fig.2.10). Where insulin containing and insulin deficient islets were juxtaposed the zonal nature of the atrophy could be readily appreciated (Fig 2.9). Thus the relative area of normal and atrophic exocrine tissue in any section was entirely related to the relative numbers of insulin containing and insulin deficient islets. These changes were most apparent in the pancreases of those patients who had died of recent-onset diabetes.

As has been mentioned above, insulin secreting islets were present in the PP lobe in very few cases. However, acini surrounding insulin containing islets in this part of the pancreas still appeared larger and better granulated than acini surrounding insulin deficient islets.

Patients with atypical pancreatic pathology

Having described the findings in the "classical" examples of type 1 diabetes it is necessary to discuss several cases which were radically different.

There were 3 patients aged 18 months or less with recent-onset diabetes in whom not only was insulitis absent, but also all the islets contained insulin, and appeared entirely normal (Fig 2.11). All presented with marked hyperglycaemia and coma. There was no clinical doubt about the diagnosis and the autopsy findings were consistent with a biochemical death. Another patient who

possibly belonged to this group was a female, aged 13 months, who had presented with neonatal diabetes, had been treated with insulin for over a year and died of ketoacidosis. In her pancreas there appeared to be a normal number of islets and all contained insulin, although a few islets appeared to have reduced numbers of B cells (Fig 2.12). Insulitis was absent.

The findings in one previously healthy 17 year old male with diabetic symptoms for less than a week were unique in this series. There was total exocrine atrophy of most lobules and here islets were aggregated in adipose tissue. However, some lobules of the pancreas had normal exocrine tissue and the ducts were normal. Superimposed upon this chronic exocrine condition were the typical features in the endocrine pancreas of recent-onset diabetes - a mixture of insulin containing and insulin deficient islets with many of the former affected by insulitis (Fig 2.13). The possibility of Shwachman syndrome (Shwachman et al. 1964) was considered but the patient had no haematological abnormalities, normal growth, no skeletal changes and no history of steatorrhoea.

One patient, a 14 year old boy with Down's syndrome, who had had diabetes for several years also had steatorrhoea of unknown cause. In his pancreas there was an extremely dense, diffuse lymphocytic infiltrate with germinal centre formation and all islets were insulin deficient. The exocrine lobules were preserved but the acini were almost totally degranulated and atrophic (Fig 2.14). While there was a resemblance to cystic fibrosis in this case the ducts and acini were not dilated, there were

no ductal concretions and there was no pulmonary pathology.

Amyloid deposits in the islet stroma are characteristic of type 2 diabetes but they have very seldom been observed in recent-onset type 1 diabetes (Gepts & LeCompte 1985). Intra-islet amyloid was present in the pancreas of a 29 year old schizophrenic who died of recent-onset diabetes (Fig 2.15). He was not being treated with any drugs. Apart from this finding the changes in the pancreas were typical of type 1 diabetes, with many of the insulin containing islets being affected by insulitis.

Another unique case in this series was that of a 15 year old girl who presented 2 years prior to death with glycosuria, haematuria and proteinuria. At this time she was on no medication and had no significant past medical history. She was diagnosed as having both systemic lupus erythematosus (SLE) and type 1 diabetes. Within two years, in spite of steroid therapy, she developed chronic renal failure secondary to the SLE (proven on renal biopsy). She died of peritonitis while being treated with chronic ambulatory peritoneal dialysis. She had required insulin therapy for her diabetes. Although there was some autolysis it was still possible to see that the pancreatic findings were atypical. Only 10% of the islets were insulin deficient and far from there being a loss of B cells there seemed to be a generalised islet hyperplasia (Fig 2.16). Insulitis was not seen. There has been a previous report of a patient with similar findings (Evans 1972). This person presented with typical type 1 diabetes at the age of 8 years and died of diabetic nephropathy 14 years later. At autopsy there were no insulin deficient

islets but here too there was a striking hyperplasia of insulin containing islets. The morphological difference between these two cases is that in the present one insulin deficient islets were also present. The fact that these two cases look alike does not of course mean that they have the same pathogenesis.

Perhaps the most exciting of these atypical cases was that of a 22 year old previously well Chinese male who was admitted to a casualty department complaining of abdominal pain and vomiting. He was discharged later that day with a diagnosis of gastroenteritis. The next day he was readmitted having had persistent vomiting. On admission he had ketoacidosis and a normal serum amylase. He had a cardiac arrest 2 hours later and died. At autopsy there were no significant abnormalities seen other than those in the pancreas. There was a diffuse and moderately heavy mixed infiltrate of lymphocytes and eosinophils in the exocrine pancreas. Acinar necrosis was present, particularly around islets. The changes in the islets were almost uniform. The majority of the endocrine cells were necrotic and there was a mild diffuse inflammatory cell reaction, also consisting of lymphocytes and eosinophils. Occasional islets were seen in which there were groups of more normal endocrine cells. On staining the islets for pancreatic hormones only two B cells were identified in the 2 sections available for study. There seemed to be no destruction of A or D cells and the more normal appearing islets were in fact 'collapsed', insulin deficient islets in which the A and D cells had come to lie together. In contrast to 'classical' type 1 diabetes, these appearances

were suggestive of an acute event affecting almost all the B cells simultaneously. The patient had a normal glycosylated haemoglobin which is further evidence that he had been diabetic for a very short time. As will be described in chapter 4, the histological appearance of exocrine inflammation, acute B cell necrosis and infiltrating eosinophils is also seen in the pancreases of neonates dying of disseminated Coxsackie B virus infection. On histological grounds therefore it seems possible that this case represents acute viral diabetes. Interestingly a serum sample taken at autopsy was islet cell cytoplasmic antibody negative.

Patients with other clinical disorders

Three patients had Down's syndrome. One is described above. Two had had diabetic symptoms for less than 2 weeks One presented at eighteen months and had no morphological abnormality in the pancreas. The other presented at 12 years and the findings in the pancreas were more typical of recent-onset diabetes (insulitis affecting insulin containing islets).

Five patients with recent-onset diabetes had evidence of other organ specific auto immune diseases (2 Addison's disease, 3 thyrotoxicosis). While qualitatively the pancreas in these five appeared similar to that of other patients there was an interesting quantitative difference. In the 'polyendocrine' patients 60% of islets contained insulin compared to 32% in the other patients with recent-onset diabetes. Also, in the polyendocrine patients only 2.6% of insulin containing islets were affected by

Figure 2.11

Lack of insulin deficient islets in a diabetic infant

This pancreas was from a 3 month old male infant with a 1 week history of diabetic symptoms. No abnormality in the pancreas was detectable.

I.P. for insulin x 90.

Figure 2.12

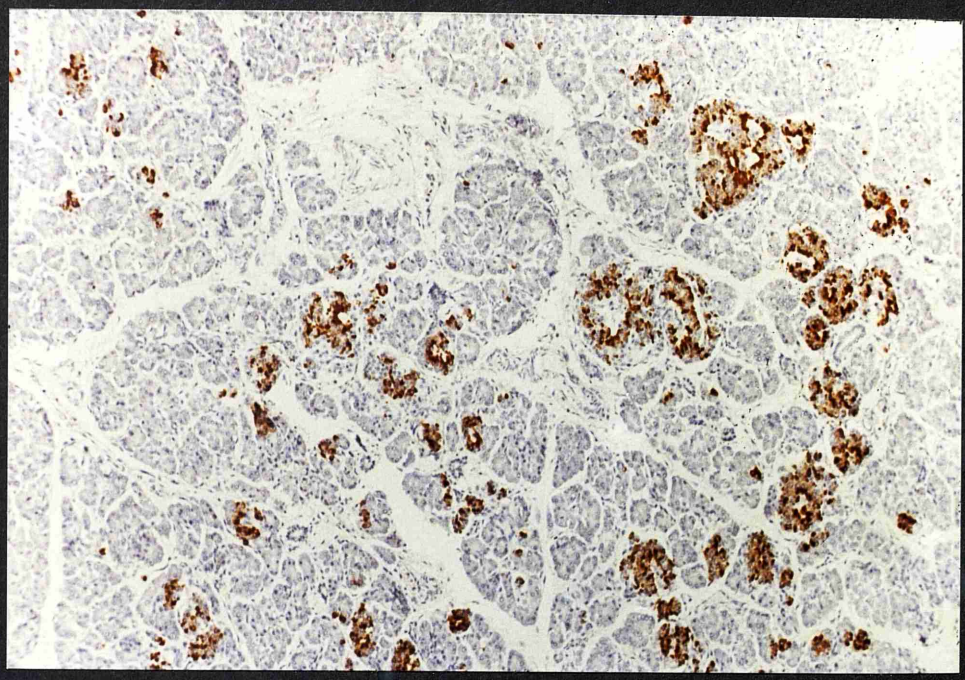
Islets of a 13 month old child who presented with neonatal diabetes

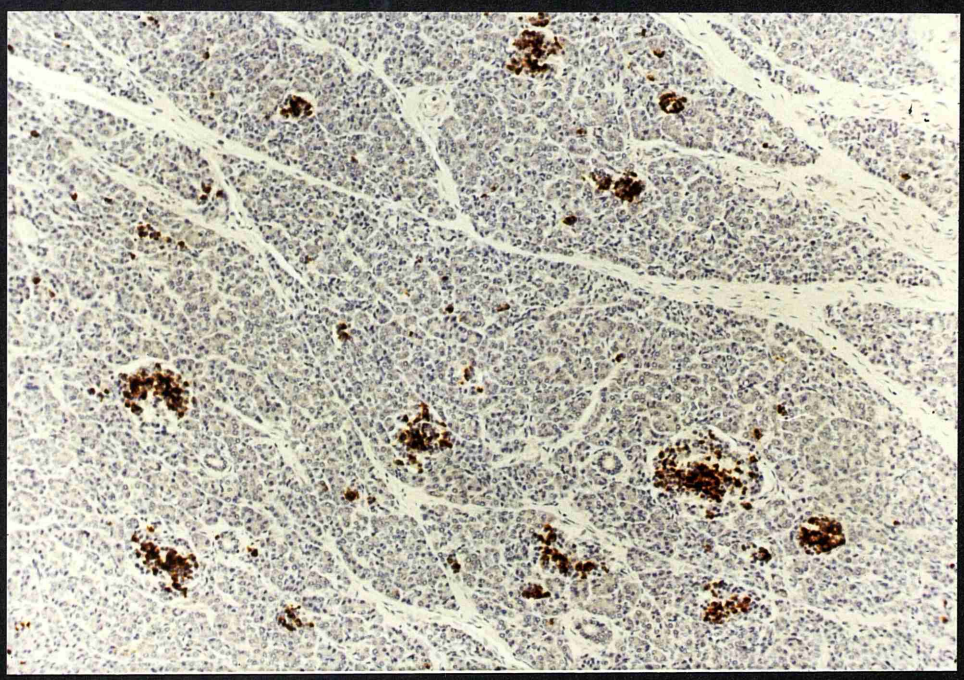
All islets contain insulin

I.P. for insulin x 100.

There is a suggestion of a slight reduction in the number of B cells in the larger islet.

I.P. for insulin x 340





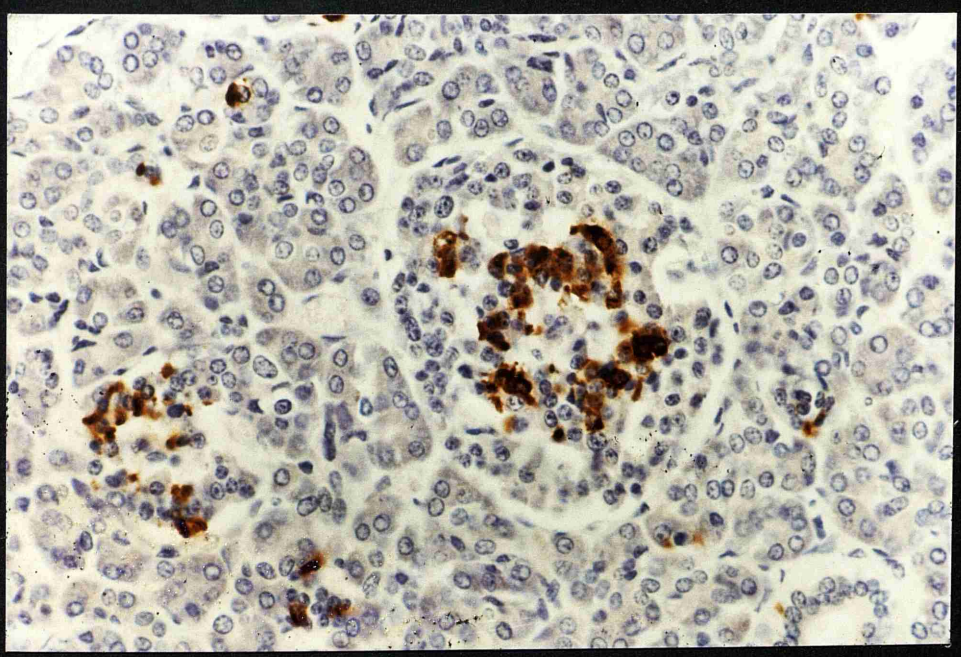


Figure 2.13

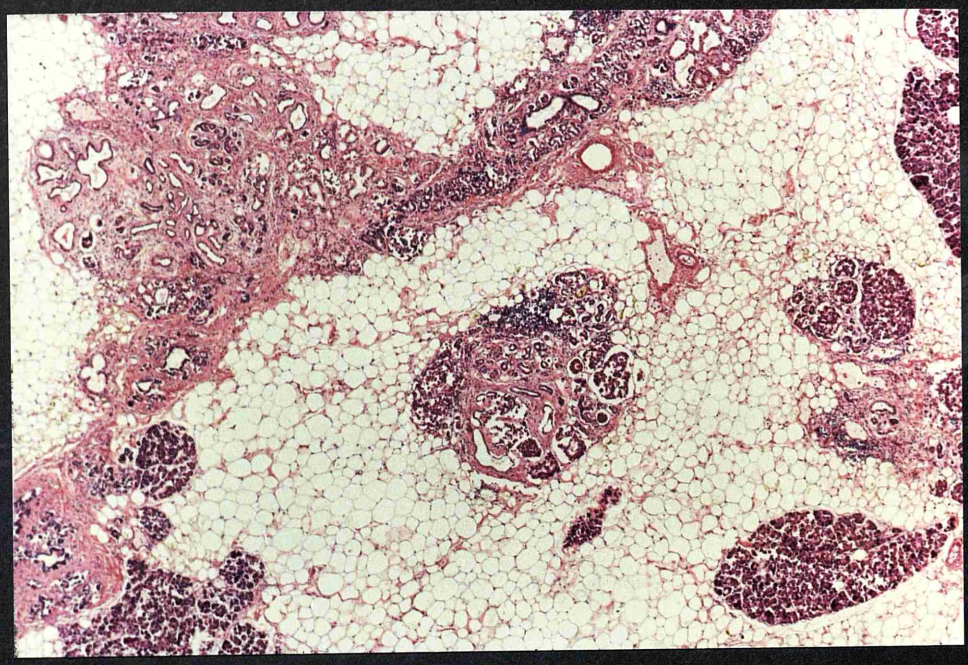
Extreme exocrine atrophy in a 17-year-old male with recent-onset diabetes

There is irregular but marked loss of exocrine acinar tissue and replacement by adipose tissue.

H & E x 35

This is a higher power of the central atrophic lobule in the photograph above. Note the lymphocytic infiltrate affecting these insulin containing islets.

I.P. for insulin x 90



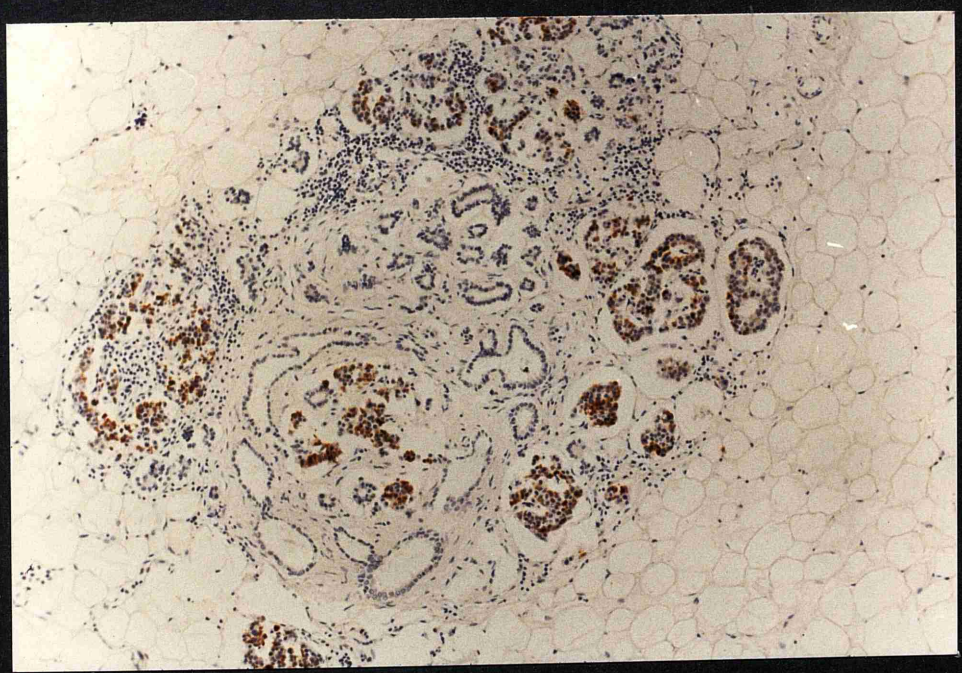


Figure 2.14

Fourteen-year-old patient with Down's syndrome, prolonged duration diabetes and steatorrhoea

All islets are insulin deficient. Note the diffuse chronic inflammatory cell infiltrate, germinal centre formation and mild atrophy of acinar tissue.

H & E x 35

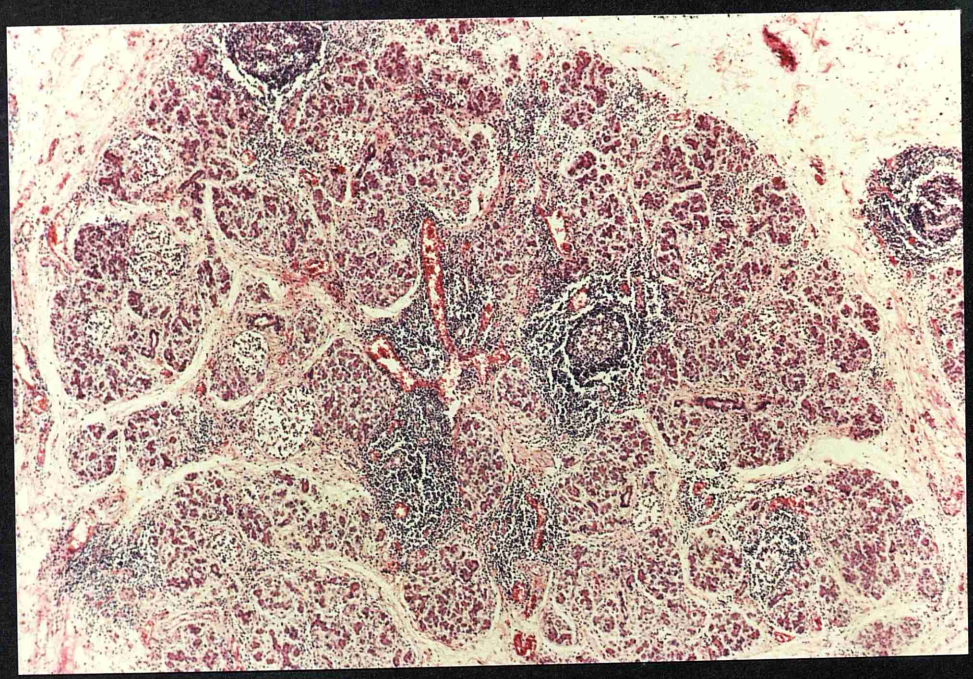


Figure 2.15

Intra-islet amyloid in recent-onset type 1 diabetes

Two islets are present in the right half of the picture and one on the left. All contain pink hyaline deposits.

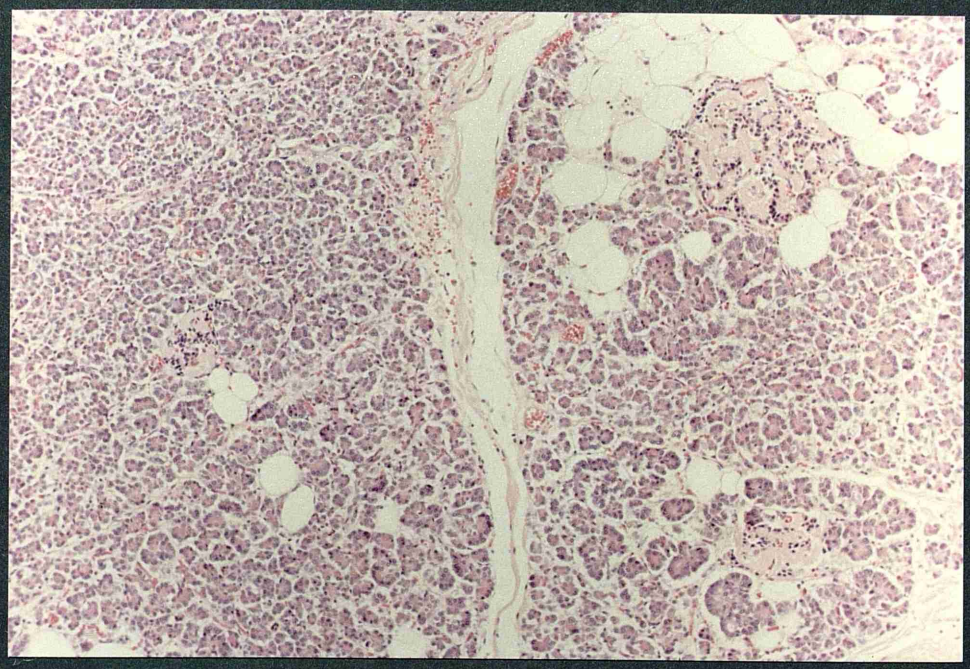
H & E x 100

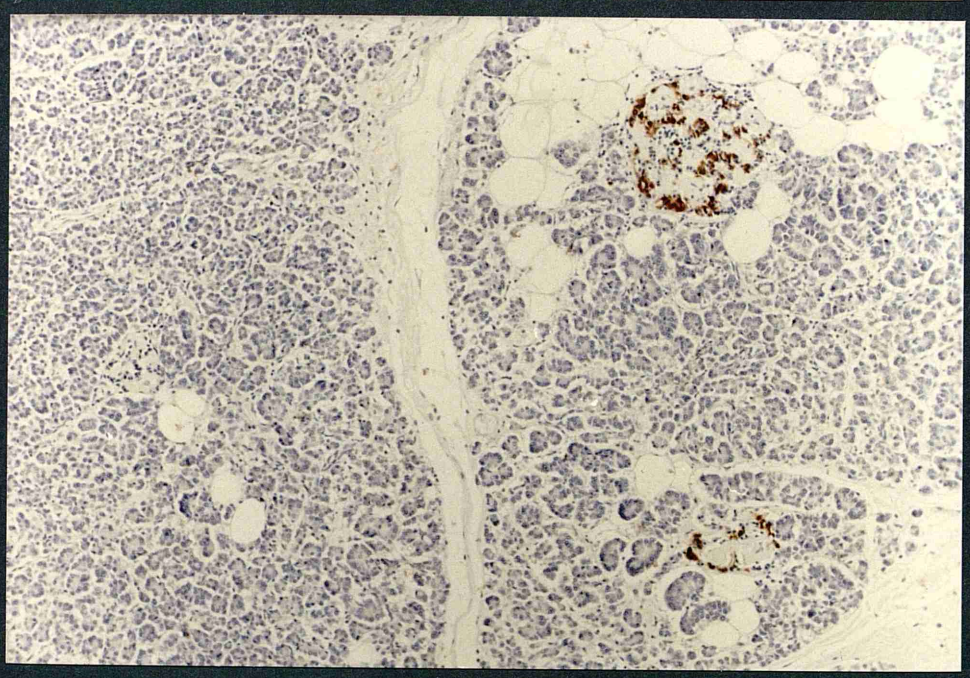
The islet on the left is insulin deficient

I.P. for insulin x 100

This is a serial section of the top right islet photographed above. Note green birefringence of amyloid deposits and the presence of insulitis.

Sirius red: photographed through crossed polarisers x 440





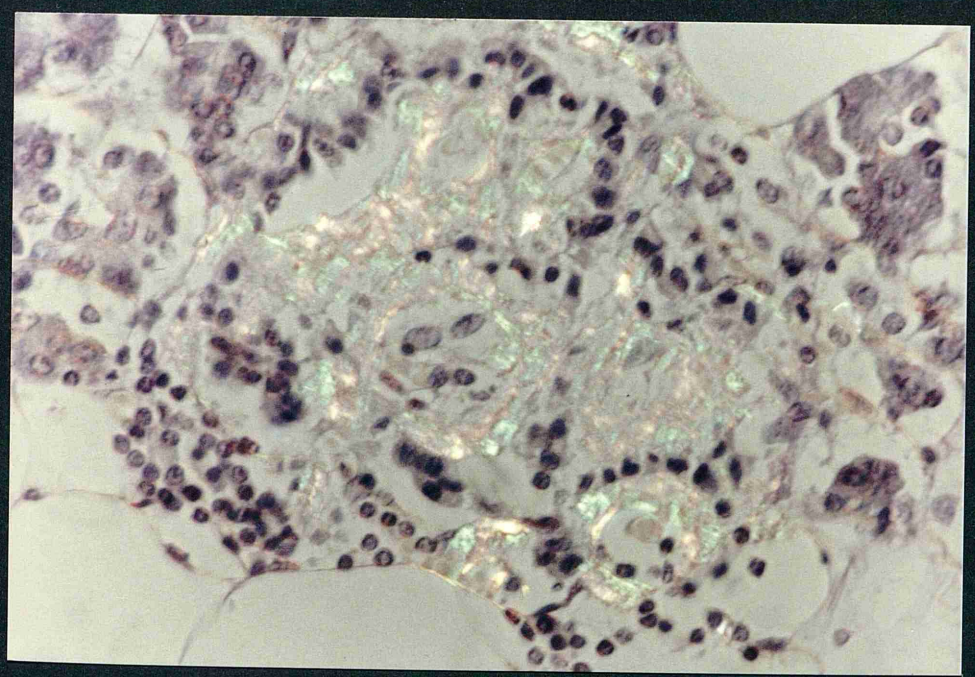


Figure 2.16

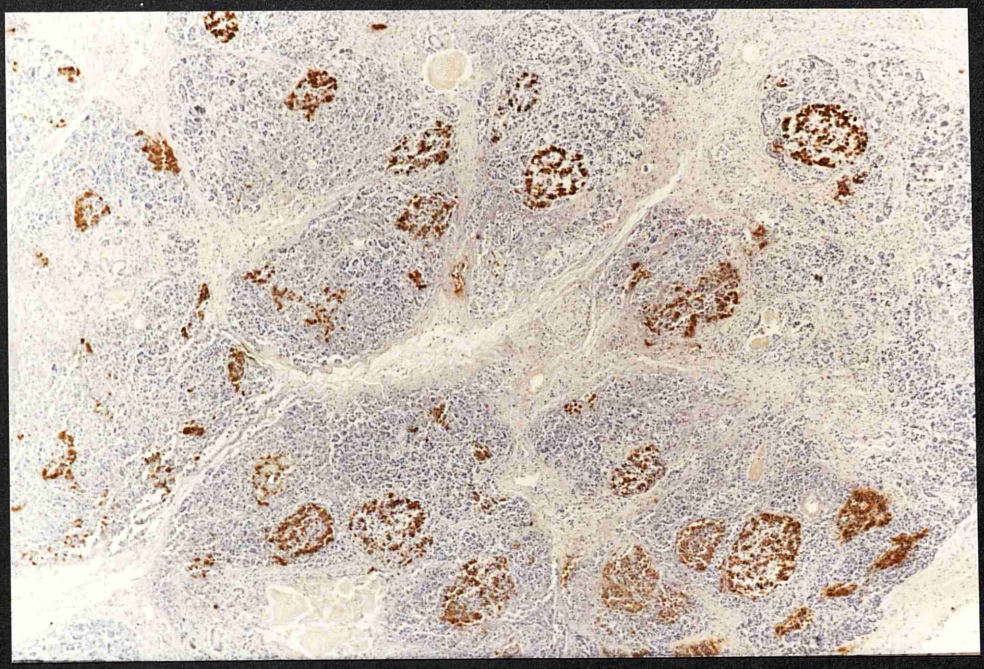
Fifteen-year-old-girl with 2 year history of lupus erythematosus and insulin-dependent diabetes

While there is obviously some autolysis it can be seen that there is apparent islet hyperplasia and marked preservation of B cells.

I.P. for insulin x 35

The islets in the left hand side of this photograph are insulin deficient. However 90% of islets in this case contained insulin.

I.P. for insulin x 120.



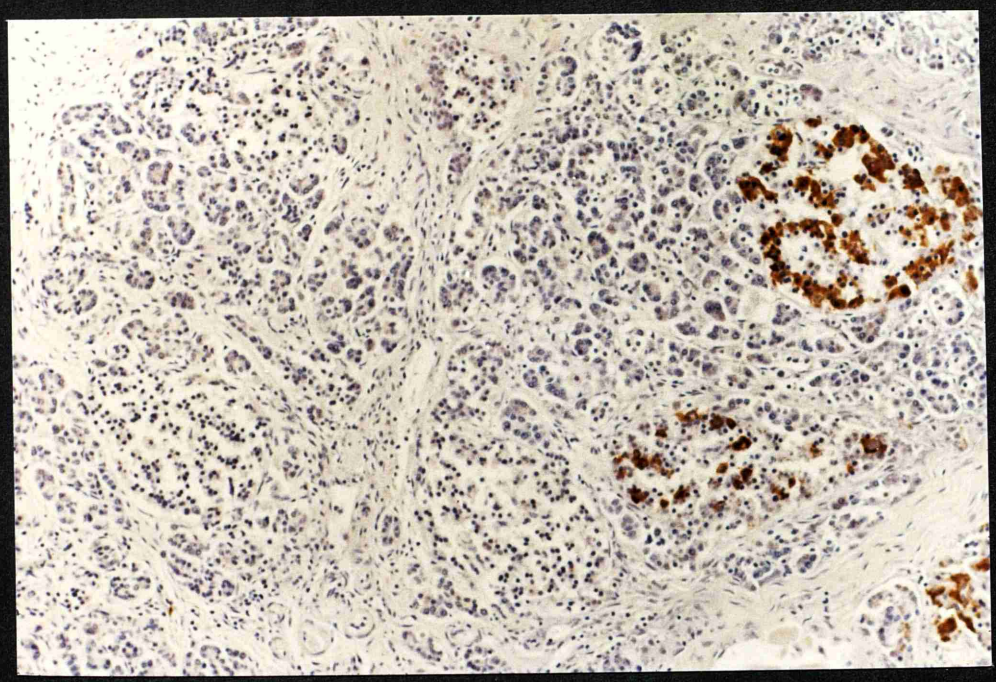


Figure 2.17

Twenty-two-year-old Chinese male with recent-onset diabetes

There is a "necrotic hole" below and to the right of the prominent intralobular duct.

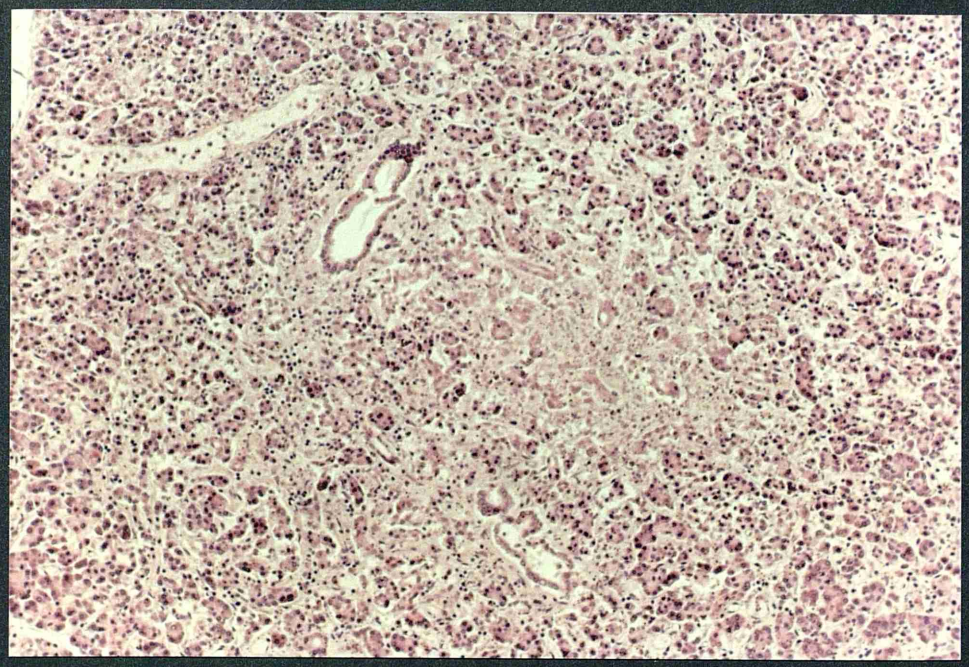
H & E x 110

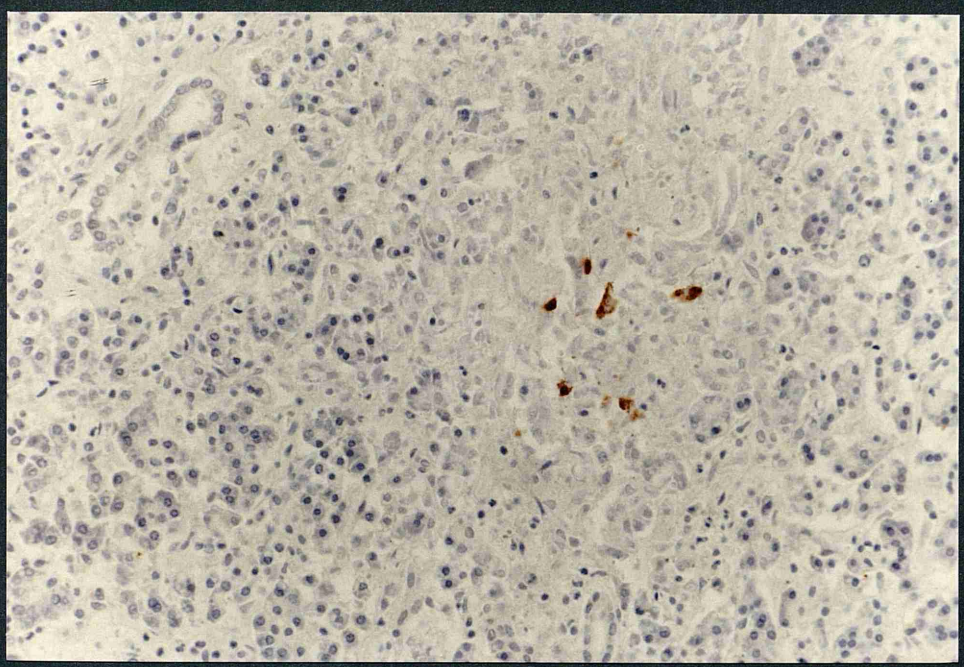
Detail of this necrotic area shows preservation of A cells. B cells were not seen.

I.P. for glucagon x 220

The inflammatory cell infiltrate in the exocrine tissue includes lymphocytes and prominent eosinophils.

H & E x 470





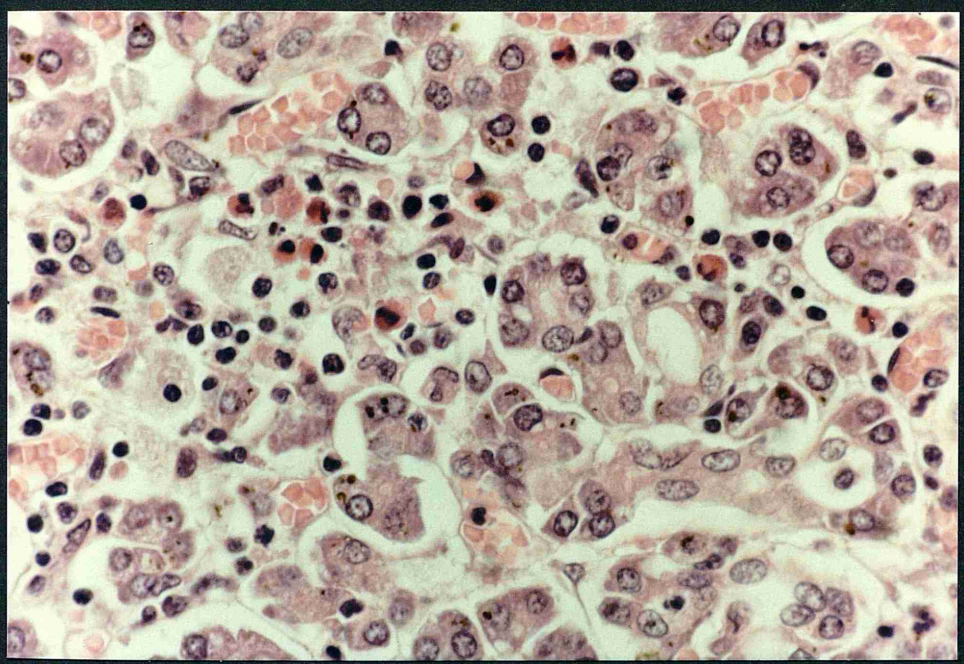


Figure 2.17 continued

Occasional islets are present which do not appear necrotic.

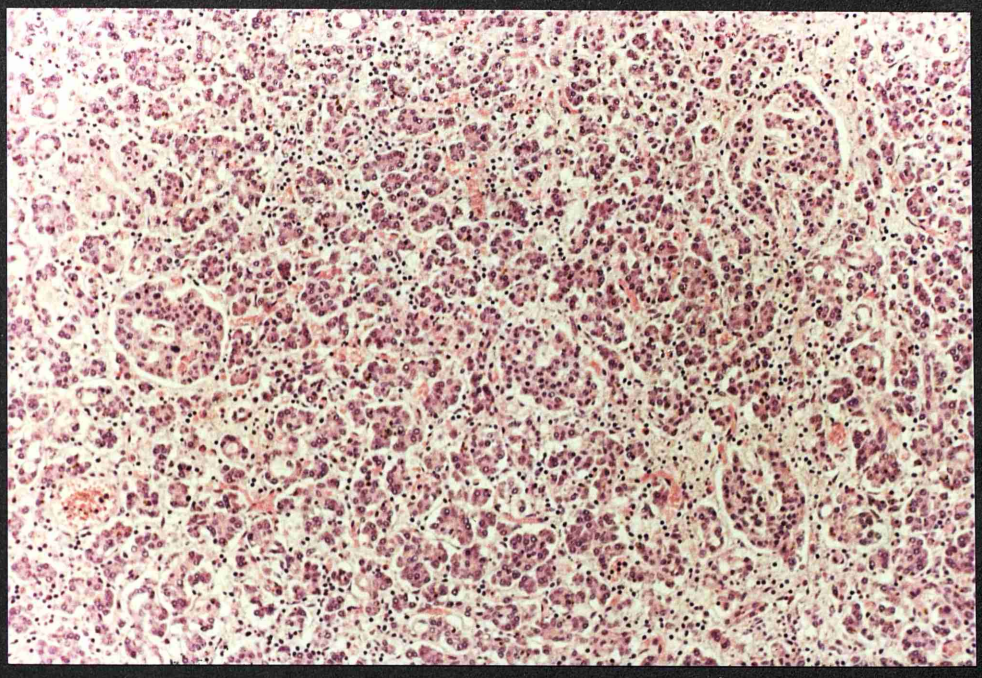
H & E x 110

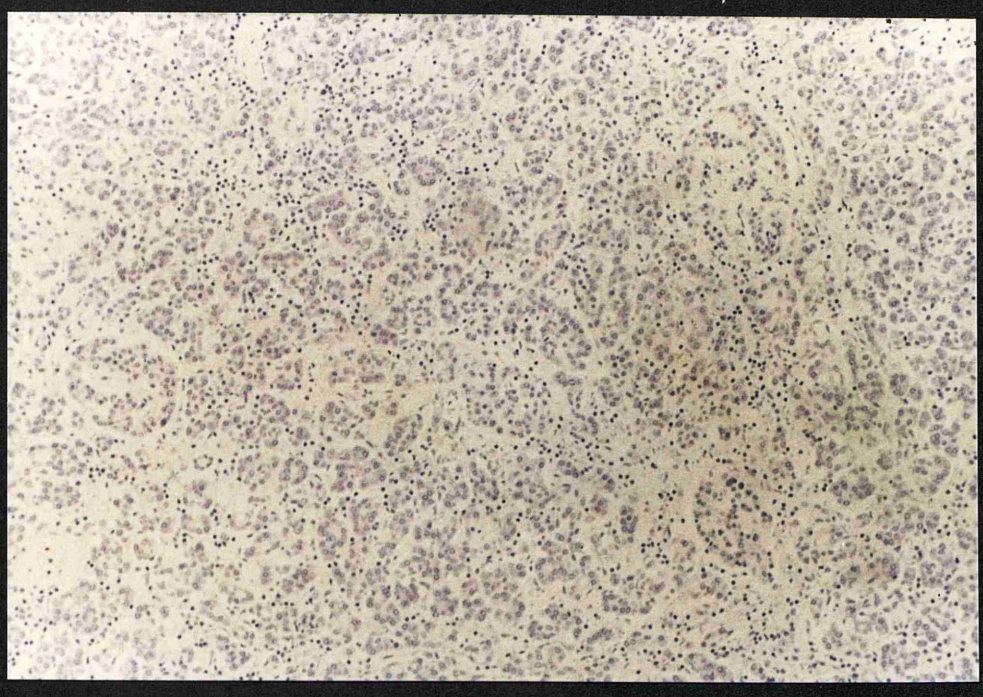
These islets are all insulin deficient.

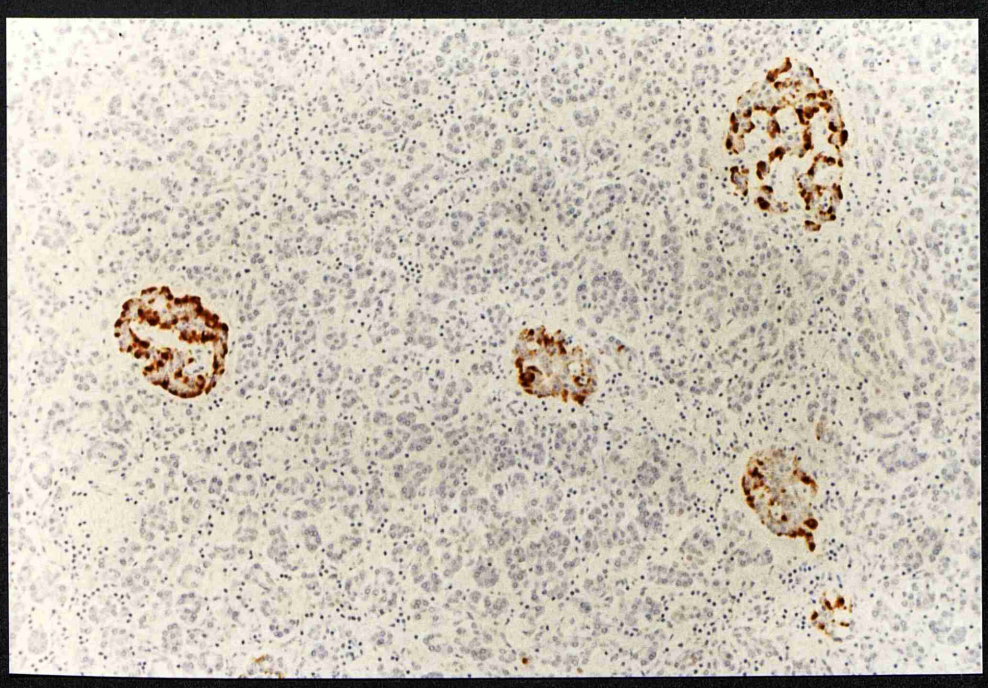
I.P. for insulin x 110

There is preservation of A cells in the islets.
(D cells were also unaffected).

I.P. for glucagon x 110







insulitis while the figure in the remainder was 23%. Although there must be considerable sampling problems the destructive process of B cells does appear to be slower in these patients with polyendocrine diabetes. This is in keeping with the finding that such patients remain islet cell antibody positive for longer than diabetics with no evidence of other autoimmune diseases (Betterle et al. 1984).

The pancreatic histology in patients with other problems (muscular dystrophy, cerebral palsy etc) did not differ significantly from the remaining patients.

DISCUSSION

There were essentially three reasons for attempting to gather a large series of cases of recent-onset diabetes. Firstly, to assess the frequency of insulitis in the disease; secondly, to establish whether this lesion primarily affected insulin containing islets, and thirdly, to have sufficient numbers to be able to identify a possible minority of cases of viral aetiology. On these three counts the study was largely successful.

As has been said, there is considerable controversy in the literature concerning the frequency of insulitis in recent-onset type 1 diabetes. While Gepts (1965) identified it in 15 out of 22 cases and Junker et al. (1977) in 6 of 11 cases it was not seen in the 13 patients studied by Doniach & Morgan (1973). Junker et al suggested that the lesion was more frequent in younger patients and indeed when the results of these studies are amalgamated they show that 15 out of 21 patients aged 14 years and under had

insulitis. By contrast in only 6 out of 25 patients older than 14 years was this lesion seen. In Doniach & Morgan's study 5 of the 13 untreated diabetics studied were under 15 years of age and two of them were thought to have only insulin deficient islets in the material examined. In Gepts' study (1965) insulitis was not found in patients with diabetes of greater than 6 months duration, yet 4 of Doniach & Morgan's 8 untreated diabetics aged 17 to 21 years at onset of diabetes had had diabetes for longer than 6 months at the time of death and one of them was thought to have no residual insulin containing islets. These factors may have contributed to the absence of insulitis in their report.

In the present study, insulitis was found in 59 of the 74 patients (80%) with known recent-onset disease and it confirms the high proportion of patients under 15 years of age with this lesion (Table 2.2). Only one block of pancreas was available for study in all 7 patients aged 15 to 19 years and in 2 of the 3 patients aged 10 to 14 years with recent-onset diabetes in whom no evidence of insulitis was found. Since the distribution of insulitis within the pancreas is very patchy (Gepts 1965) there must be a serious risk of sampling error, particularly in an adult sized pancreas, if only one block of pancreas is available for study. This may well have contributed to the reduced incidence of insulitis in the older patients studied. Only prospective studies, where multiple blocks of pancreas are available, can satisfactorily answer the question of whether insulitis is present more commonly in this age group than indicated by retrospective studies.

Gepts & De Mey (1978) studied 16 patients with recent-onset type 1 diabetes, 11 of whom had insulitis. In one case they noted that insulitis affected only islets containing B cells. Since this observation was not repeated in the other 10 cases with insulitis this case has represented the only evidence that insulitis is associated with destruction of B cells. In the present study, in a sample of 62 patients, it was shown that 20% of insulin containing islets were affected by insulitis while only 1% of insulin deficient islets were thus affected (Table 2.1). A complete range of morphological appearances was seen from "early insulitis", where there was little evidence of B cell loss in the islet accompanied by a peripheral inflammatory cell infiltrate, to "advanced insulitis" where there was a marked reduction in the number of B cells often associated with a more prominent infiltrate of lymphocytes. In a case report of a patient who died of recent-onset diabetes in whom fresh frozen pancreas was available for study with specific monoclonal antibodies, it was shown that the majority of infiltrating lymphocytes involved in insulitis were of T cytotoxic/suppressor phenotype (Bottazzo et al. 1985). Taken together these findings support the concept that insulitis represents an immunologically mediated destruction of B cells. They do not resolve the question as to whether this is an autoimmune destruction of B cells or destruction of B cells infected by virus. The situation could be analogous to the liver in chronic active hepatitis. Some cases are due to Hepatitis B virus infection and some appear to be autoimmune, but the histology of both is similar, and in both cytotoxic T

lymphocytes are involved in the destruction of hepatocytes.

The presence of complement fixing islet cell antibodies in the serum of patients several years before clinical presentation with type 1 diabetes has suggested that B cell destruction may take place during this time (Gorsuch et al. 1981). The present study has shown that B cell destruction, as witnessed by insulitis, can be present up to 6 years after diagnosis, which is further evidence that the disease process in the pancreas can have a very protracted course. There is, however, obviously a considerable range within the group studied, as Table 2.1 shows. For example, in the youngest patient, 80% of the remaining insulin containing islets were affected by insulitis whereas only 0.28% of such islets were affected in the oldest patient. Thus the destruction of B cells may take place relatively rapidly in some patients and extremely slowly in others.

Excluding a very young patient with no demonstrable pancreatic abnormality, insulin containing islets were seen in the PP rich lobe in 2 cases and insulitis was observed in this area in only one. There were another 7 patients who had insulitis in the glucagon rich lobe but who had neither insulin containing islets nor evidence of insulitis in the PP lobe. While acknowledging that normal PP islets contain fewer B cells than islets in the glucagon rich lobe the relative increase in amount of endocrine tissue compared to exocrine tissue in the PP lobe means that the number of B cells per unit volume of the PP lobe is only reduced by approximately 50% when compared to the glucagon rich lobe (Stefan et al. 1982). It is not thought that

this is sufficient to explain the absence of B cells from the PP lobe in the majority of these cases. It has been suggested, on the evidence of one case report where B cells and insulitis were absent from the PP lobe, that the destruction of B cells is completed sooner in this part of the pancreas than in the glucagon rich lobe (Klöppel et al. 1984). The findings in the majority of the present cases would lend support to this idea.

Another anatomical unit affecting the apparently non-random topographical destruction of B cells was the pancreatic lobule. Exocrine pancreatic lobules are surrounded by connective tissue septa and are apparently supplied by a single artery (Cadete-Leite 1973). As such they represent the smallest complete vascular anatomical unit. As has been observed before (Gepts 1965), residual insulin containing islets tended to be grouped together within a few lobules in which there were few insulin deficient islets. Such lobules were surrounded by many other lobules in which only insulin deficient islets were present. An equally striking observation was that islets affected by insulitis tended to be grouped together. Taken together these findings suggest that the destruction of B cells often takes place simultaneously throughout a lobule but that different lobules are affected at different times.

Given that the destructive process affecting the B cells is usually a chronic one, it is not surprising that there was evidence of regeneration of residual B cells. While mitoses in B cells could be observed, albeit rarely, a more frequent finding was the presence of enlarged hyperplastic islets with polypoid endocrine cells. In some

cases most residual insulin containing islets were thus affected. Gepts (1965) originally thought that these hyperplastic islets were formed by a proliferation of cells from duct epithelium. In support of this he photographed an islet with a central cavity said to be lined by duct-like epithelium. It is notable that in the many reviews he has published since then this remains the sole photograph to support his concept of islet neoformation. It seems more likely that what B cell regeneration there is takes place in residual insulin secreting islets. This is supported by the fact that there was no evidence in the present study of an increase in the number of insulin containing islets within lobules composed entirely of these islets. There was, however, often an apparent increase in the number of B cells within such islets. Equally when serial sections were done through the entire thickness of residual insulin containing islets (as part of the studies in chapter 4) no evidence of a central duct was ever seen.

The findings in the cases where the pancreatic pathology differed substantially from the majority of classical cases have mostly been discussed in the results section. However two examples deserve further attention.

Three children with recent-onset disease, aged 18 months or less, had no insulitis and apparently normal numbers of islets with a normal proportion of B cells. Since there seemed little doubt clinically that these children had diabetes it has to be suggested that they may have a different disease from the classical type 1 diabetes which is accompanied at presentation by loss of B cells. The child with neonatal diabetes who died aged 13 months

and who had required insulin therapy during this time may also belong to this pathogenetic group. It is not meant to be implied that all children who present with diabetes at age less than 2 years have a different disease. There were 8 patients in the present survey with diabetes of prolonged duration who had presented under 2 years of age. At the time of death all islets were insulin deficient in these patients. The combination of a histologically unremarkable pancreas and insulin dependent diabetes in very young children has not previously been described.

It was suggested that the Chinese male with recent-onset diabetes in whom the glycosylated haemoglobin concentration was normal had an extremely acute form of the disease. This was supported by the fact that all islets seemed to have experienced a simultaneous destruction of B cells associated with an infiltrate of lymphocytes and eosinophils. This case, in whom no islet cell antibodies were found, may represent an acute viral infection of B cells. It is always difficult to draw conclusions from an autopsy study as to the prevalence of a normally non-fatal disorder. However, because of the very acute nature of this man's condition clinically, it could be argued that diseases such as his would have been over-represented, rather than the opposite, in an autopsy series such as this. Thus it seems likely that if up to 30% of type 1 diabetics are associated with a B cell specific viral infection, as has been proposed (King et al 1983, Banatvala et al. 1985), the pathogenesis of the B cell destruction in these cases would have to be different from that seen in this exceptional case where an acute cytopathic viral effect may have been involved.

Changes in the exocrine pancreas

The presence of acute inflammation within ducts and among the pancreatic acini in recent-onset diabetes has been noted before (Maclean & Ogilvie 1959, Gepts 1965). The pattern of the inflammation in which there was prominent neutrophil infiltrate within and around ducts, is not typical of a terminal pancreatitis secondary to shock (Foulis 1980). However, there is no clinical or experimental work which offers a plausible explanation for the occurrence of this pattern of exocrine pancreatic inflammation in diabetes. Equally the mild diffuse lymphocytic infiltrate seen in some cases lacks a satisfactory aetiology.

The appearance of acinar cell degranulation confined to the vicinity of insulin deficient islets has not been previously reported. The proposed explanation for this histological observation is based on a knowledge of the islet blood supply and the action of islet hormones on pancreatic exocrine tissue (Henderson, Daniel & Fraser, 1981). As has been demonstrated and discussed in Chapter 1, pancreatic islets are supplied by arterioles which form relatively wide sinusoids within the islet. In place of a single vein draining the islet, abundant capillaries emanate from the islet to supply the surrounding exocrine tissue. It has been postulated, therefore, that pancreatic acini around islets are exposed to concentrations of islet hormones which are several hundred times higher than concentrations in the systemic circulation (Fraser & Henderson 1980).

Insulin is a trophic hormone for the exocrine

pancreas. Studies on both intact animals (Adler & Kern 1975) and isolated pancreatic acini (Korc et al 1981) showed that insulin stimulated total protein synthesis by pancreatic acini. This stimulatory effect was particularly important in amylase synthesis: in severely diabetic animals the amylase content of the exocrine pancreas was reduced almost to zero (Adler & Kern 1975). By contrast, insulin tended not to inhibit synthesis of chymotrypsinogen and lipase (Henderson et al. 1981). Insulin has also been shown to stimulate cell division in pancreatic acini (Henderson et al. 1981).

Glucagon and somatostatin, on the other hand, both have an inhibitory effect on the secretion of the exocrine pancreas (Henderson et al. 1981). Prolonged exposure of rats to high levels of glucagon caused exocrine pancreatic atrophy and extreme zymogen degranulation (Lazarus & Volk 1958). Even small doses of somatostatin injected into an intact animal completely abolished the production of pancreatic juice (Wilson et al. 1977).

Less is known about the effect of pancreatic polypeptide on exocrine function. It has been shown to increase DNA synthesis in rat pancreatic acinar cells (Greenberg, Mitznegg & Bloom 1977). However, when given to a variety of animals, including man, it had a marked inhibitory effect on pancreatic secretion (Williams & Goldfine 1985).

It was demonstrated in chapter 1 that in a case of type 1 diabetes the capillary connections between insulin deficient islets and the surrounding exocrine acini were not disturbed. Capillary blood leaving insulin deficient

islets in this disease will therefore presumably contain high concentrations of only inhibitory hormones (glucagon, somatostatin and pancreatic polypeptide). This is the probable cause of the acinar atrophy and degranulation observed around insulin deficient islets. By contrast, blood draining insulin containing islets, where there is evidence of B cell hypertrophy and hypersecretion (Gepts 1965), will presumably contain abundant insulin in addition to the other hormones, and hence no atrophy is seen around these islets. The difference observed between acini around insulin containing and insulin deficient islets was most marked in the patients with recent-onset diabetes who all died in ketoacidosis. This is perhaps due to the very high glucagon output in ketoacidosis (Unger 1981) which would be expected to exaggerate the degranulation observed around insulin deficient acini.

There is a functional counterpart to the pancreatic atrophy found in type 1 diabetes. The stimulated release from the pancreas of amylase, bicarbonate and trypsin in response to intravenous secretin and cholecystokinin-pancreozymin was reduced in type 1 diabetics when compared to normals. Exocrine pancreatic function appeared to decline progressively within the first decade after diagnosis reaching a plateau at approximately 30% of normal function (Frier et al. 1976). Insulin is stored as a prohormone, pro-insulin, in the pancreatic B cells. When it is released pro-insulin is cleaved into insulin and C-peptide in equimolar amounts. Measurement of C-peptide release is therefore a measure of endogenous insulin secretion, even in treated insulin-dependent diabetics.

Endogenous insulin reserve, assessed by C-peptide status, was compared to exocrine pancreatic function in a group of type 1 diabetics (Frier et al. 1978). The stimulated maximal output of amylase and bicarbonate correlated significantly with the maximal stimulated level of C-peptide. Thus the better preserved the endogenous insulin secretion the better preserved was the exocrine function, with respect to amylase and bicarbonate production. There was no correlation however between C-peptide concentrations and trypsin secretion. These clinical observations are in keeping with the morphological appearances described in recent-onset diabetes - the more insulin containing islets there were the less the degree of exocrine atrophy.

The pancreas in longstanding type 1 diabetes is reduced considerably in size, with almost all the reduction occurring in the glucagon rich lobe (Rahier et al 1983). The fact that there is a differential between the two lobes suggests that the atrophy has a hormonal aetiology rather than an ischaemic one, as had been proposed in the past. It seems likely therefore that the atrophy observed in the glucagon rich lobe is at least partly due to prolonged exposure of the exocrine pancreatic acini to high levels of inhibitory hormones. It is not exactly clear why the acini in the PP lobe should be protected from atrophy. No obvious difference was observed in the present study between acini in the two lobes. In the one case where insulin containing islets and insulitis were present in the PP lobe, acini around insulin deficient islets in this lobe appeared smaller and degranulated when compared to acini around insulin containing islets. As has been said, the

effects of pancreatic polypeptide on the exocrine pancreas have not yet been studied in any detail but it has been observed that the hormone has a stimulatory effect on acinar DNA synthesis (Greenberg et al. 1977). Thus pancreatic polypeptide may have a trophic effect on the exocrine pancreas in this respect, similar to that of insulin. Perhaps this partially explains the lack of atrophy in this lobe in long term type 1 diabetes.

Functional implications of pancreatic endocrine - exocrine interactions

In classical endocrinology, a disease state caused by lack of a hormone can reveal much about the action of that hormone in the normal situation. Studies of the pancreas in type 1 diabetes have helped confirm that insulin is a major trophic hormone for the exocrine pancreas. With evolution, it appears that the 'islet organ' has been subdivided into a million parts, and that the development of the blood supply has ensured very high concentrations of this trophic hormone in capillaries in the exocrine tissue around the islets. It is possible to tentatively suggest why such a structure may have evolved. The principal action of the exocrine pancreas is to secrete protein, in the form of digestive enzymes. Indeed the exocrine pancreas synthesizes considerably more protein weight for weight, than any of the other viscera (Hansson, 1959, Kukral, Adams & Preston, 1965). One of the principal actions of insulin is to promote synthesis of protein, and high concentrations of the hormone would ensure the massive synthesis of protein required by the exocrine pancreas.

A high concentration of insulin in most tissues causes 'down regulation' of insulin receptors i.e. a decrease in the density of insulin receptors on a cell (Kahn et al. 1981). If pancreatic acinar cells had a low density of insulin receptors the effect of the high local concentration of insulin might be negated. However, the opposite appears to be true. The density of insulin receptors on pancreatic acinar cells is extremely high, having been estimated to be twice that found on the hepatocyte (Bergeron et al. 1980). Thus the islets of Langerhans may have evolved partly to support the digestive function of the pancreas.

CHAPTER 3

CHAPTER 3

The partially diabetic pancreas: a histological study of a new animal model

INTRODUCTION

In chapter 2 it was shown that in the pancreas of patients who died of recent-onset diabetes there were striking changes in the morphology of exocrine acini in different parts of the gland. Thus acini surrounding insulin deficient acini appeared small and degranulated while acini around insulin containing islets appeared normal. The proposed explanation was that because of the peculiar islet blood supply, in which the drainage is via capillaries which ramify in the exocrine tissue, rather than by way of a vein, acini around islets are supplied by blood containing very high levels of islet hormones. Glucagon and somatostatin both inhibit exocrine pancreatic secretion and the former causes acinar cell degranulation. Acini surrounding insulin deficient islets will therefore be supplied by high levels of inhibitory hormones only and will thus atrophy. Acini around insulin containing islets remain normal because of the trophic effect of insulin on the exocrine pancreas.

An animal model was designed to study this process further by producing a "partially diabetic" pancreas. If all insulin secreting B cells were selectively destroyed in the head of the pancreas, the morphological and functional effects of insulin on the exocrine pancreas could be studied by comparing the differences between the "diabetic" head of pancreas and the normal tail of pancreas. If the

islet vascular anatomy produces the effect proposed then the mean concentration of insulin in exocrine capillaries in the former area would be considerably lower than in the normal area. The major advantage of such a model over existing models of totally diabetic animals would be that if sufficient B cell mass was preserved the animal would not become diabetic. This would allow the effect of insulin on adjacent acinar tissue to be studied in the absence of the systemic metabolic effects caused by the diabetic state. In addition each animal could act as its own control, having both "diabetic" and normal portions of pancreas.

Such a model, in rabbits, is described, based on a modification of previous work by Gomori and Goldner (1945).

MATERIALS AND METHODS

Male New Zealand white rabbits (Hacking & Churchill Ltd., Cheshire, U.K.), each 2.5 to 3Kg in weight, were used. The animals were fasted for 12 hours pre-operatively and one hour prior to laparotomy 0.5ml of Hypnorm, a neuroleptanalgesic, was given intramuscularly. The animals were anaesthetised with a combination of Halothane (1-2%) and Nitrous Oxide. Antibiotic prophylaxis (Cefotaxime) was administered pre- and post-operatively and a nasogastric tube was passed prior to laparotomy.

An upper left transverse abdominal incision was used. The spleen and tail of pancreas were identified and delivered through the incision, following division of the lienorenal ligament. The vascular supply to the tail of the pancreas and the spleen, via the splenic artery and the

short gastric arteries, was temporarily occluded by positioning two vascular clamps at right angles - one across the junction of body and tail of pancreas and one across the short gastric vessels (Fig 3.1).

Following vascular occlusion, 200 mg/Kg of a 5% freshly prepared solution of alloxan (BDH Chemicals, Gillingham, UK) in normal saline was injected into a peripheral vein and, after 4 minutes, 0.5 g/kg of dextrose (25% solution) was injected by the same route. The vascular clamps were released 2 minutes later, after a total of 6 minutes occlusion time. The abdomen was then closed. (The dosage of alloxan required to destroy all the B cells in the head was established in a pilot study of ten animals. This study also showed that if no dextrose was given after the alloxan then there was also massive B cell necrosis in the tail of pancreas).

During the 24 hours post-operative period the animals were nursed in a warm environment and allowed free access to water. Blood glucose levels were checked intermittently. A total of 10g/kg/24 hours of dextrose was administered in divided doses during the first 12 hours post-operatively to prevent hypoglycaemic episodes. This was given either as a 50% solution nasogastrically or as a 15% solution (diluted with 0.9% saline plus 40 mmol/l KCL) subcutaneously if the nasogastric tube was blocked.

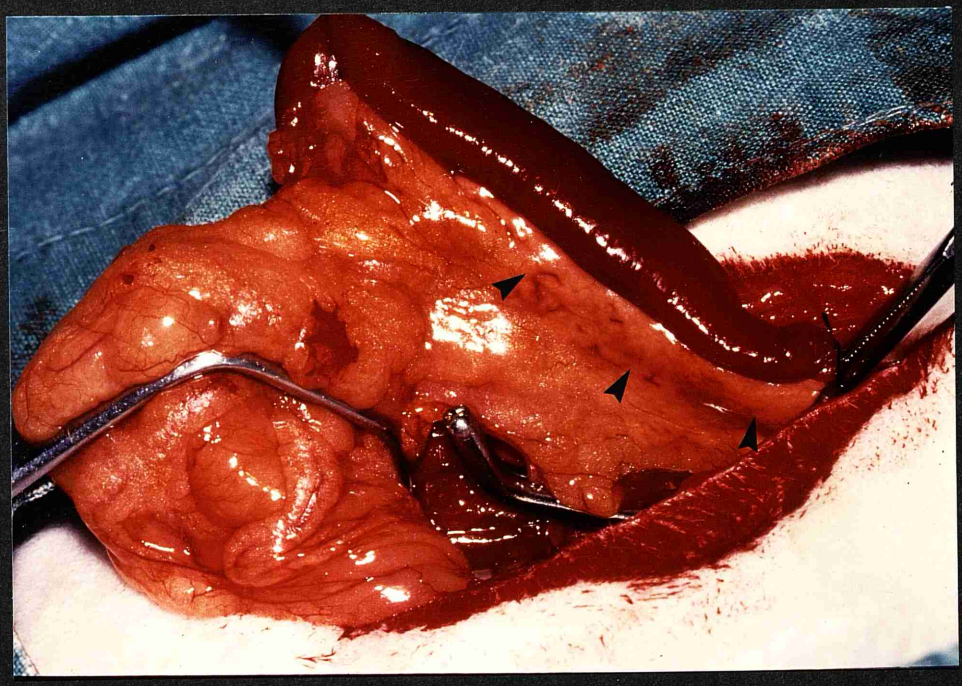
Histology

Post mortem examinations were performed on all animals except one which died pre-operatively. Samples of pancreas, liver and kidney were fixed in neutral buffered formalin. The material was processed in a standard fashion

Figure 3.1

Operative procedure

The spleen is raised up and the tail of pancreas is present along its lower border (arrow heads). The left hand clamp has occluded the short gastric arteries. The right hand clamp has included the junction between body and tail of pancreas (marked by suture). The spleen and tail of pancreas are thus isolated from the circulation.



and embedded in paraffin wax. Five micron sections were cut and stained by haematoxylin and eosin and by indirect immunoperoxidase techniques for pancreatic hormones. The following primary polyclonal antisera were used: guinea pig anti-insulin (Wellcome, Dartford, UK), rabbit anti-glucagon (Guildhay, Guildford, UK) rabbit anti-somatostatin (RIA UK, Tyne and Wear, UK) and rabbit anti-pancreatic polypeptide (Metachem Diagnostics, Northampton, UK). The following bridges for the indirect techniques were used: peroxidase conjugated rabbit anti-guinea pig and swine anti-rabbit immunoglobulins (Dako, High Wycombe, UK). The reactions were developed using diaminobenzidine as substrate.

Glucose Tolerance

After an overnight fast a venous blood sample was taken. The animals were then given an oral glucose load of 1.75g/Kg. Venous blood samples were taken at 30, 60, 90 and 120 minutes for blood glucose estimation.

RESULTS

Early Mortality

Seventeen out of 34 operated animals died in the first post-operative week. Apart from four animals who died of direct surgical complications there was a high mortality due to toxic side effects of alloxan. Eight animals died between 8 hours and 56 hours of severe periportal liver necrosis (Fig 3.2) and 3 between 19 hours and 5 days of acute tubular necrosis of the kidney (Fig 3.3). No obvious cause of death could be found in the remaining two post-operative deaths.

Islet histology in animals which died in the first post-operative week

Apart from a minor degree of necrosis and inflammation at the clamp site, the exocrine pancreas was unaffected by alloxan administration. Necrosis of most islet endocrine cells was evident in the head of pancreas in animals which died more than 5 hours after the operation (Fig 3.4). While islets containing a few necrotic cells could usually be found in the tail, the degree of necrosis was consistently less than that seen in the head of pancreas. (Table 3.1). Three animals died five days post-operatively. In these animals no normal islets were seen in the pancreatic head - only small groups of glucagon containing A cells and somatostatin containing D cells being present. Many islets were present in the tail of pancreas and here mitoses were readily seen (Fig 3.5). Immunohistochemistry showed that the dividing endocrine cells were B cells (Fig 3.6).

Late Deaths

Seventeen animals survived the first post-operative week. One was sacrificed at 5 weeks because of severe diabetes. This animal suffered a respiratory arrest during the operation and as a result no dextrose was given before releasing the clamp to the tail of pancreas four minutes after the alloxan injection. Post mortem histology of the pancreas in this animal showed no surviving B cells in either head or tail. One other non-diabetic animal died of an unknown cause 9 weeks post operatively. The remaining 15 animals were sacrificed between 4 and 12 weeks after surgery and the histology of the head and tail of pancreas compared.

TABLE 3.1 **Islet histology in animals dying within 60 hours of operation.**

Interval between surgery and time of death	Head of Pancreas Islets	Tail of Pancreas Islets
At operation	No histology	No histology
1 hour	+	0
5 hours	+++++	++++
7 hours	+++++	+
8 hours	+++++	+++
8 hours	+++	0
16 hours	+++++	++
19 hours	+++++	++++
20 hours	+++++	+++
27 hours	+++++	+++
40 hours	++++	+
40 hours	+++++	+++
48 hours	++++	0
56 hours	+++++	+

Key to histology table

0 - Normal
\+ - A few islets contain some necrotic cells.
++ - Most islets contain some necrotic cells.
+++ - Some islets are completely necrotic except for a few surviving cells; many islets contain a few necrotic cells and some islets are normal.
++++ - Many islets are completely necrotic, other islets contain some necrotic cells.
+++++ - All islets are completely necrotic except for a few cells.

Islet histology in animals which survived the first post-operative week

That part of the head of pancreas which lies within the duodenal loop was selected to identify the number of surviving B cells seen immunohistochemically in one 5 micron section (Table 3.2). While the minimum number of B cells seen in this area in 3 control animals was 1250 the maximum number identified in the experimental animals was 30. By contrast, although no detailed morphometry was performed on the tail of pancreas there seemed to be no significant loss of B cells in this region (Fig 3.7). Table 3.2 also shows that there was no convincing evidence of regeneration of B cells within the head of pancreas in that the number of B cells seen in animals which were killed more than 10 weeks after the operation was no different from the number seen in animals sacrificed after 4 weeks. The distribution of A and D cells appeared normal in the tail of pancreas. In the head the small groups of endocrine cells which were found consisted primarily of these cells (Figs 3.8 and 3.9). PP cells were only rarely identified in either area.

The acinar tissue was normal with no evidence of atrophy in either head or tail of pancreas in all but one animal. In contrast to the others this exceptional animal had lost weight following the operation. At autopsy, five weeks after surgery, it was found to have a large abdominal abscess. In this animal the acini in the head of pancreas were small and contained fewer zymogen granules than acini in the tail (Fig 3.10).

TABLE 3.2 Surviving B cells in a standard section of pancreas contained within the duodenal loop.*

Number of weeks post-operative	Number of B cells identified
4	1
4	1
4	0
4	28
4	16
5	0
6	6
7	0
8	6
9	4
10	12
11	16
11	30
11	2
12	0

* Minimum number found in control animals was 1250

Figure 3.2

Periportal liver cell necrosis

The pale zones contain necrotic hepatocytes.

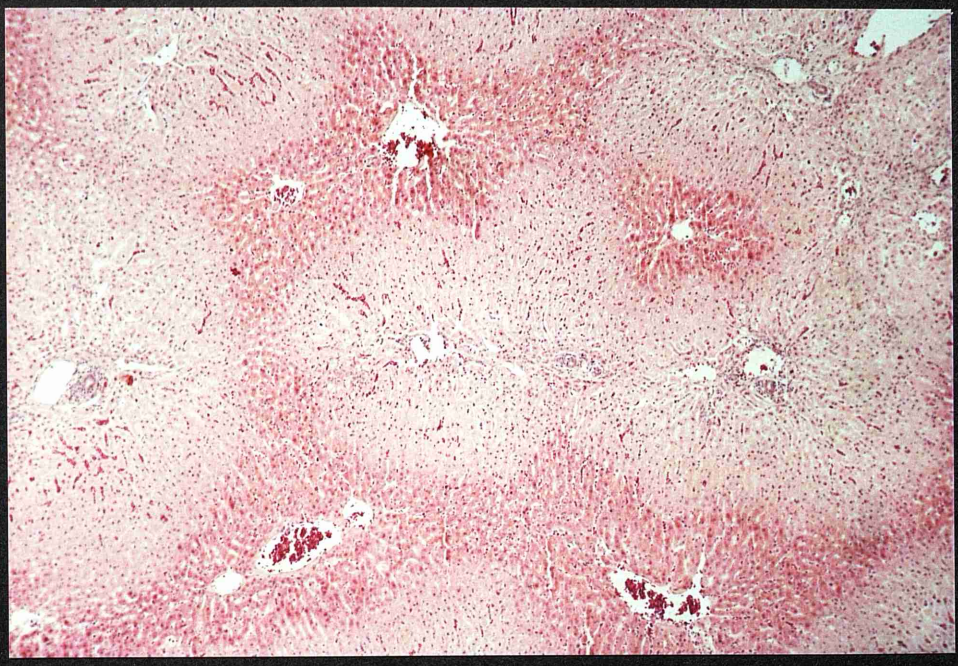
H & E x 44

Figure 3.3

Acute tubular necrosis in the kidney

Several tubules are lined by necrotic epithelial cells which have dark pink cytoplasm and pyknotic nuclei.

H & E x 350



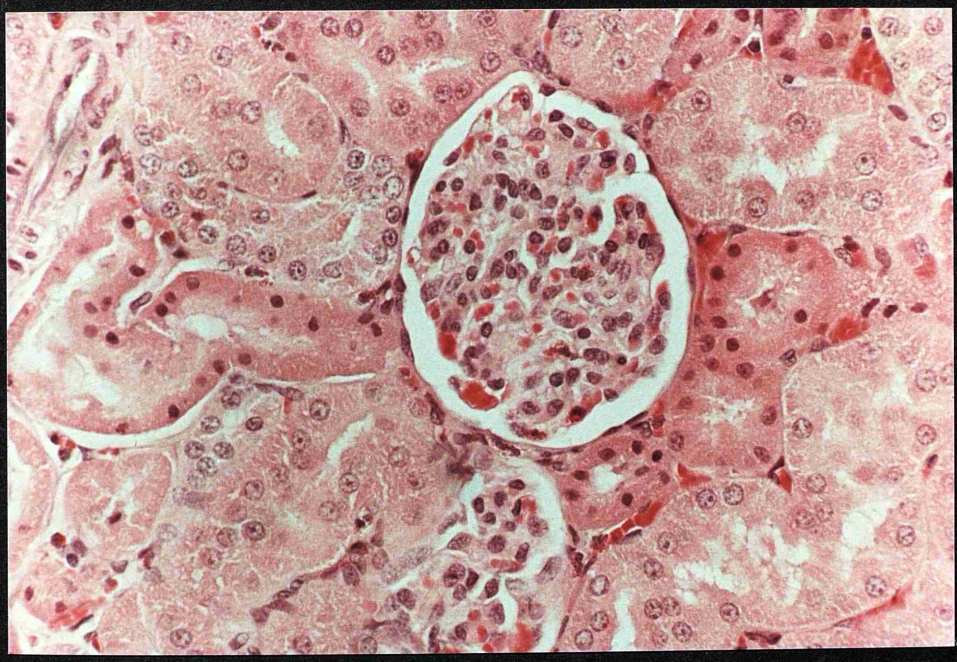


Figure 3.4

Necrosis of endocrine cells in head of pancreas

This animal died 24 hours after surgery. Most of the endocrine cells in these islets are necrotic.

Figure 3.5

Endocrine cell regeneration in tail of pancreas

Mitotic figures can be seen in five endocrine cells in this islet. The animal died five days after surgery.

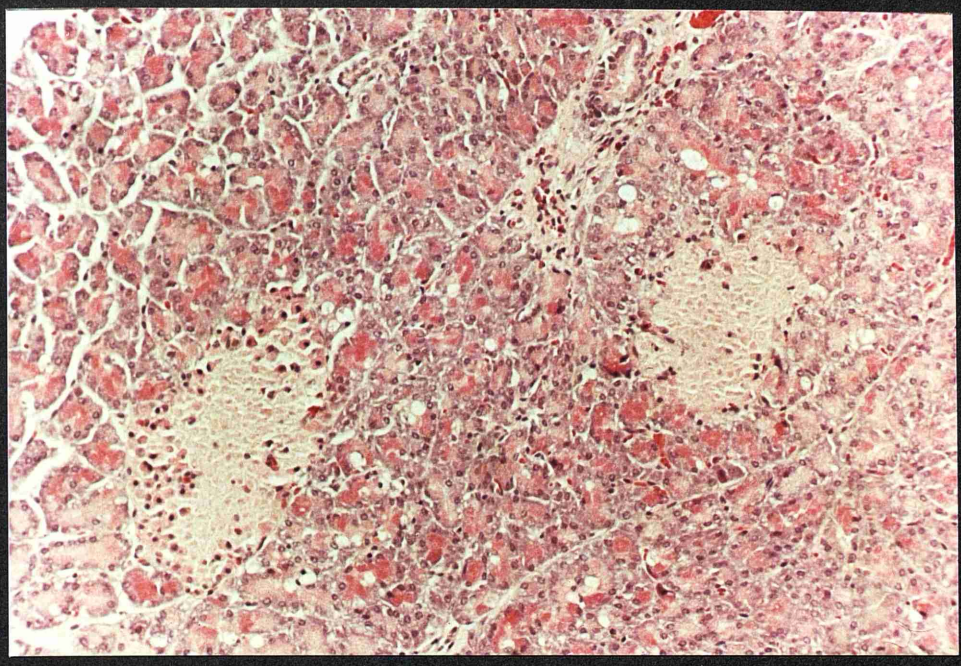
H & E x 550

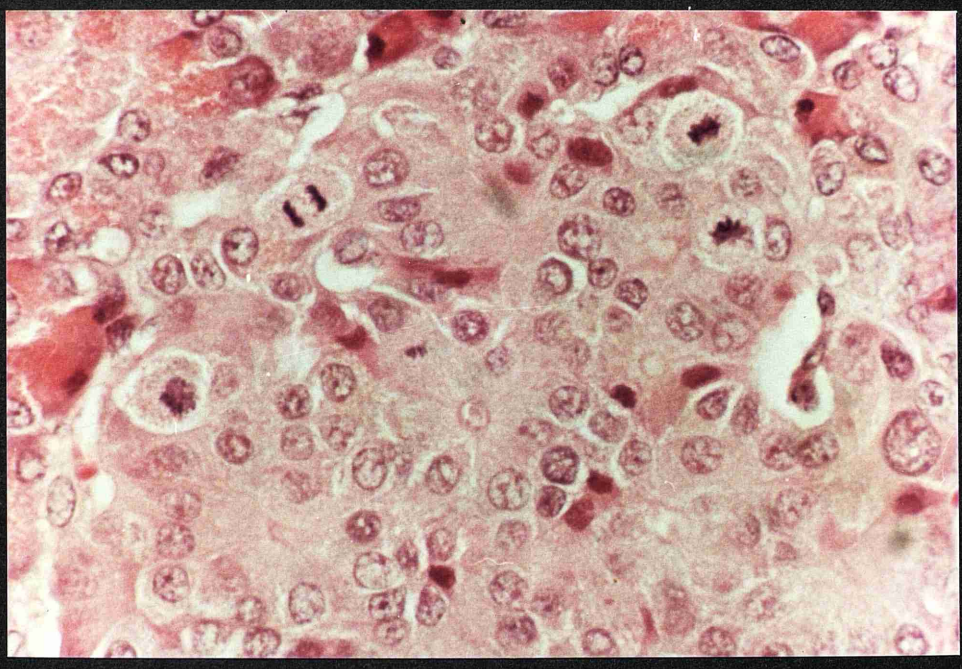
Figure 3.6

B cell regeneration in tail of pancreas

Two mitotic figures are present. Both these cells contain insulin granules. Same animal as that in figure 3.5.

I.P. for insulin x 550





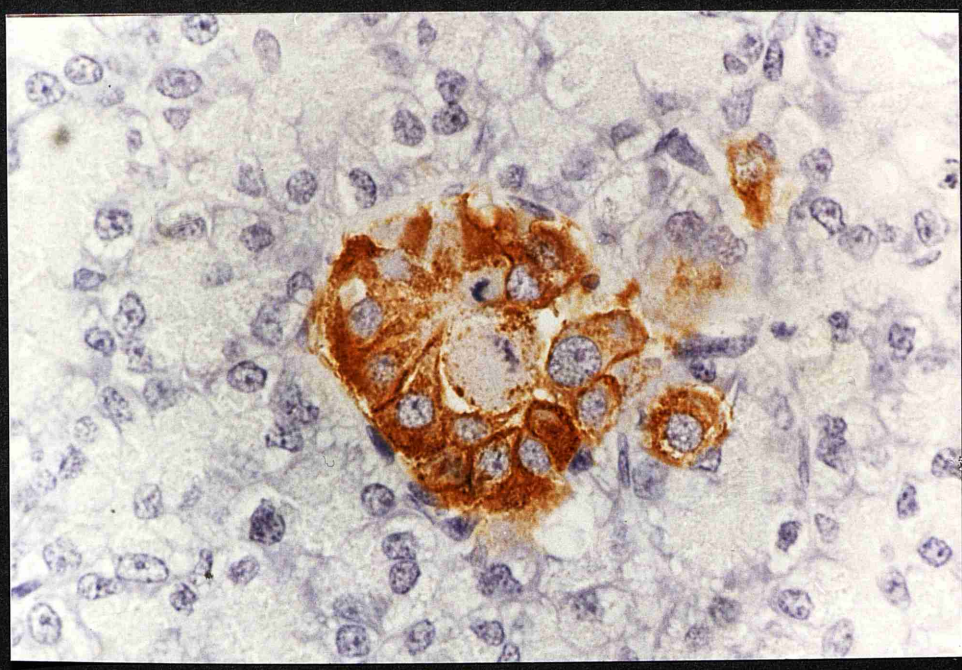


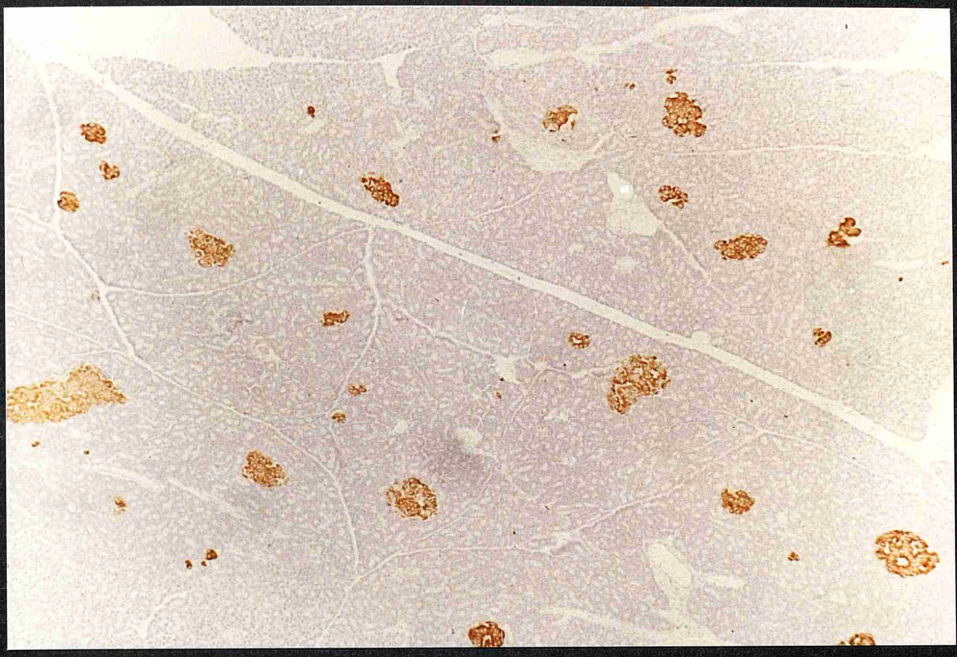
Figure 3.7

The partially diabetic pancreas: distribution of B cells

B cells are well preserved in the tail of pancreas.

B cells are absent from the head of pancreas.

Both I.P for insulin x 55



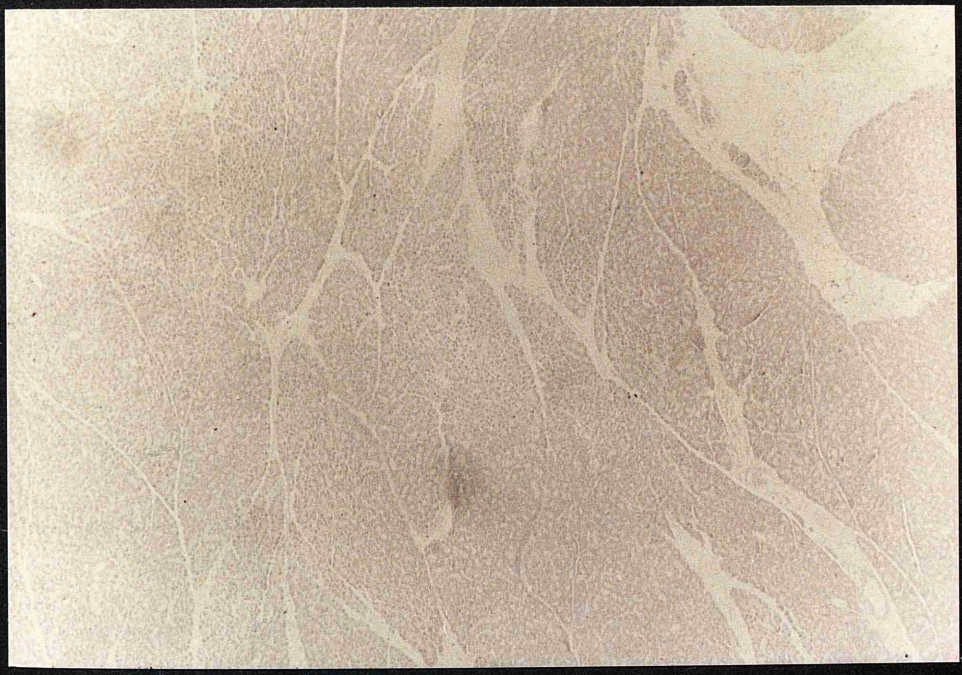


Figure 3.8

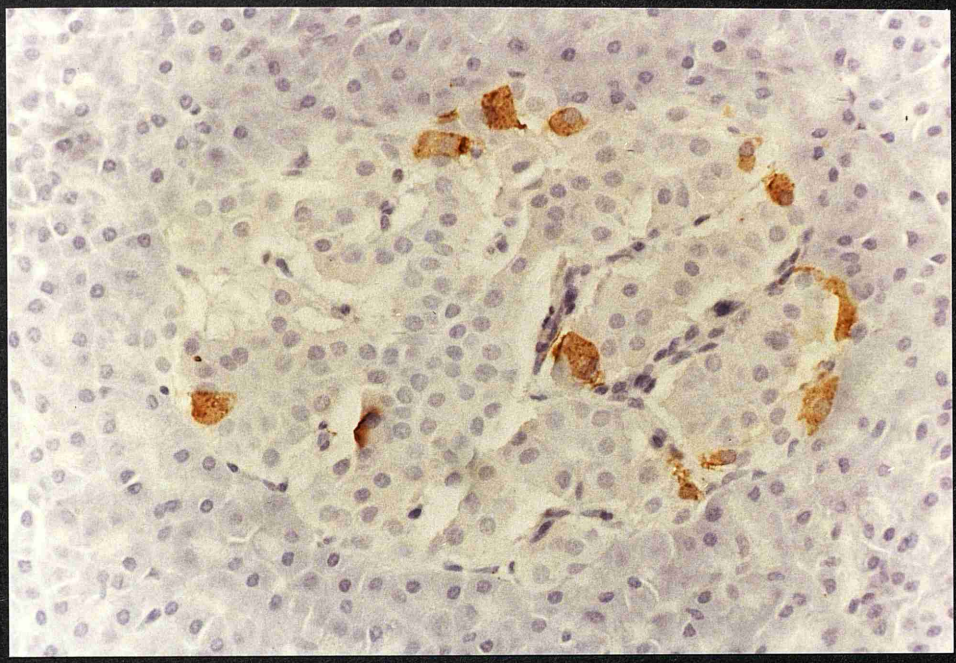
The partially diabetic pancreas: distribution of A cells

Normal islet in the tail of pancreas.

I.P. for glucagon x 330

Residual A cells in head of pancreas. No insulin was detectable.

I.P. for glucagon x 350.



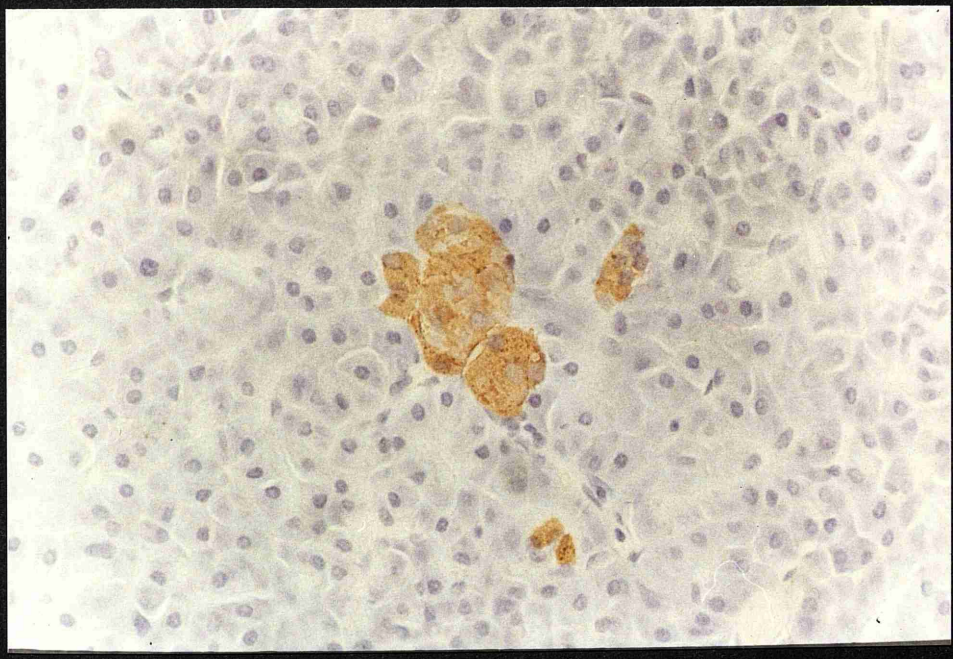


Figure 3.9

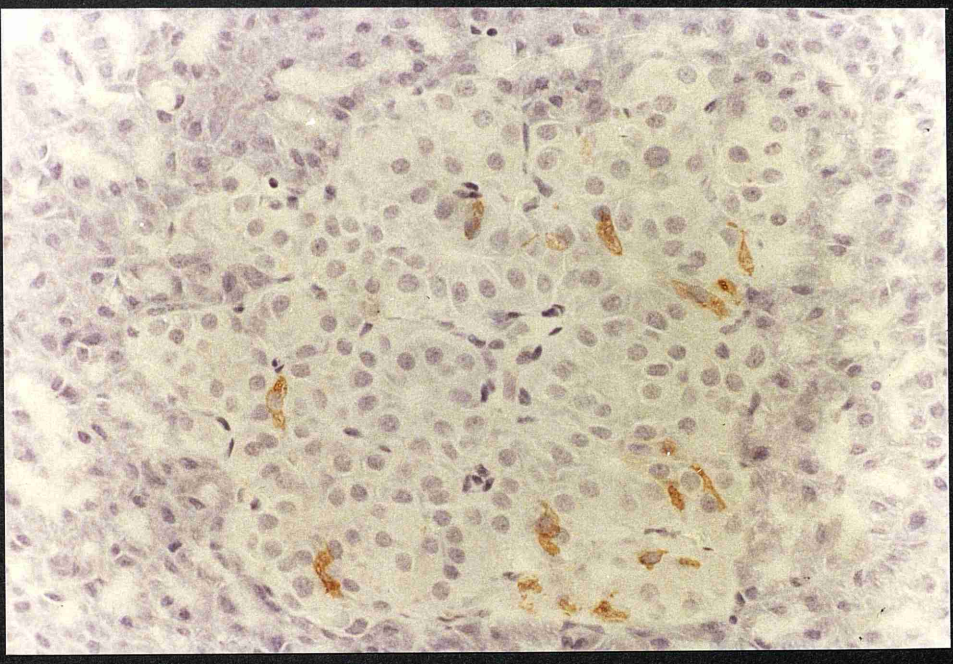
The partially diabetic pancreas: distribution of D cells

D cells in tail of pancreas.

I.P. for somatostatin x 270.

D cells in head of pancreas. No insulin was detectable.

I.P. for somatostatin x 340.



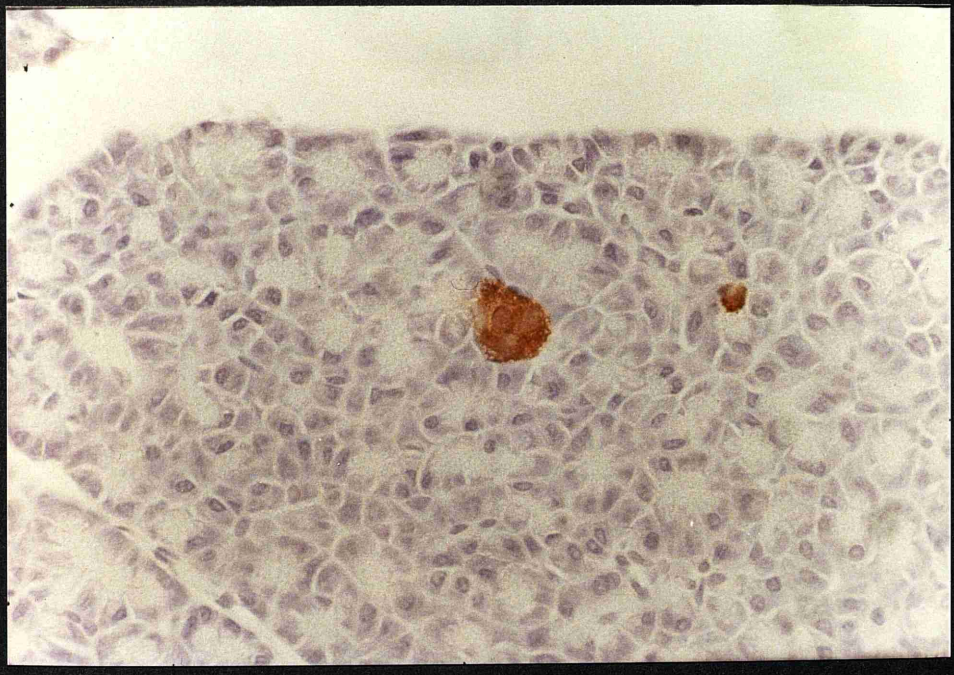


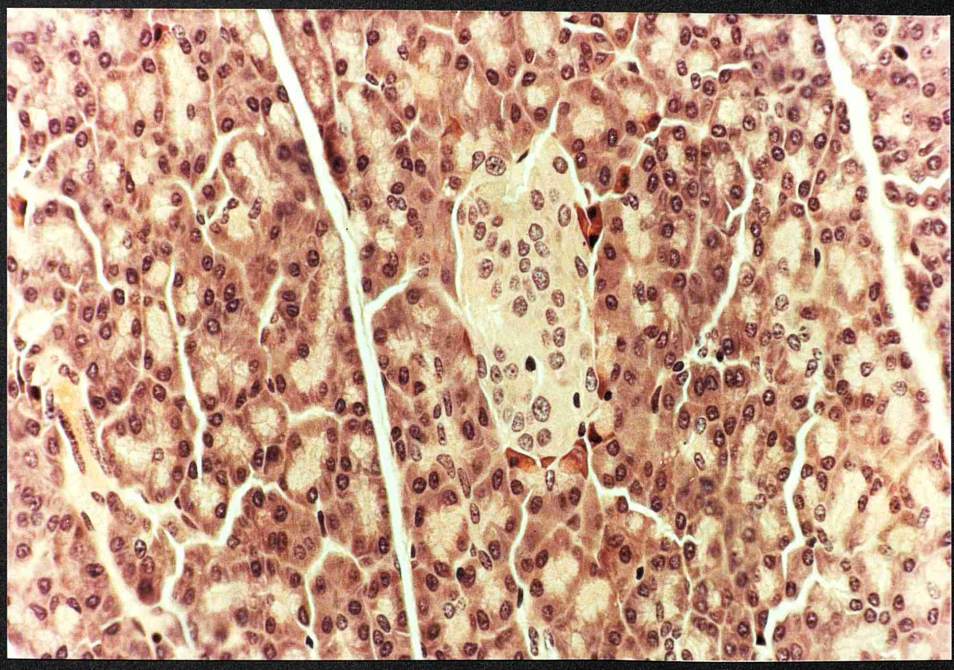
Figure 3.10

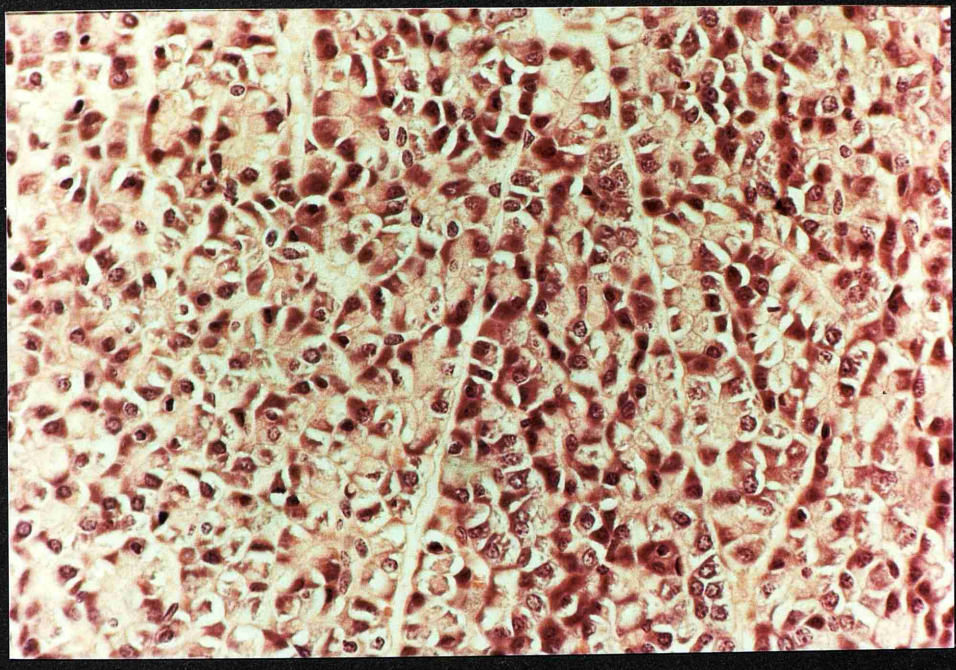
Exocrine atrophy in insulin deficient area of pancreas in catabolic animal

Normal acinar tissue in insulin containing tail of pancreas.

Acinar degranulation in insulin deficient head of pancreas.

Both H & E x 340





Biochemical findings

The fifteen experimental animals described above had normal fasting blood glucose concentrations prior to sacrifice (3.7 - 6.0 mmol.litre). The range in 15 control rabbits was 4.1 - 6.3 mmol/litre. An oral glucose tolerance was performed on 3 experimental animals and there was no evidence of diabetes. The maximum blood glucose achieved in these animals was 10.8 mmol/litre one hour following the oral glucose. At 2 hours the maximum was 8 mmol/litre.

DISCUSSION

The rabbit was the animal of choice for this model for two reasons. The blood supply of the rabbit islets of Langerhans has been studied carefully and shown to be similar to that of the human (Vandamme et al 1968, Henderson and Daniel 1979). In addition, the size of the animal made the operative technique and post-operative management relatively straightforward.

Since individual animals have varying susceptibility to alloxan (Rerup 1970) a high dose (200mg/Kg) of the drug was required to consistently destroy the vast majority of B cells in the desired area. In the canine experiments described by Gomori and Goldner (1945) a lower, subdiabetogenic dose of alloxan ($\leqslant$ 100mg/Kg) was given during the period when part of the pancreas was clamped. Thus in only 3 out of 13 dogs were all the B cells said to be destroyed in the unclamped part of pancreas. Since this assessment was made prior to the use of sensitive immunohistochemistry even this may have been an overestimate. In five dogs no abnormality was seen in any part of the pancreas so these

authors did not succeed in producing a partially diabetic pancreas such as is described here.

The unpredictable nature of alloxan was exemplified by the presence of severe hepatic and renal damage in some animals but not others. While alloxan-induced renal damage is well recognised (Rerup 1970) liver damage due to alloxan has been infrequently recorded (Lazarow & Palay 1946). The latter was the principal cause of early post-operative death in the present series. Dextrose has been shown to inhibit alloxan induced B cell necrosis (Zalawich & Biedler 1973) and the dextrose infusion following alloxan was designed to limit the degree of B cell damage in the tail of pancreas after release of the clamp. (Dextrose was not used in Gomori & Goldner's model). One animal which did not receive dextrose at this time developed severe diabetes post-operatively (blood sugar 34 mmol/litre) and histology showed no residual B cells anywhere in the pancreas. A possible strategy to overcome the alloxan induced hepatic necrosis may therefore be to establish a low dose dextrose infusion into the portal vein during the alloxan administration. This may allow a sufficiently high intrahepatic dextrose concentration to inhibit the liver damage without raising the systemic blood sugar to a level which would interfere with the B cell necrosis in the pancreatic head.

The results in the animals which survived the post-operative period confirmed that alloxan specifically damaged B cells within the pancreas and that while the tail of pancreas was histologically indistinguishable from normal, there was a virtual total ablation of B cells from

the head and body of pancreas. In this respect, therefore, the head and body resembled the pancreas of patients with long-standing type 1 diabetes while the tail was normal. Thus a partially diabetic pancreas was produced. While it is estimated that B cells were destroyed in about 70% of the pancreas the animals appeared to have normal glucose tolerance.

With one exception, the exocrine tissue in all animals was histologically normal, with no difference between insulin containing and insulin deficient areas. This contrasts with the findings described in the previous chapter where insulin deficient islets were surrounded by small degranulated acini. Exocrine atrophy was found in the insulin deficient part of the pancreas in one animal, which was catabolic due to a large abdominal abscess. This animal had lost weight following the operation while the other animals had increased their weight by up to 50%. Catabolic states, such as sepsis, result in a very high glucagon output. (Unger 1981). Contrary to what might initially be expected, diabetic ketacidosis is also accompanied by very high glucagon output (Unger 1981). Thus it seems that the acinar degranulation and atrophy, seen in the children dying of diabetes and the partially diabetic rabbit with sepsis, may have been due to a combination of high local glucagon and low local insulin rather than to low local insulin per se. No attempt was made to make any other animals catabolic deliberately as this was thought to be unethical.

Assuming that one can extrapolate from rabbit to man, the findings here suggest that the degranulation and

shrinkage of acini seen around insulin deficient islets in type 1 diabetes may not be exactly the same phenomenon as the long term weight loss in the type 1 diabetic pancreas. The former may be reversible if the stimulus to high glucagon output (ketoacidosis) is corrected. It still remains possible that local insulin deficiency occurring over years could be the cause of the loss of weight in the glucagon rich lobe. This could be investigated in a long term experiment using the present animal model.

This histological study was limited to showing that a true partially diabetic pancreas had been produced. The model has many possible uses. It should firstly prove suitable for studying the role of locally produced insulin on the synthesis of amylase and other exocrine proteins. The rate of synthesis of the enzymes could be measured in both fasting (low insulin secretion) and fed (high insulin secretion) states. Any differences observed between the head and tail of the pancreas should be insulin dependent.

There was probably a minor degree of B cell necrosis in the tail of pancreas in most animals in this study (Table 3.1). In the animals which died 5 days after surgery numerous mitotic figures in B cells in the tail of pancreas were seen, suggesting significant regeneration of these cells at this time. Interestingly, there was virtually no evidence of regeneration of B cells in the head of pancreas during the period of the experiment (Table 3.2). Taken together these findings suggest that in the young rabbit B cells can regenerate from other differentiated B cells but that if all B cells are destroyed in an area then new B cells cannot be produced

from either other endocrine cells or uncommitted stem cells. The model could therefore be used to formally study B cell regeneration, but as with all animal experimentation the risk remains that any findings would not necessarily be applicable to man.

Finally, by altering the position of the vascular clamps on the body of the pancreas it should be possible to produce animals with varying degrees of residual B cell mass. At present there are no well established clinical tests which can measure the B cell mass of a patient. Such a test would have a role, for example, in studying at what point B cell destruction takes place in the prediabetic period in complement fixing islet cell antibody positive individuals included in family studies of type 1 diabetes. Any novel biochemical tests designed to examine B cell mass could be standardised using animals such as are described here, in which a variable and measurable reduction in B cell mass had been produced.

CHAPTER 4

CHAPTER 4

Major histocompatibility complex antigens and type 1 diabetes.

Part 1

Introduction - Class II Major Histocompatibility Complex and Organ Specific Autoimmunity in Man

The class II major histocompatibility complex (MHC) genes code for the membrane bound glycoproteins HLA-DP, HLA-DQ and HLA-DR in man. (Since the separate functions of these different proteins is not known they will be referred to collectively as HLA-DR). There is an epidemiological link between specific HLA-DR antigens and particular autoimmune diseases. As has been mentioned 98% of patients with type 1 diabetes are of DR3 and/or DR4 genotype (Wolf et al. 1983). The relative risk of Sjögren's disease and thyrotoxicosis in DR3 individuals is x 9.7 and x 3.7 respectively (Roitt 1984). The present introduction explores some possible mechanisms which might explain this genetic linkage.

While class I MHC antigens (HLA-A, B and C in man) are present on most nucleated cells in the body, cellular expression of HLA-DR is more restricted. DR antigens are found on B lymphocytes, activated T lymphocytes, antigen presenting cells (eg interdigitating reticulum cells, macrophages and Langerhans cells) endothelial cells, Kupffer cells and certain epithelial cells including mammary gland ducts, small intestinal, lingual and tracheal epithelium, and bronchial glands (Natali et al. 1981, Daar et al. 1984).

It is now thought that a major function of MHC products is to facilitate antigen presentation (Alberts et al. 1983). T helper lymphocytes, which will initiate an immune response, only recognise the antigen to which they are directed if it is "presented" to them by a cell expressing the same class II MHC protein as themselves. Such antigen presenting cells normally phagocytose foreign antigen, "process" it - a means by which the antigen is subjected to proteolytic digestion into smaller units - and then transport the antigenic fragments onto the cell surface adjacent to their own HLA-DR molecules. Similarly cytotoxic T lymphocytes only recognise the antigen to which they are directed if it is expressed on a cell surface adjacent to a class I MHC protein which they share with the presenting cell.

It has recently been proposed that lack of reactivity to certain cell surface antigens is due to lack of effective antigen presentation (Cowing 1985). If these surface antigens were restricted to HLA-DR negative cells they would not be presented to potentially autoreactive T helper lymphocytes. The corollary of this is that if a cell which did not normally express HLA-DR on its surface were induced to do so, then it might be converted into a functioning antigen presenting cell, purely by virtue of its HLA-DR expression. It might then present one of its own cell surface antigens, for which there was no tolerance, and this may be recognised by potentially autoreactive specific T helper lymphocytes (Bottazzo et al. 1983).

This hypothesis was prompted by observations on

the thyroid (Hanafusa et al. 1983). Thyroid epithelial cells did not normally express HLA-DR, as assessed by immunohistochemistry. A proportion of thyrocytes from thyrotoxic glands and most thyrocytes from glands affected by Hashimoto's thyroiditis did express HLA-DR (Hanafusa et al. 1983, Jansson, Karlsson & Forsum 1984, Aichinger, Fill & Wick 1985, Lloyd et al. 1985). (Figs 4.1, 4.2, 4.3). Since thyroid cells are known to express unique surface antigens, they could have been presented for the first time as a result of the induced HLA-DR expression. The end result of stimulating such an autoreactive T cell response, with its associated T and B lymphocyte co-operation, might be the production of either cytotoxic T cells and destruction of thyrocytes, as in Hashimoto's thyroiditis or primary myxoedema, or production of autoantibodies to, for example, the TSH receptor with resulting thyrotoxicosis. The hypothesis offers possible explanations for the genetic linkages observed between particular HLA-DR groups and autoimmune diseases. For example, certain HLA-DR antigens may be more readily expressed than others on a target cell following an appropriate stimulus. Alternatively the particular shape, charge or other property of a given class II MHC protein could make it act as an "immune response gene" i.e. once expressed it might be more capable of presenting particular autoantigens on the target cell than other class II MHC proteins.

The questions raised by these observations include 1) What agents are known which can cause aberrant expression of HLA-DR on thyroid cells ? 2) Are thyrocytes expressing HLA-DR capable of acting as functional antigen

Figure 4.1

Class II MHC and thyroid autoimmunity: Ashkenazy cells

Focal thyroiditis and Ashkenazy cells were present in this thyroid., Membrane staining for class II MHC is seen on these large Ashkenazy cells.

I.P. using TAL-1B5 x 350

Figure 4.2

Class II MHC and Thyroid Autoimmunity: Focal thyroiditis

Note the staining of thyroid follicular cells adjacent to the focus of inflammation (arrowed), but absence of such staining away from the inflammatory infiltrate.

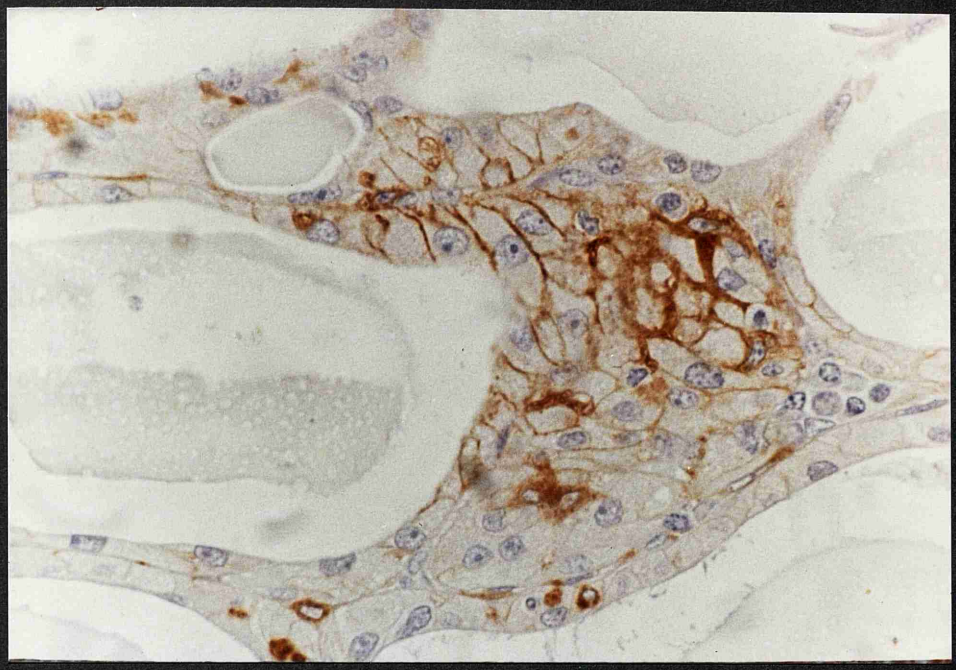
I.P. using TAL-1B5 x 100

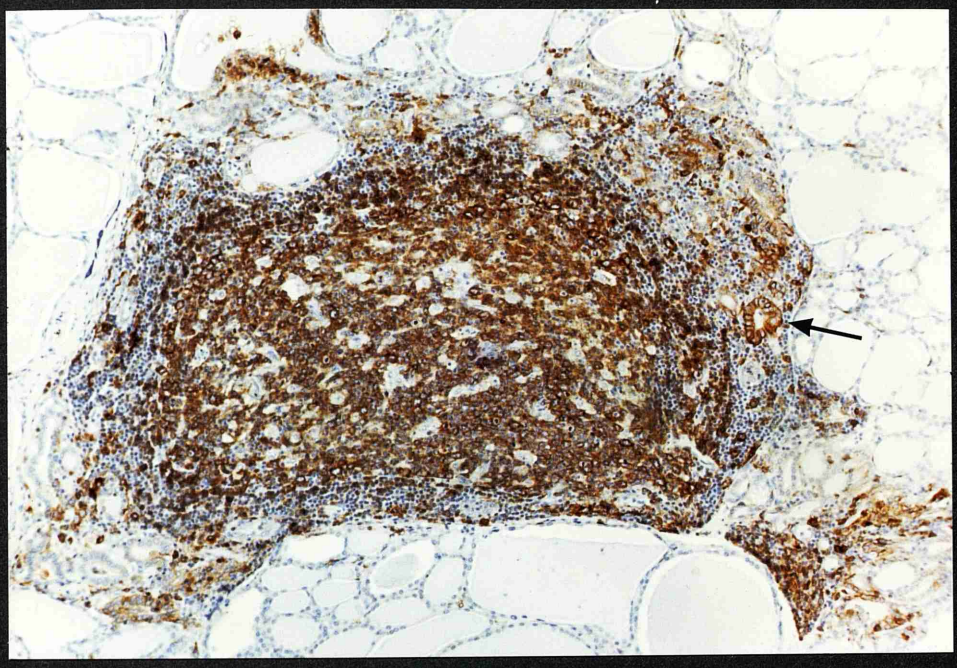
Figure 4.3

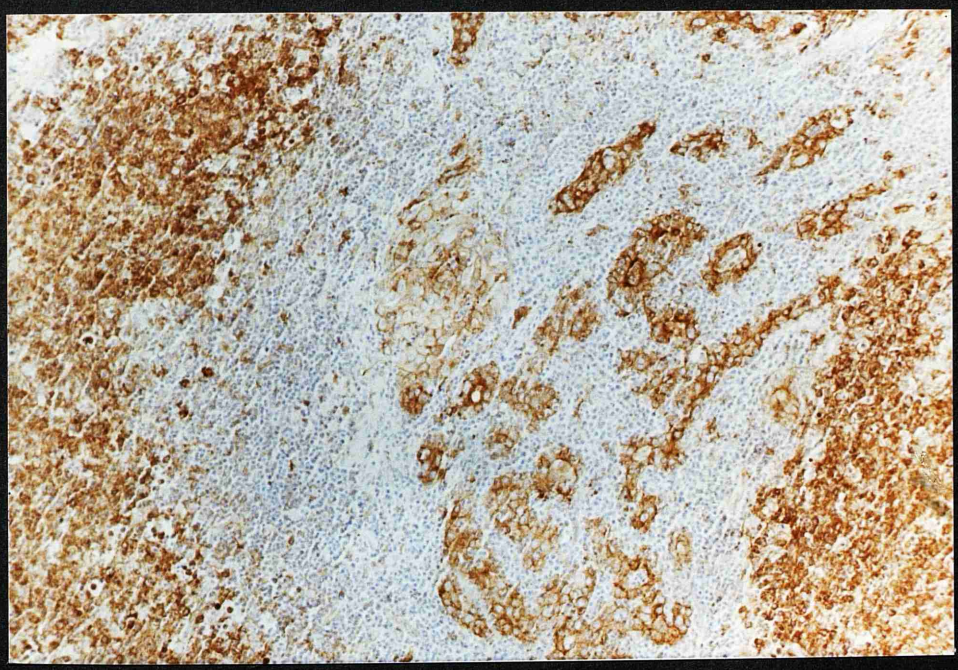
Class II MHC and Thyroid Autoimmunity: Hashimoto's Thyroiditis

Class II MHC positive lymphocytes are present at the lateral margins of the photograph. In the centre are class II MHC positive thyroid follicular cells.

I.P. using TAL-1B5 x 110







presenting cells, presenting either exogenous antigen or cell surface antigen to T helper lymphocytes in vitro and in vivo ? 3) Is aberrant expression of HLA-DR seen on the target cells in other organ specific autoimmune diseases ?

In vitro induction of HLA-DR on thyrocytes

When normal human thyroid cells were cultured in the presence of the lectins phytohaemagglutinin, concanavalin A, pokeweed mitogen or leucoagglutinin, a proportion of thyrocytes were induced to express HLA-DR (Pujol-Borrell et al. 1983, Davies 1985). Of perhaps more biological significance, interferon-γ, a lymphokine produced by T lymphocytes, also induced HLA-DR expression. Interferon-α and interferon-β did not possess this property (Todd et al. 1985).

Antigen presentation by HLA-DR positive thyrocytes

A T lymphocyte clone (HA 1.7) which reacts specifically with a defined peptide, p20, part of the HA-1 molecule of influenza strain A haemmagglutinin, has been described. Purified thyrocytes from a patient with Graves' disease were cultured in the presence of the antigen p20, and the clone HA 1.7. The thyroid cells were spontaneously HLA-DR positive in culture and there was a proliferative response by T lymphocytes, showing that the p20 antigen had been presented by the HLA-DR positive thyroid cells to the T cell clone. When killed whole virus was added instead of p20 the T cell clone did not proliferate, indicating that the thyroid cells were incapable of "processing" the whole virus and presenting the specific peptide p20 (Londei et al. 1984).

Presentation of thyroid cell surface antigens by HLA-DR positive thyrocytes

Thyrocytes and lymphocytes were cultured from two glands removed because of Graves' disease. T lymphocyte clones were prepared from the intrathyroidal lymphocytes and a proliferative assay was performed to see whether any such clones reacted with autologous thyroid follicular cells. Several of the isolated clones proliferated in the presence of the spontaneously HLA-DR positive cultured autologous thyroid cells. These clones were T helper lymphocytes and the proliferative reaction was inhibited when monoclonal antibodies to class II MHC antigens were included in the the system (Londei, Bottazzo et Feldmann, 1985). Thus it appeared that T helper lymphocytes in thyrotoxic glands were capable of responding to autologous surface antigens on HLA-DR positive thyrocytes.

A further related experiment using thyrocytes from normal individuals has been reported. Normal thyrocytes were cultured *in vitro* in the presence of the lectin leucoagglutinin and induced to express class II MHC antigen. HLA-DR positive (but not negative) thyrocytes caused proliferation of autologous T lymphocytes prepared from peripheral blood. Again, the reaction was inhibited by monoclonal antibodies to HLA-DR (Davies 1985). This appeared to show that potentially autoreactive T cells were present in the peripheral blood and that they could be activated by aberrant HLA-DR expression on thyroid cells.

On the basis of this evidence it has been suggested that a focus of inflammation within the thyroid, perhaps due to viral infection, could result in release of

interferon-γ by activated T cells. This might cause HLA-DR expression on adjacent thyrocytes which in turn could result in presentation of thyroid specific surface antigens to autoreactive T helper lymphocytes (Bottazzo et al. 1983). These lymphocytes could release more interferon-γ resulting in more aberrant HLA-DR expression on thyrocytes and so a vicious circle could be initiated.

Other organ-specific autoimmune diseases

Aberrant expression of class II MHC antigens was seen on bile ducts in 8 out of 10 cases of primary biliary cirrhosis (Ballardini et al. 1984) (Fig 4.4). Such expression was only rarely observed on bile ducts in a variety of other liver disorders and then only in the presence of marked periductal inflammation. It has been argued that HLA-DR expression on target cells in autoimmune disease may be the result of an autoimmune response and not the cause of it (Mowat, 1985). Support for this view was the finding of such expression on bile ducts in a rejected liver transplant (Takacs et al. 1983).

HLA-DR expression on salivary ductal and acinar epithelium was seen adjacent to areas of lymphocytic infiltration in patients with Sjögren's syndrome (Lindahl et al. 1985). However, control tissue from glands in which there was sialadenitis but no serological evidence of Sjögren's syndrome was not included in this study, and thus it is not certain whether aberrant expression of HLA-DR on salivary epithelium is specific for the disease.

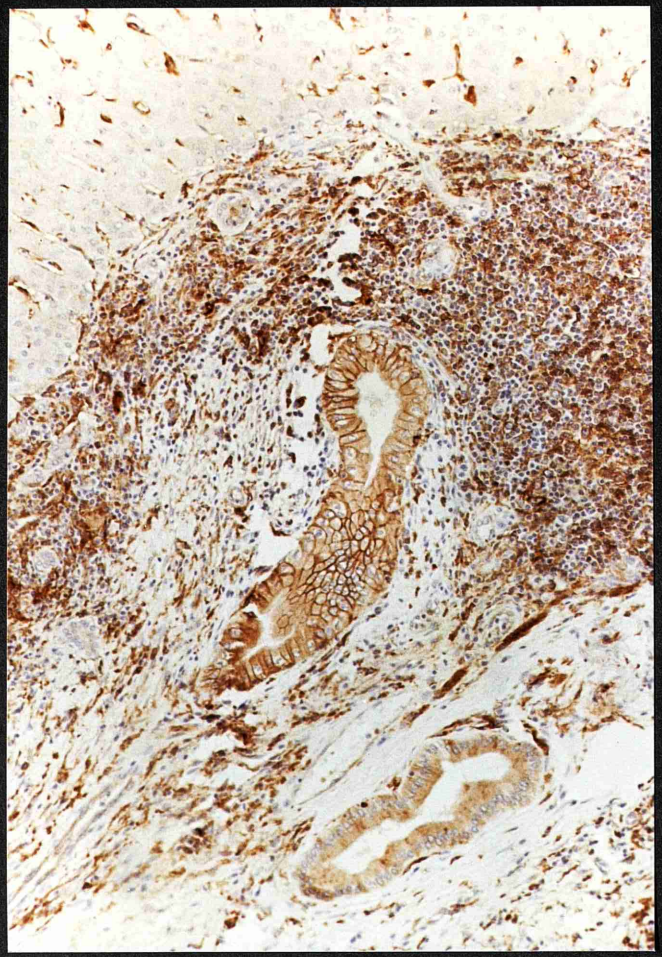
While it has been argued that HLA-DR expression on certain cells may trigger autoimmunity the subsequent immune response will involve proliferation of suppressor T

Figure 4.4

Class II MHC in Primary Biliary Cirrhosis

There is marked positivity for class II MHC with membrane accentuation on the epithelial cells of the right hand duct.

I.P. using antiserum A II x 350



lymphoctyes as well as cytotoxic T lymphocytes and B lymphocytes. Whether the initiated autoimmune reaction progresses to cause disease or not will depend on the presence or absence of suppressor cell activation. If this is deficient then autoimmune disease may result. If not, there may be a transient appearance of autoantibodies but no long lasting disease. Indeed it has been argued that the initial trigger of aberrant HLA-DR expression may not necessarily lead to tissue destruction or disease but to active tolerance through the generation of T lymphocyte suppressor clones (Iwatani, Row & Volpé. 1985).

The pancreas in type 1 diabetes offers perhaps a unique opportunity to test the hypothesis that aberrant expression of HLA-DR is involved in the induction of autoimmunity since of the four hormone producing cell types in the endocrine pancreas only the B cells are destroyed. Thus it might be predicted, that since pancreatic endocrine cells do not normally express HLA-DR (Natali et al. 1981, Alejandro et al. 1982), only the B cells would express HLA-DR aberrantly in type 1 diabetes. In one published case report of a child who died of recent-onset diabetes in whom frozen pancreas was available for study, such aberrant expression of HLA-DR was seen (Bottazzo et al. 1985). However this study was far from conclusive since few islets were found which showed this phenomenon and only one normal control pancreas was studied.

The remainder of this chapter is devoted to the study of aberrant expression of HLA-DR on pancreatic endocrine cells in type 1 diabetes.

Part 2

Aberrant expression of HLA-DR antigens by insulin containing B cells in recent-onset type 1 diabetes.

INTRODUCTION

The aims of this study have been outlined in part 1 of this chapter. A monoclonal antibody, TAL-1B5, which is directed against a formalin resistant epitope of the non-polymorphic region of the alpha chain of the HLA-DR glycoprotein molecule (Adams, Bodmer & Bodmer. 1983), was used to detect HLA-DR expression on tissue sections. The epitope which this antibody recognises is not preserved if the tissue has been fixed in neutral buffered formalin. This problem does not apply to tissue fixed in Bouin's fluid or formol saline with or without added mercuric chloride. Thus cases of diabetes had to be selected from those described in Chapter 2 on the basis of their fixation. There were a considerable number of suitable cases available from one hospital (Royal Hospital for Sick Children, Glasgow) so it was decided to study them alone, since most of the appropriate control pancreases could be selected from the same source.

PATIENTS AND METHODS

The autopsy records of the Royal Hospital for Sick Children in Glasgow were searched for patients who had died of type 1 diabetes. Fouteen cases were identified in which pancreatic material was available (Table 4.1). The cause of death was ketoacidosis in all cases except patient 4 who died of lobar pneumonia. Twenty-six control patients aged 6 weeks to 12 years who had died of diseases unrelated to the

pancreas and twelve patients aged 2-14 years who had died of cystic fibrosis were selected from the same hospital. Five pancreatic resections for chronic pancreatitis, two autopsy pancreases with pancreatitis and six autopsy pancreases from type 2 diabetic patients (non-insulin dependent) were also studied. All the material from children had been fixed in formol saline with added mercuric chloride while the adult material was fixed in formol saline. The pancreatic specimens had been embedded in paraffin wax. Five micron serial sections were cut from the type 1 diabetic pancreases. These were stained using an indirect immunoperoxidase technique with diaminobenzidine as substrate. The following primary polyclonal antisera were used: guinea pig anti-insulin (Wellcome), rabbit anti-glucagon (Guildhay antisera), rabbit anti-somatostatin (RIA UK Ltd), rabbit anti-pancreatic polypeptide (Milab), rabbit anti-human muramidase (Dako). The monoclonal antibodies PD 7/26 (gift from D.Y. Mason, Oxford) which is directed against the T200 leucocyte common antigen (Warnke et al. 1983), and TAL-1B5 (gift from ICRF, London) were also used. The following bridges for the indirect techniques were used: peroxidase conjugated rabbit anti-guinea pig, swine anti-rabbit and rabbit anti-mouse immunoglobulins (all Dako).

Stains for insulin, HLA-DR and the T200 leucocyte common antigen were done on adjacent serial sections of the diabetic pancreases. Control pancreases were also stained with the hormone antisera and antibodies PD7/26 and TAL-1B5.

Detailed studies of the specificity of the antibody

TAL-1B5 for HLA-DR have been published (Adams et al. 1983, Epenetos et al. 1985). Specificity was established using a solid phase radioimmunoassay on purified HLA-D alpha chains and subsequently by immunoprecipitation analysis and reactivity on a variety of cell lines and tissues (Adams et al. 1983). The distribution of staining in formalin fixed spleen, lymph node and tonsil from the diabetic and control patients used in the present study was compared with staining of normal fresh frozen spleen and lymph node by both TAL-1B5 and a separate monoclonal to HLA-DR (DK 22 from Dako). The results were identical. TAL-1B5 is a mouse IgG1 antibody. When the unrelated IgG1 mouse monoclonal antibody, anti-neurofilament 160KD (Boehringer), was substituted for TAL-1B5 only nerve fibres and smooth muscle cells were stained. In particular no staining was seen on pancreatic endocrine cells in either diabetic or control pancreases and lymphoid cells and endothelial cells were consistently negative. The specificity of PD7/26 for leucocytes and lymphoid cells had been tested in our laboratory on a wide range of normal tissues and tumours.

Double staining techniques were developed to show which hormone producing cells expressed HLA-DR. Following an overnight incubation at 4°C of the monoclonal antibody TAL-1B5, the sections were incubated for 30 minutes with peroxidase conjugated rabbit anti-mouse immunoglobulins. The reaction was developed using diaminobenzidine as substrate. Double staining for insulin was done using a subsequent 4 hour incubation of the guinea pig anti-insulin antiserum followed by a 30 minute incubation of rabbit anti-guinea pig immunoglobulins. This was followed by a 30

minute incubation of rhodamine conjugated swine anti-rabbit immunoglobulins (Dako). Repeated washings were done between steps using tris buffered saline. Double staining for HLA-DR and the other hormones utilised the same method up to the development with diaminobenzidine. This was followed by a 15 minute incubation with normal swine serum, a 4 hour incubation with the rabbit anti-hormone antibodies (see above) and finally a 30 minute incubation with rhodamine conjugated swine anti-rabbit immunoglobulins (Dako). Double staining of sections for insulin and muramidase were also performed, the latter being an established marker for macrophages in formalin fixed tissues (Meister, Huhn & Nathrath 1980). Following a 15 minute incubation with normal swine serum, sections were incubated with rabbit anti-human muramidase (Dako) diluted 1/100 for 4 hours. A standard indirect immunoperoxidase technique was then performed with peroxidase conjugated swine anti-rabbit immunoglobulins (Dako) and diamino-benzidine as substrate. Subsequently, guinea pig anti-insulin (Wellcome) diluted 1/4000 was placed on the sections for 4 hours and this was followed by incubation with fluorescein conjugated swine anti-guinea pig immunoglobulins (Nordic). Again, repeated washings with tris buffered saline were done in between incubations. Double stained sections were viewed using a Leitz Orthomat microscope. Control pancreases and spleens were tested on all occasions and there were no cross reactions observed between the two steps of these double techniques.

RESULTS

Normal autopsy pancreas

The distribution of endocrine cells within islets and within different regions of the pancreas was normal in all cases. No expression of HLA-DR was seen on endocrine cells. Endothelial cells both in endocrine and exocrine pancreas were consistently positive and in 9 of the 26 cases scattered positive duct epithelial cells were also observed. Acini were negative (Fig 4.5).

The pancreas in type 1 diabetes (Table 4.1).

In 2 patients, both of whom had had diabetes for 2 years, all islets were insulin deficient, and no HLA-DR positive endocrine cells were observed in these islets in sections stained using TAL-1B5. While most islets in the remaining 12 cases were also insulin deficient, a proportion did contain insulin. HLA-DR staining on endocrine cells was observed in pancreases from all these patients, including those in whom diabetes had been present for several years. Positive cells showed uniform cytoplasmic staining with no evidence of membrane accentuation. The number of positive endocrine cells seen per section of islet varied from ones with only individual positive cells to ones in which the majority of endocrine cells appeared positive (Fig 4.6, 4.7). Adjacent serial sections were stained for insulin and HLA-DR. This showed that HLA-DR expression on endocrine cells was confined to islets containing insulin (Table 4.1). Using double staining techniques on all the cases it was established that HLA-DR positive endocrine cells did not contain glucagon, somatostatin or pancreatic polypeptide. Only

TABLE 4.1 Type 1 diabetic patients: insulin content of islets, insulitis and expression of HLA-DR on endocrine cells as assessed from consecutive serial sections stained for insulin, leucocyte common antigen and HLA-DR.

CASE	No. of sections	Age	Sex	Duration of Diabetes	No.of ICI's	No.of IDI's	No. of ICI's with HLA-DR positive endocrine cells	No. of IDI's with HLA-DR positive endocrine cells	No. of HLA-DR endocrine positive islets with insulitis	No. of HLA-DR endocrine positive islets without insulitis	No. of ICI's with insulitis	No.of IDI's with insulitis
1	2	5	M	2 yrs	0	252	0	0	0	0	0	0
2	6	10	M	3 wks	165	392	22	0	1	21	5	1
3	1	10	F	2 yrs	0	54	0	0	0	0	0	0
4	1	9	F	2 yrs	2	22	2	0	1	1	1	0
5	2	12	F	3 wks	39	45	10	0	3	7	11	3
6	1	3	F	6 wks	87	66	63	0	15	48	23	1
7	2	8	M	Few mths	72	69	18	0	3	15	4	0
8	1	11	M	2 wks	32	16	14	0	0	14	0	0
9	2	2	M	2 wks	19	224	1	0	0	1	2	0
10	4	11	M	6 yrs	14	267	7	0	1	6	1	0
11	2	3	M	4 dys	23	191	2	0	2	0	5	1
12	3	3	M	3 mths	41	82	13	0	5	8	7	1

TABLE 4.1 continued

CASE	No. of sections	Age	Sex	Duration of Diabetes	No.of ICI's	No.of IDI's	No. of ICI's with HLA-DR positive endocrine cells	No. of IDI's with HLA-DR positive endocrine cells	No. of HLA-DR endocrine positive islets with insulitis	No. of HLA-DR endocrine positive islets without insulitis	No. of ICI's with insulitis	No.of IDI's with insulitis
13	3	6	M	3 dys	114	375	18	0	14	4	64	8
14	1	5	F	12 wks	22	5	1	0	0	1	5	0
					630	2060	171	0	45	126	128	15

ICI = Insulin containing islet
IDI = Insulin deficient islet

insulin containing B cells were shown to double stain for HLA-DR. (Figs 4.8, 4.9, 4.10).

The following experiment was done to demonstrate that cells identified as HLA-DR positive B cells were not endocrine cells which had been engulfed by HLA-DR positive macrophages. Sections from 2 cases in which many HLA-DR positive endocrine cells were present were chosen. One section was stained by the indirect immunoperoxidase technique for HLA-DR. The adjacent serial section was then double stained for muramidase (a macrophage marker), using a similar indirect immunoperoxidase technique, and insulin, using an indirect immunofluorescence technique. Thirty-six islets, each of which was shown to contain multiple HLA-DR positive endocrine cells, were studied. Insulitis was present in 17 of these islets and, in keeping with previous experience, macrophages were scanty in the inflammatory infiltrate (Bottazzo et al. 1985). A single macrophage adjacent to one of the islets stained also for insulin. No cells which were morphologically endocrine were muramidase positive and all the islets which were assessed contained insulin positive cells (Fig 4.11).

HLA-DR expression on endocrine cells and insulitis.

The results in table 4.1 represent the findings from three serial sections stained for the presence of the T 200 leucocyte common antigen (to identify insulitis), insulin and HLA-DR. This showed that there was no evidence of insulitis in the majority of islets in which HLA-DR expression on endocrine cells was seen. The inflammatory infiltrate of insulitis may be quite focal within an islet and thus insulitis could be missed in 3 sections of an

islet. Thirty further serial sections were cut from the pancreas of case 8. The middle section was stained for HLA-DR and this identified 12 islets with multiple positive endocrine cells. Focal insulitis was identified in only one of the 12 islets when the remaining sections were stained for the leucocyte common antigen. Table 4.1 also shows that the majority of islets with insulitis, even though they contained insulin, did not appear to contain HLA-DR positive endocrine cells. A further 25 serial sections were cut from 1 block of case 5 and insulitis was identified immunohistochemically in 10 islets in the middle section. HLA-DR positive endocrine cells were identified in the remaining sections in all 10 islets, although in some of these islets very few such cells were seen.

In the majority of islets with HLA-DR positive endocrine cells the endothelial cells of the islet sinusoids appeared swollen and stained more heavily for HLA-DR than controls. A similar finding was present in the islets where there was insulitis. Many of the inflammatory cells in the islets were HLA-DR positive (Fig 4.7).

HLA-DR expression in pancreatic diseases other than type 1 diabetes

No HLA-DR positive endocrine cells were identified. The majority of pancreases with residual acinar tissue from cystic fibrosis patients had scattered positive duct and acinar cells; this was also observed in some of the pancreases with pancreatitis. No aberrant HLA-DR expression was seen in pancreases from type 2 diabetic patients.

Figure 4.5

Class II MHC in normal islet

All endocrine cells are negative. Capillary endothelial cells are positive.

I.P. using TAL-1B5 x 250

Figure 4.6

Class II MHC in type 1 diabetes

Two positive endocrine cells are present lying in a position characteristic of B cells. Note the uniform cytoplasmic staining.

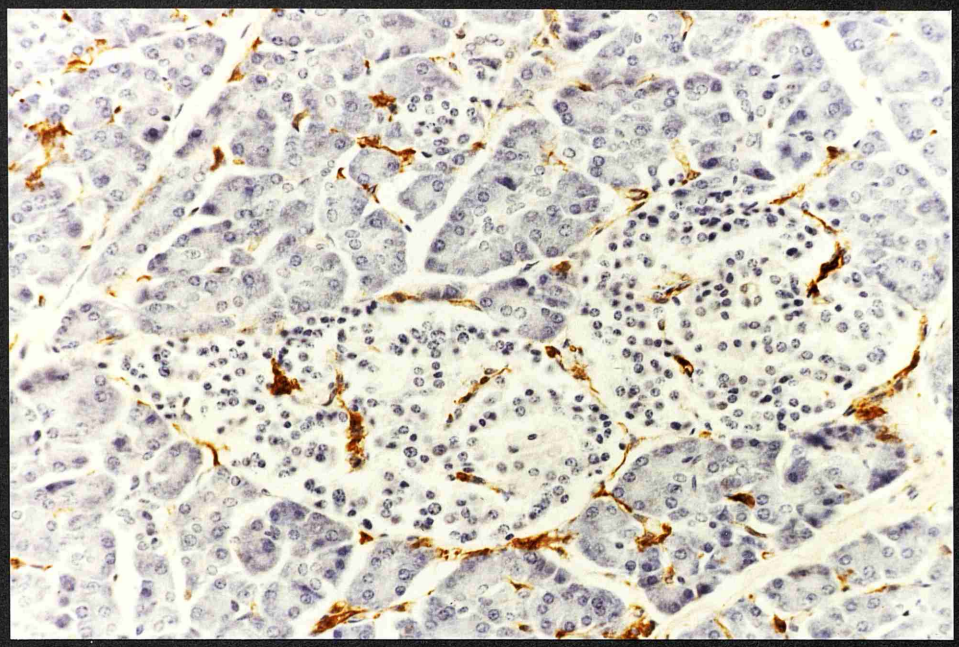
I.P. using TAL-1B5 x 550

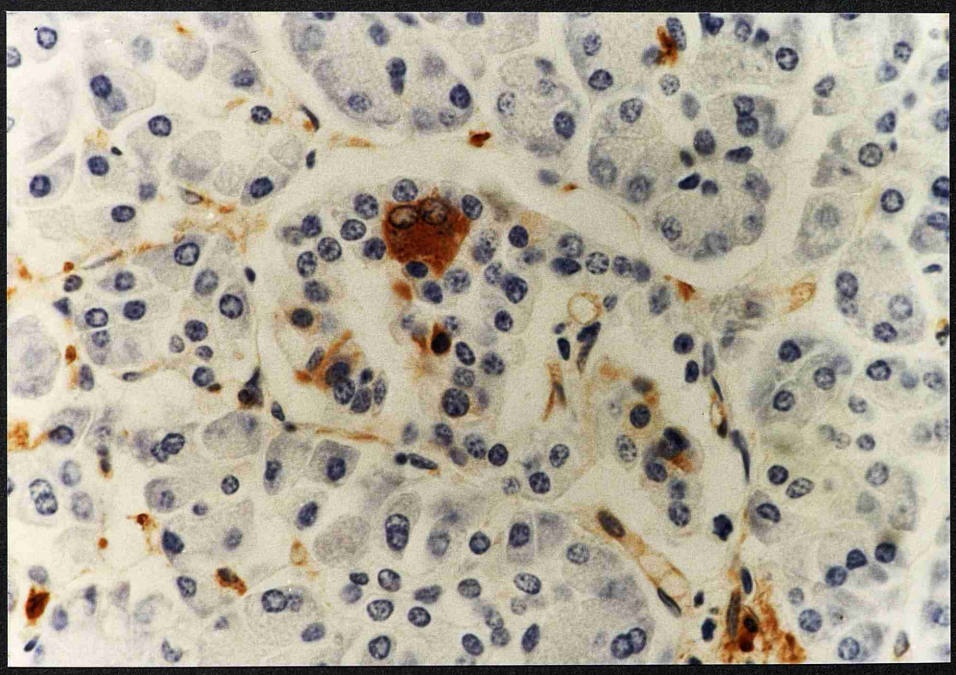
Figure 4.7

Class II MHC and insulitis

There are multiple positive endocrine cells and the lymphocytes of the inflammatory infiltrate are also stained.

I.P. using TAL-1B5 x 480





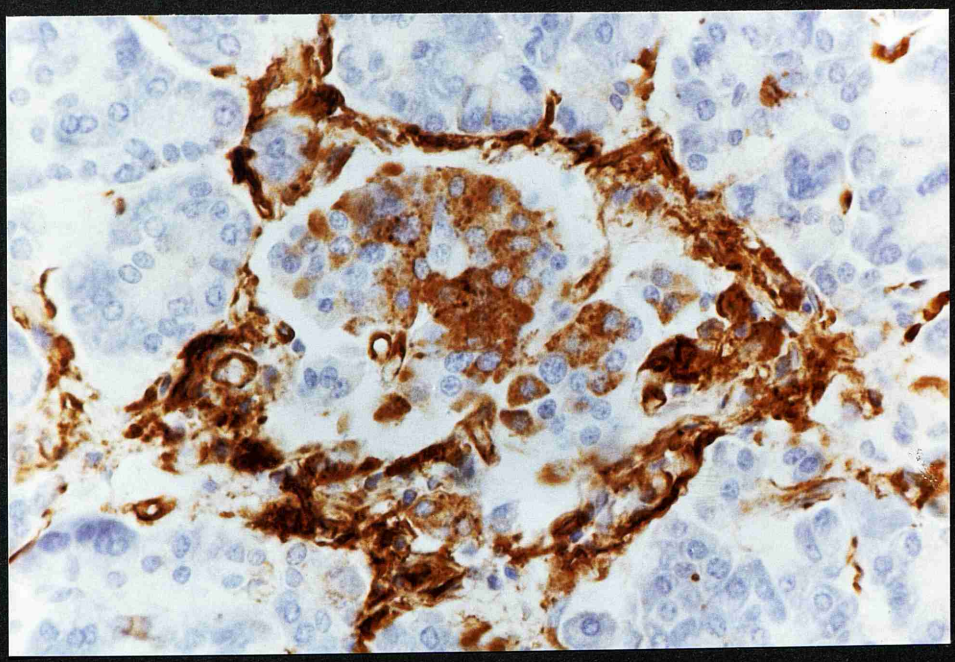
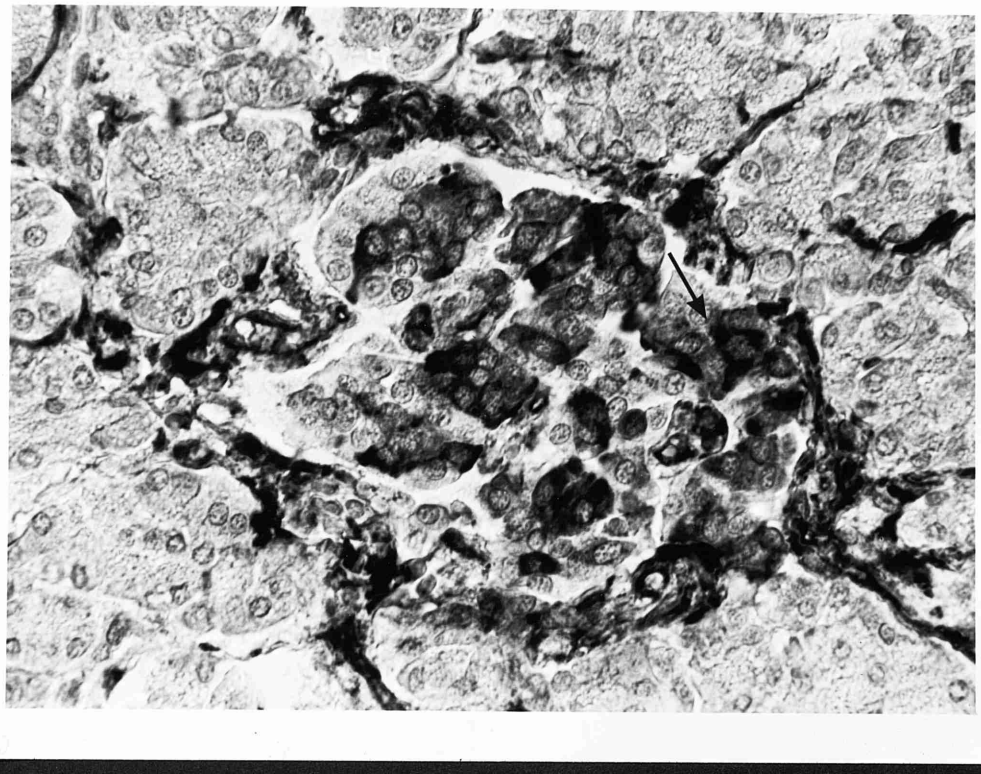


Figure 4.8

Class II MHC and B cells in Type 1 diabetes

This section has been double stained for class II MHC (top) and insulin (bottom). The class II positive endocrine cells also stain for insulin. An example is arrowed.

I.P.using TAL-1B5 followed by immunofluorescence (IF) for insulin x 480.



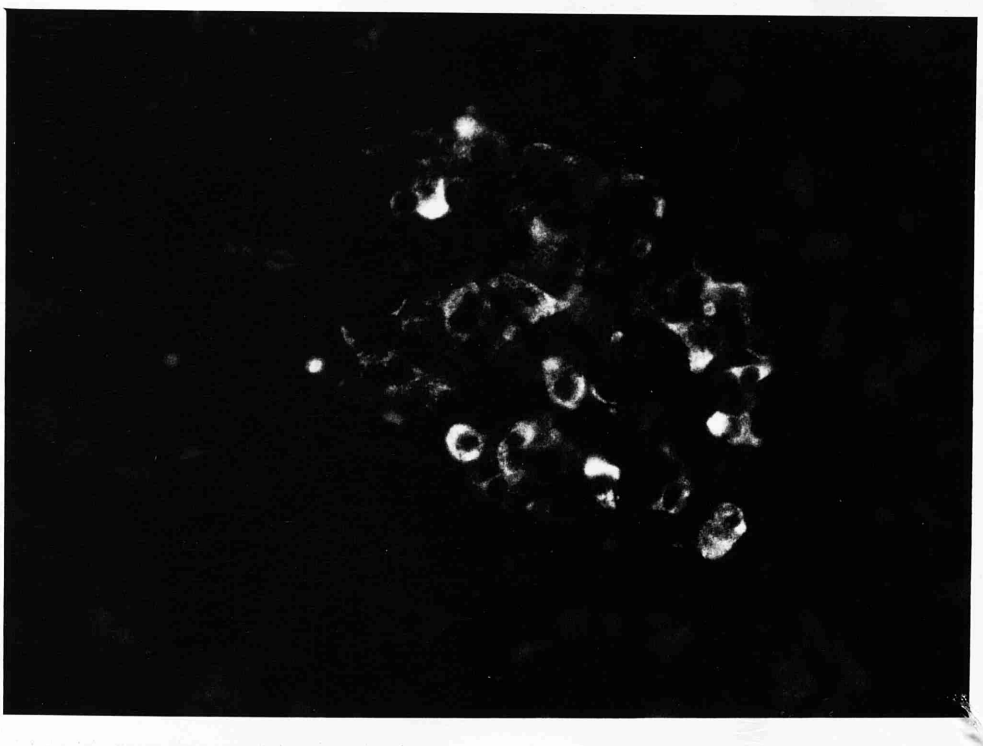
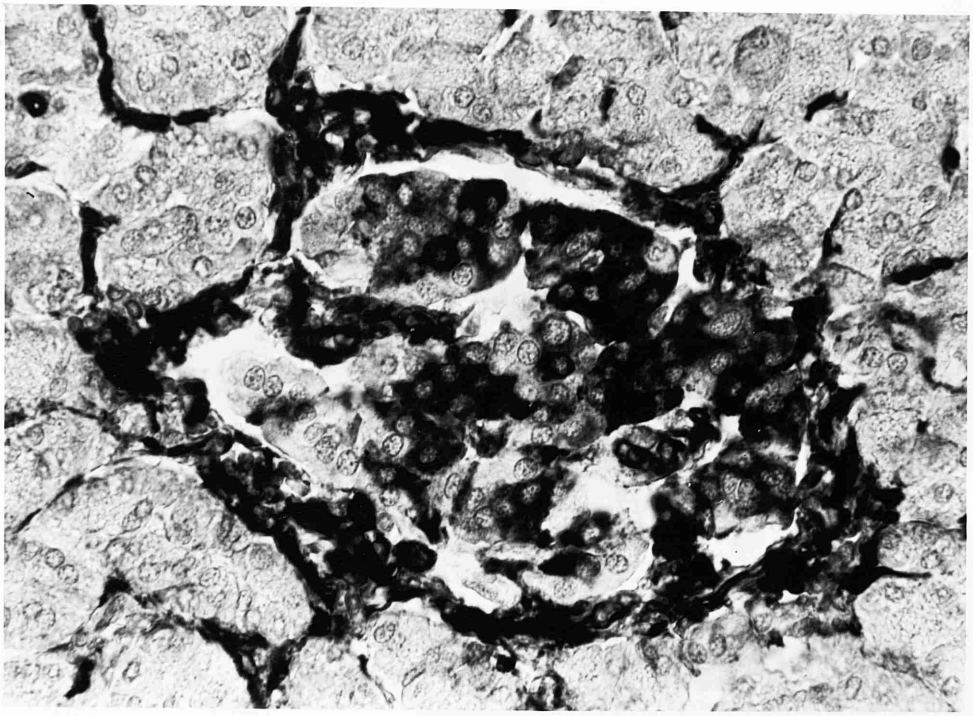


Figure 4.9

Class II MHC and A cells in Type 1 diabetes

This section has been double stained for class II MHC (top) and glucagon (bottom). No glucagon cells are convincingly positive for class II MHC.

I.P. using TAL-1B5 followed by I.F. for glucagon x 480



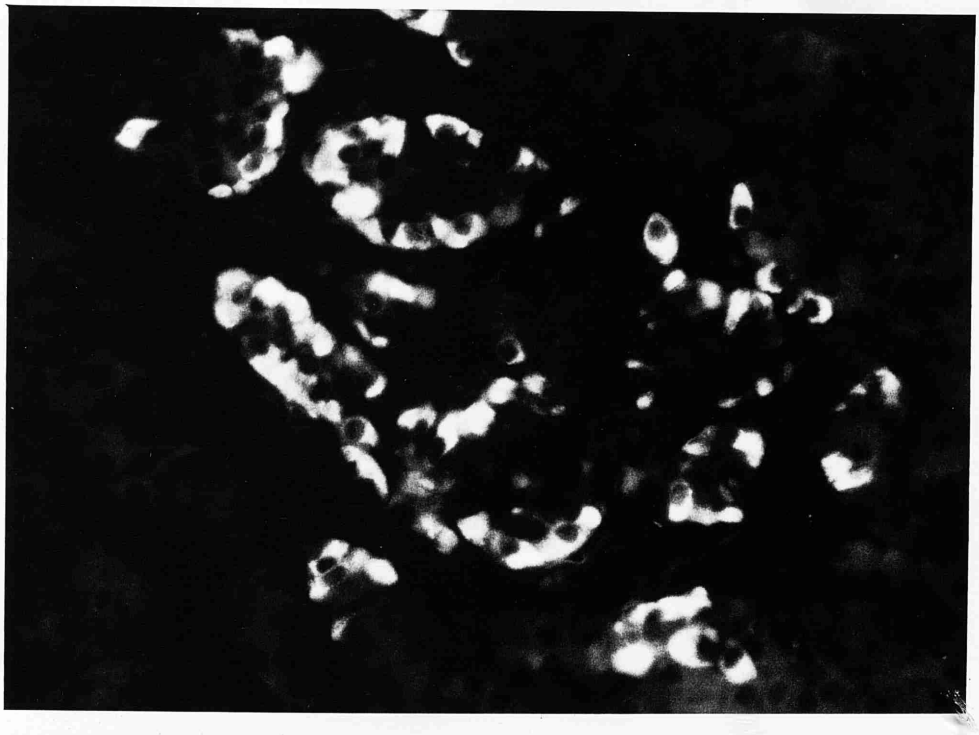
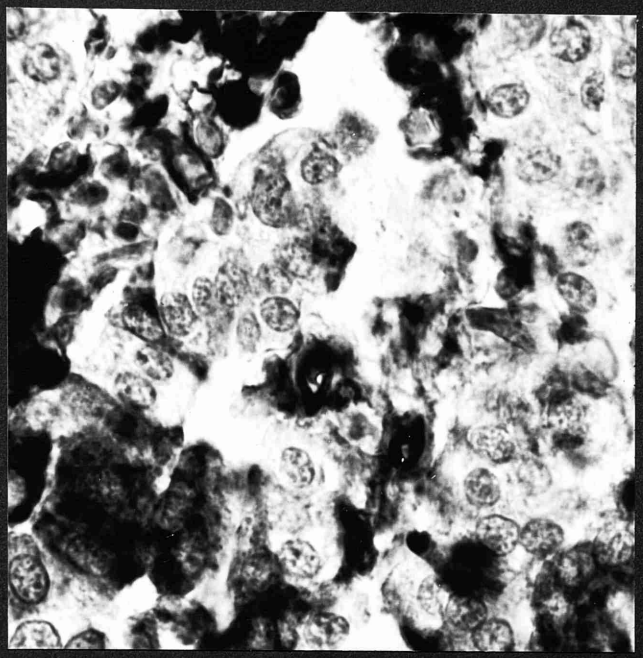


Figure 4.10

Class II MHC and D cells in Type 1 diabetes

This section has been double stained for class II MHC (top) and somatostatin (bottom). Three somatostatin cells are clearly seen and all are negative for class II MHC. Endocrine cells staining for class II MHC are present in the bottom left of the field.

I.P. using TAL-1B5 followed by I.F. for somatostatin x 1100.



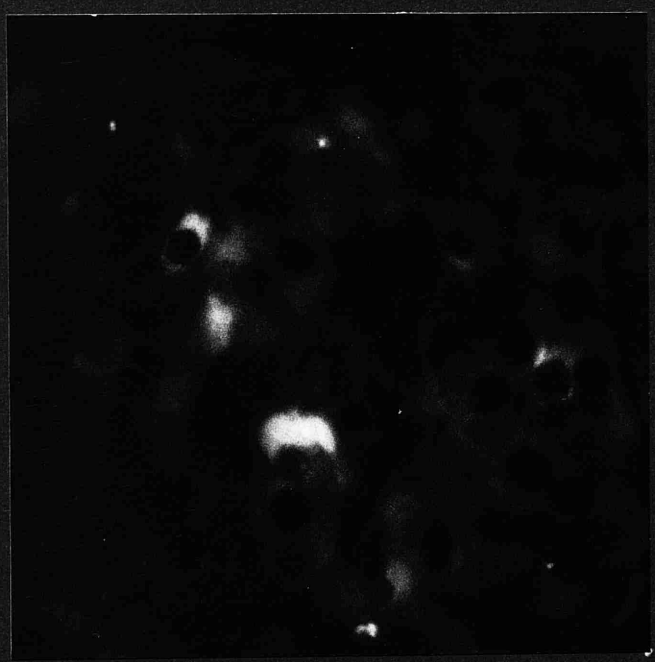


Figure 4.11

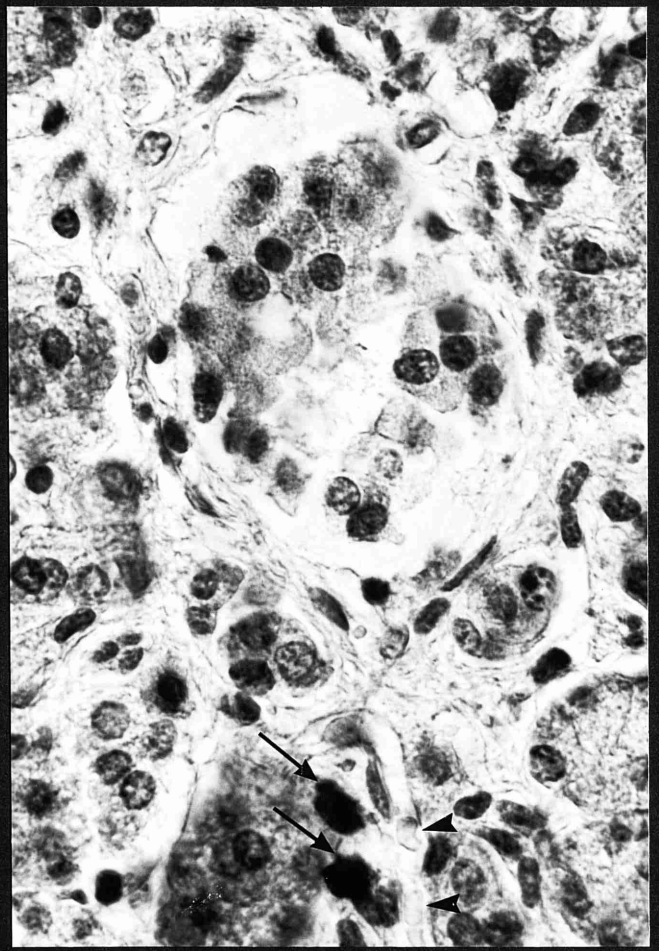
Class II MHC positive endocrine cells, insulin and macrophages

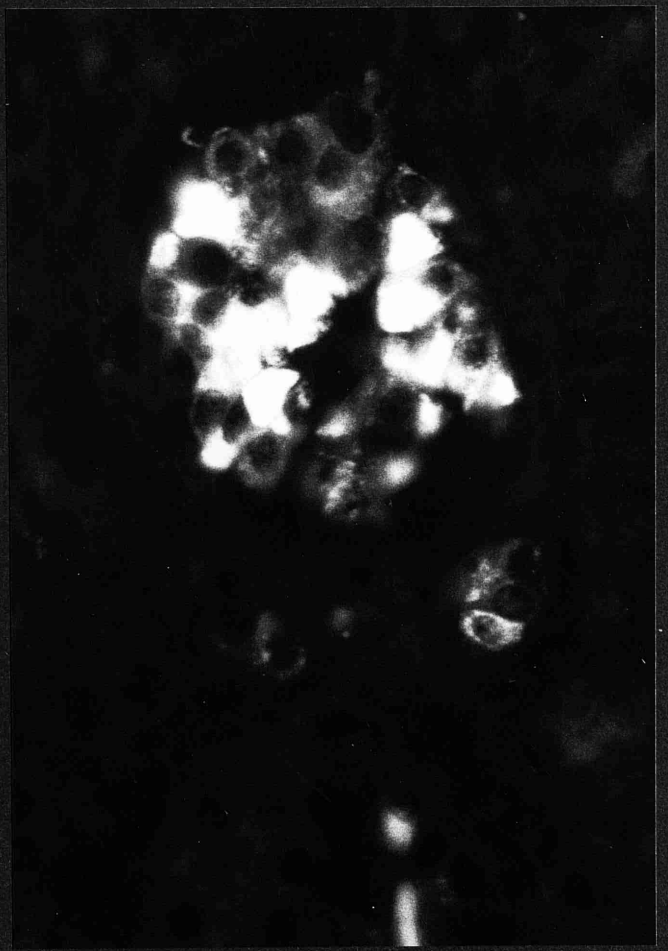
Top photograph: This section shows multiple class II positive endocrine cells.

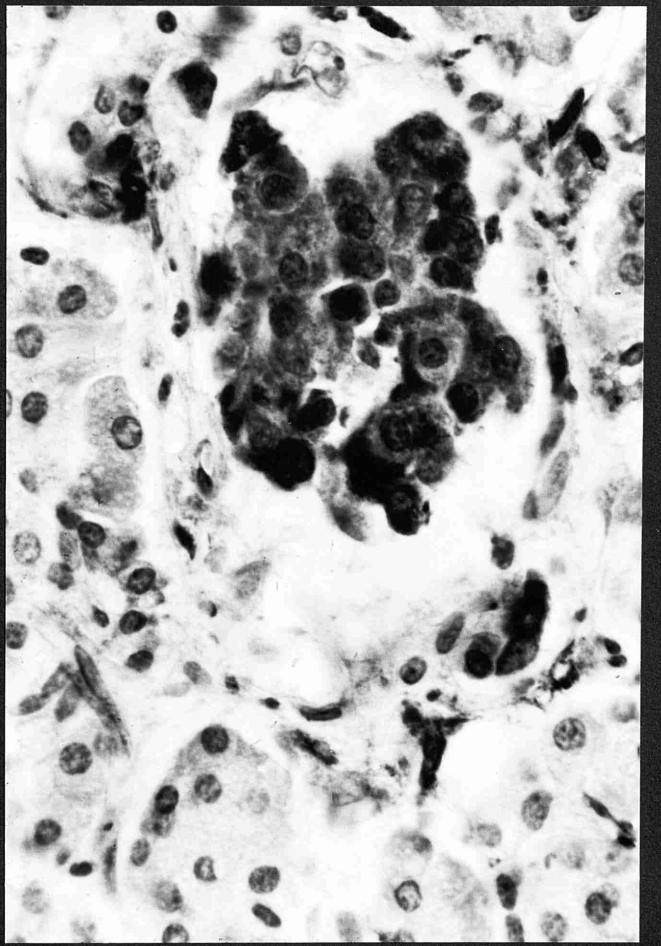
I.P. using TAL-1B5 x 700.

The adjacent serial section has been double stained for insulin (middle) and muramidase (bottom). Note that most of the endocrine cells stain for insulin but that macrophages are not present in the islet. Two macrophages are seen in the lower photograph in exocrine tissue (arrowed). The fluorescence seen in the exocrine tissue in this area is due to autofluorescing red blood cells (arrow heads).

I.P. for muramidase followed by I.F. for insulin x 700.







DISCUSSION

As was discussed in chapter 2, the pancreas at clinical presentation of type 1 diabetes shows a characteristic picture in which there are three distinct populations of islet. The commonest islet is insulin deficient with no evidence of inflammation. Insulin containing islets, apparently unaffected by any destructive process, are generally found and these are often grouped together in a non-random fashion in certain pancreatic lobules. Finally, insulin containing islets affected by insulitis have been shown to be the characteristic lesion of the disease. Thus in the one pancreas, often even within the one section, it is possible to view islets found in long standing diabetes, apparently normal islets and islets in which there is evidence of selective damage to B cells.

From family studies it has been shown that complement fixing cytoplasmic islet cell antibodies may be present for years before presentation (Gorsuch et al, 1981), suggesting a long pre-diabetic period during which B cells are destroyed. In this study the destructive process has also been shown to continue for many years after presentation; cases 4 and 10 having evidence of insulitis two and six years after presentation respectively. Apart from an increased proportion of insulin deficient islets the histological features in these two pancreases closely resembled that seen in those who died at presentation. By extrapolation it could be supposed that the pancreas in the pre-diabetic phase, when complement fixing cytoplasmic antibodies are present, would also be qualitatively similar

but have an increased proportion of uninvolved insulin containing islets. If this argument is correct, then abnormalities detected in the pancreas at clinical presentation may be similar to abnormalities present at the onset of B cell destruction.

In the present study HLA-DR expression in islets from normal autopsy pancreases was confined to endothelial cells. This confirms previous work on fresh frozen material (Natali et al. 1981, Alejandro et al. 1982). Aberrant expression of HLA-DR on islet endocrine cells was found only in type 1 diabetes and only in islets containing insulin. Double stains for HLA-DR and hormone provided further evidence that of all the endocrine cells only B cells expressed HLA-DR. B cells expressing HLA-DR showed no morphological evidence of necrosis and there was no evidence that they had been phagocytosed by macrophages. Indeed macrophages were scanty within islets even in the presence of marked inflammation. These findings confirm those of the single case report of recent-onset type 1 diabetes where this was studied (Botazzo et al, 1985).

It is inherent in the hypothesis of Bottazzo et al (1983) that HLA-DR expression by the target cell precedes the autoimmune and inflammatory response. The alternative view, that aberrant HLA-DR expression is the consequence of these responses has also been proposed (Mowat 1985). Thus it is pertinent to try and suggest whether aberrant expression of HLA-DR on B cells precedes or follows insulitis within a given islet.

Table 4.1 shows that most islets in which aberrant expression of HLA-DR on B cells was present did not have

evidence of insulitis. The serial sections done on case 8 further supported this, in that when many sections were studied focal insulitis was only found in one of 12 islets where there was aberrant HLA-DR expression on endocrine cells. The control pancreases included material from patients with cystic fibrosis and pancreatitis where there was a widespread inflammatory infiltrate in the pancreas. HLA-DR expression was not observed in islet endocrine cells in these cases even when islets were completely surrounded by lymphocytes.

Gamma interferon, a lymphokine produced by T lymphocytes, which are known to be present in insulitis (Bottazzo et al. 1985), has been shown to induce aberrant expression of class II major histocompatability antigens on astrocytes (Wong et al. 1984) and thyrocytes (Todd et al. 1985), and hyperexpression on many other cells (Rosa & Fellous 1984). Thus it has been argued that class II expression on thyrocytes seen in thyroid autoimmune disease may be a consequence of adjacent inflammation and not the primary abnormality. However the situation in the pancreas appears to be different. While gamma interferon induced class II expression on cultured pancreatic exocrine cells in vitro no such expression was seen on cultured pancreatic endocrine cells (Campbell & Harrison 1985, Pujol-Borrell et al, 1986). Equally, alpha interferon, beta interferon, interleukin 2 and supernatants from activated lymphocytes derived from islet cell antibody positive pre-diabetic family members failed to induce class II expression on islet endocrine cells (Pujol-Borrell et al, 1986). Recently it has been shown that pancreatic endocrine cells

cultured in the presence of both tumour necrosis factor and gamma interferon can be induced to express class II MHC (Pujol-Borrell, personal communication). However, this aberrant expression was seen on A cells as well as B cells. Thus, to date, no chemical mediators of inflammation, liable to be released in the insulitis process, have been shown capable of causing selective expression of class II MHC on B cells in vitro. This is in keeping with failure to observe class II MHC expression on islet endocrine cells in chronic inflammatory diseases of the exocrine pancreas.

In what is liable to be a unique study, Sibley et al (1986) reported the recurrence of diabetes in 4 patients who had received pancreatic organ grafts from their normal identical twins. The recipients had had diabetes for at least 17 years. Because these were isografts no suppressive immunotherapy was given in three of the patients. As soon as six weeks after transplant insulin depleted islets and insulitis were present on biopsy of the grafted pancreases. Interestingly, aberrant class II MHC expression on islet cells was sought but was not found. A possible interpretation of this finding is that while aberrant class II MHC expression on B cells may be involved in the induction of autoimmunity (and may have been present on the recipients' own pancreases when they originally presented with diabetes), once a sufficient number of cytotoxic T cells has been generated and tolerance overcome, B cells can be destroyed and insulitis be present without induction of more aberrant class II MHC expression.

Thus the finding of aberrant HLA-DR expression on B

cells in islets unaffected by insulitis in recent-onset diabetes, the absence of such expression on B cells in pancreatic inflammatory diseases or when B cells are cultured in the presence of gamma interferon and the lack of the phenomenon in the transplanted twin pancreases all suggest that within a given islet aberrant class II MHC expression on B cells may precede insulitis.

Two of the patients with cystic fibrosis were diabetic. HLA-DR expression was not seen on endocrine cells in their pancreases, nor in pancreases from patients with type 2 diabetes, providing evidence that aberrant expression of HLA-DR on B cells is not a consequence of the metabolic derangement of diabetes.

While it has been shown that aberrant HLA-DR expression on B cells may be involved in the pathogenesis of type 1 diabetes, its exact role remains speculative. Since B cells do not normally express HLA-DR, any cell specific surface antigens they possess have presumably never been 'presented' for surveillance by T helper lymphocytes, and thus immunological tolerance to them has not been established. If aberrant expression of HLA-DR on the B cell surface were present it could theoretically convert the cell into a functional antigen presenting cell, as has been shown for HLA-DR positive thyrocytes derived from patients with Graves' disease (Londei et al. 1984, 1985). An autoimmune reaction to a B cell specific autoantigen could then be initiated. Such a pancreatic B cell specific membrane antigen has been described, and appears to be the target of the islet cell surface antibodies found in the sera of newly diagnosed patients with type 1 diabetes (Lernmark 1985).

An inherent disadvantage of the present study is that only formalin fixed tissue was available. This severely limited the range of antibodies which could be used and thus the results of only one monoclonal antibody to HLA-DR have been presented. The antibody, TAL-1B5, is directed against the alpha chain of HLA-DR (Adams et al. 1983). For the HLA-DR molecule to function in antigen presentation both alpha and beta chains need to be present in the cell membrane and protrude onto its outer surfaces (Alberts et al. 1983). In the present study only the alpha chain has been demonstrated and the immunohistochemical staining appeared to fill the entire cytoplasm up to the area of the cell membrane. A detailed demonstration of the exact position of membrane staining in the presence of such strong cytoplasmic staining is beyond the scope of a light microscopic study such as this on fixed, autopsy pancreas. Thus more evidence will be required to prove that the HLA-DR antigens demonstrated in this study are capable of converting the B cell into an antigen presenting cell.

It is generally held that the pathogenesis of type 1 diabetes involves both an inherited susceptibility and as yet unknown environmental agents. The strongest established genetic linkage so far demonstrated has been with the HLA-DR group of the individual. In one study 98% of type 1 diabetics were positive for either DR3 or DR4 and 51% possessed both these antigens (Wolf et al. 1983). Theoretically DR 3 and DR 4 antigens could be more readily inducible on B cells than others. Alternatively once expressed their particular shape or charge could make them more capable of presenting particular autoantigens on B cells than other class II antigens.

PART 3

Aberrant expression of class II MHC and hyperexpression of class I MHC on islets in type 1 diabetes.

INTRODUCTION

In the previous part of this chapter it was shown that aberrant expression of class II MHC (HLA-DR) on B cells was a feature of type 1 diabetes. It was argued that this was an important finding since knowledge of agents which could cause this phenomenon could elucidate the pathogenesis of the disease. Normally, one would wait for other workers to confirm such a finding, but, because of the extreme rarity of the material on which the observations were made, this may not happen quickly. For this reason it was felt important to repeat the study using different reagents on a greater number of cases.

The monoclonal antibody, TAL-1B5, which was used in the previous study failed to bind to the HLA-DR molecule if the tissue had been fixed in buffered formalin. Many of the cases which were obtained for the study in chapter 2 had been fixed in buffered formalin and so a search was made for an antibody or antiserum which could be used on tissue fixed in this and other fixatives. In spite of considerable effort, which involved information on, or study of, more than 100 monoclonal antibodies to class II MHC, no suitable monoclonal antibodies were found. There are many epitopes on the non-polymorphic regions of the class II molecule, any of which could be altered during fixation. In view of the lack of success with monoclonal antibodies, a search was made for suitable polyclonal antisera, since they should detect a large number of

epitopes. After a further extensive search, two suitable, well characterised polyclonal antisera were obtained, one of which forms the basis of this study. It is perhaps worth emphasising that this is the first report of an antiserum capable of detecting class II antigens in buffered formalin fixed autopsy tissue.

Bottazzo et al (1985), in their study of a child who died of recent-onset diabetes in whom fresh frozen pancreas had been obtained at autopsy, noted that endocrine cells in some islets hyperexpressed class I MHC products as assessed by increased staining with immunofluorescence. However no attempt was made to show which endocrine cells hyperexpressed class I MHC and, as has been noted before, the only control studied was one normal pancreas. No monoclonal antibodies or polyclonal antisera to class I MHC suitable for the study of the distribution of class I MHC in formalin fixed tissues have been described. In view of the paucity of useful monoclonal antibodies to class II MHC in this situation, a search was made from the outset for a polyclonal antiserum to class I MHC. This was finally obtained and proved to be satisfactory. An indirect way of looking at class I MHC expression in tissue is to study the distribution beta 2-microglobulin. This protein, which is coded for by a gene on a different chromosome from that which codes for class I MHC, associates non-covalently with class I MHC in the plasma membrane (Alberts et al. 1983). Thus, in addition to studying class I MHC expression in the diabetic pancreases, the distribution of beta 2-microglobulin was also examined in an attempt to confirm the results.

with first world laboratory services. Two cases were found as the result of a 15 year computer survey of deaths in England and Wales. Two cases were found in the autopsy records of the Royal Hospital for Sick Children in Glasgow and one was supplied by Dr. Liz Gray of Aberdeen, who kindly reviewed her files. Finally, one case was sent by Dr. Andrew Abraham of Washington D.C. His case had been the subject of a published report. (Ahmad & Abraham 1982).

All the tissues studied had been fixed in either buffered formalin or formol saline with or without added mercuric chloride. Six micron serial sections were cut from the paraffin embedded blocks of the pancreases from patients with type 1 diabetes and those with Coxsackie virus infection. Adjacent serial sections from these cases, and three non serial sections from all others, were stained with an indirect immunoperoxidase technique in which diaminobenzidine was the substrate, using the following primary polyclonal antisera: rabbit anti-class I MHC heavy chain ("AI", gift from J.J. Neefjes, Amsterdam) guinea pig anti-insulin (Wellcome) and rabbit anti-class II MHC ("AII", gift from J.J. Neefjes, Amsterdam). A minority of the type 1 diabetic pancreases were stained using the same techniques with two other primary polyclonal antisera- rabbit anti- beta 2-microglobulin (Dako) and a second rabbit anti-class II MHC (UUII, gift from L. Klareskog, Uppsala University). These two antisera were tested to confirm the findings using the antisera AI and AII. The following bridges for the indirect techniques were used: peroxidase conjugated rabbit anti-guinea pig, swine anti-rabbit and rabbit anti-mouse immunoglobulins (all

Dako). In addition to the sections described, one pancreatic block from each of 3 cases (Nos. 8, 12 and 19, Table 4.2) was selected and 40, six micron serial sections cut. The middle two sections of these series were stained for class I MHC (using antiserum AI) and insulin respectively. The remaining sections were all stained for class II MHC (using antiserum AII). Insulin containing islets were identified on the section stained for insulin and the presence or absence of hyperexpression of class I MHC and aberrant expression of class II MHC noted by viewing each islet on the remaining serial sections. Over 90% of the insulin containing islets identified were assessed in each of these cases.

Detailed studies of the specificity of these various antisera have been published. The rabbit antiserum to class I MHC, AI, was prepared against a mixture of purified heavy chains of HLA-B7 and HLA-B40 (Neefjes et al. 1986a). In an immunoblot analysis of white cells from 21 different HLA blood type donors this antiserum reacted with all the HLA-B locus specificities tested, in addition to a number of A-locus products. The distribution of staining on formalin fixed pancreas and tonsil using this antiserum and the antiserum to beta-2-microglobulin, was compared to staining on fresh frozen pancreas and tonsil using the same antisera, and also the mouse monoclonal antibody W6/32 (Sera-Lab, Sussex) which recognises all HLA-A and -B antigens (Parham, Barnstable and Bodmer 1979). The staining patterns were identical.

The rabbit antiserum, AII, was raised against purified human class II antigens, DR and DQ (Neefjes et al. 1986b).

Part of the antigen had been denatured by heat. The serum recognised human class II alpha chains and a number of class II beta chains in immunoblot analysis and it reacted with DP, DQ and DR. The specificity of the second anti-class II MHC antiserum (UUII) has also been previously described in detail (Klareskog et al. 1978). The staining patterns using immunohistochemistry on formalin fixed pancreas and lymph node with these two antisera and TAL-1B5 were compared with their staining on fresh frozen pancreas and lymph node. The staining was identical in all cases. All these rabbit antisera worked satisfactorily on tissue fixed in any of the commonly used fixatives.

Double staining techniques were developed to show which hormone producing cells expressed class II MHC or hyperexpressed class I MHC. Following an overnight incubation at 4°C with either AI or AII the sections were incubated with peroxidase conjugated swine anti-rabbit immunoglobulins for 30 minutes at room temperature. The reaction was developed using diaminobenzidine as substrate. Double staining for insulin was done using a subsequent 4 hour incubation of the guinea pig anti-insulin antiserum followed by a 30 minute incubation in rabbit anti-guinea pig immunoglobulins (Dako). Finally, there was a 30 minute incubation with fluoroscein conjugated donkey anti-rabbit immunoglobulins (Scottish Antibody Production Unit). Repeated washings were done between steps using tris buffered saline. Double staining for class I and II MHC and the other hormones utilised the same method up to the development with diaminobenzidine. This was followed by a 15 minute incubation in normal swine serum, a four

hour incubation with the rabbit anti-hormone antibodies and finally a 30 minute incubation with fluoroscein conjugated donkey anti-rabbit immunoglobulins. Again, repeated washings with tris buffered saline were done in between incubations. Double-stained sections were viewed using a Leitz Orthomat microscope. Control pancreases and spleen were tested on all occasions and there were no cross reactions observed between the two steps of these double techniques.

RESULTS

Normal autopsy pancreas

The distribution of class II MHC in the normal autopsy pancreas, as assessed by the antiserum AII, was the same as that described previously, using the antibody, TAL-1B5. Thus, in the islets, only the endothelial cells stained, while in exocrine tissue occasional ducts were positive but acini were negative. There was a low level of staining for class I MHC antigens on islet endocrine cells using the antiserum AI. Endothelial cells and nerve fibres were strongly positive and the exocrine tissues stained relatively weakly. (Fig 4.12).

The pancreas in type 1 diabetes

Four principal variables were analysed : 1) insulin content of islets, 2) presence or absence of insulitis, 3) presence or absence of aberrant expression of class II MHC on islet endocrine cells, 4) degree of expression of class I MHC antigens on islet endocrine cells. Tables 4.2 and 4.3 give the results of the analysis of islet staining on 3 consecutive serial sections from each case, stained for

class I MHC, insulin and class II MHC respectively. As will be described, the degree of expression of class I MHC and the insulin content of an islet could be assessed accurately from a single section. However, aberrant class II MHC expression on endocrine cells and the presence of an inflammatory infiltrate, could be quite focal within an islet, and the presence of these is liable to be underestimated if only one section from each case is examined. Thus three cases (8,12,19) were chosen for more detailed study on the basis that blocks were available and that the phenomena under investigation were present. Forty serial sections were cut from one block of each case and the middle two sections stained for class I MHC antigens and insulin respectively. The remaining sections were stained for class II MHC antigens. From this, the presence of focal insulitis, or infrequent aberrant expression of class II MHC on endocrine cells, could be assessed for any islet identified on the middle two sections. The results of this analysis are given in Table 4.4.

Aberrant expression of class II MHC antigens on islet endocrine cells

Insulin containing islets were present in all 23 cases of recent-onset diabetes and in 8 out of 12 cases where the disease had been present for more than one year. Aberrant expression of class II MHC on endocrine cells was seen in 21 of the recent-onset cases and 6 of the prolonged duration cases. The maximum duration of diabetes where this phenomenon was observed was 9 years. Endocrine cells expressing class II MHC were seen in 377 out of 2744 insulin containing islets but not in 6842 insulin deficient

TABLE 4.2 **Insulin content of islets, insulitis, aberrant expression of class II MHC and hyperexpression of class I MHC on islet endocrine cells in type 1 diabetes - detail of cases.**

CASE	AGE	SEX	Duration of Diabetes	No. of ICI	No. of IDI	No. of ICI with insulitis	No. of IDI with insulitis	No. of ICI with class II MHC positive endocrine cells	No. of IDI with class II MHC positive endocrine cells	No. of class II MHC (endocrine) positive islets with insulitis	No. of class II MHC (endocrine) positive islets without insulitis	No. of ICI with hyper expression of class I MHC	No. of IDI with hyper-expression of class I MHC
1	18mths	F	1 wk	116	1239	112	8	9	0	9	0	115	6
2	21mths	F	2 wks	16	69	11	1	2	0	2	0	16	2
3	2yrs	M	2 wks	19	177	1	3	0	0	0	0	16	7
4	3yrs	F	6 wks	88	43	30	1	4	0	0	4	88	3
5	3yrs	M	4 dys	59	292	17	1	5	0	4	1	59	0
6	3yrs	M	3 mths	45	100	6	2	16	0	4	12	45	0
7	5yrs	F	12 wks	29	21	2	0	1	0	1	0	27	0
8	6yrs	M	6 dys	128	359	35	6	22	0	20	2	128	4
9	6yrs	F	few wks	36	79	4	1	4	0	1	3	36	0
10	7yrs	M	3 dys	37	148	19	6	7	0	7	0	37	4
11	8yrs	M	few mths	118	69	2	1	8	0	1	7	79	1
12	10yrs	M	3 wks	117	150	0	0	15	0	0	15	111	3

TABLE 4.2 continued

CASE	AGE	SEX	Duration of Diabetes	No. of ICI	No. of IDI	No. of ICI with insulitis	No. of IDI with insulitis	No. of ICI with class II MHC positive endocrine cells	No. of IDI with class II MHC positive endocrine cells	No. of class II MHC (endocrine) positive islets with insulitis	No. of class II MHC (endocrine) positive islets without insulitis	No. of ICI with hyper-expression of class I MHC	No. of IDI with hyper-expression of class I MHC
13	11 yrs	M	2 wks	29	13	0	0	5	0	0	5	29	2
14	12 yrs	F	3 wks	36	37	5	6	1	0	0	1	36	0
15	14 yrs	F	few wks	135	353	61	7	10	0	10	0	134	1
16	16 yrs	M	few wks	18	29	4	0	4	0	4	0	16	1
17	17 yrs	M	2 dys	377	211	226	6	28	0	23	5	369	2
18	17 yrs	M	1 wk	358	217	117	28	84	0	40	44	357	36
19	21 yrs	M	3 wks	329	278	35	1	113	0	24	89	325	10
*20	8 yrs	F	3 wks	114	99	5	0	0	0	0	0	80	0
*21	13 yrs	F	2mths	101	26	5	0	1	0	0	1	101	0
*22	18 yrs	F	few wks	185	18	7	0	8	0	0	8	140	0
*23	18 yrs	M	few wks	61	35	4	0	4	0	1	3	54	0
24	5 yrs	F	15mths	3	142	0	0	0	0	0	0	3	0

TABLE 4.2 continued

CASE	AGE	SEX	Duration of Diabetes	No. of ICI	No. of IDI	No. of ICI with insulitis	No. of IDI with insulitis	No. of ICI with class II MHC positive endocrine cells	No. of IDI with class II MHC positive endocrine cells	No. of class II MHC (endocrine) positive islets with insulitis	No. of class II MHC (endocrine) positive islets without insulitis	No. of ICI with hyper-expression of class I MHC	No. of IDI with hyper-expression of class I MHC
25	15 yrs	F	2 yrs	73	63	0	0	4	0	0	4	45	0
26	3 yrs	F	2 yrs	0	278	0	0	0	0	0	0	0	0
27	9 yrs	F	2 yrs	3	26	1	0	2	0	1	1	3	0
28	10 yrs	F	2 yrs	0	50	0	0	0	0	0	0	0	0
29	5 yrs	M	2 yrs	0	252	0	0	0	0	0	0	0	0
30	16 yrs	F	4 yrs	12	142	0	0	2	0	0	2	8	0
31	8 yrs	F	5 yrs	12	550	6	1	10	0	5	5	12	0
32	11 yrs	M	6 yrs	32	342	1	0	6	0	0	6	32	0
33	18 yrs	F	6 yrs	23	306	0	0	0	0	0	0	2	0
34	20 yrs	M	6 yrs	0	449	0	0	0	0	0	0	0	0
35	18 yrs	F	9 yrs	35	180	0	0	2	0	0	2	14	0
				2744	6842	716	79	377	0	157	220	2517	82

* POLYENDOCRINE CASES. ICI = insulin containing
IDI = insulin deficient islets

TABLE 4.3 **Insulin content of islets, insulitis, aberrant expression of class II MHC and hyperexpression of class I MHC on islet endocrine cells in type 1 diabetes - summary of cases.**

	Non poly-endocrine, recent-onset diabetes	polyendo-crine recent onset diabetes	Prolonged duration diabetes (more than) 1 year
No. of cases	19	4	12
No. of cases with ICI	19	4	8
No. if ICI	2090	461	193
No. of IDI	3884	178	2780
No. of cases with insulitis	17	4	3
No. of cases with aberrant expression of class II MHC on ICI	18	3	6
No. of ICI with aberrant expression of class II MHC	338	13	26
No. of IDI with aberrant expression of class II MHC	0	0	0
No. of ICI with insulitis	687	21	8
No. of islets with aberrant expression of class II MHC and insulitis	150	1	6
No. of islets with aberrant expression of class II MHC but no insulitis	188	12	20
No. of cases with hyper-expression of class I MHC on ICI	19	4	8
No. of ICI which hyper-express class I MHC	2023 (97% of ICI)	375 (81% of ICI)	119 (61% of ICI)
No. of IDI which hyper-express class I MHC	82 (2% of IDI)	0	0
No. of islets with aberrant expression of class II MHC but no hyperexpression of class I MHC	0	0	0

ICI = insulin containing islets. IDI = insulin deficient islets

TABLE 4.4

Insulitis, aberrant expression of class II MHC and hyperexpression of class I MHC on islet endocrine cells of insulin containing islets - results of study of 40 serial sections on 3 cases.

	CASE No. 19	CASE No. 8	CASE No. 12
Number of ICI counted	50	40	40
Number of ICI with insulitis	16	39	2
Number of ICI with aberrant expression of class II MHC	44	26	18
Number of ICI with aberrant expression of class II MHC and insulitis	16	26	2
Number of ICI with aberrant expression of class II MHC and no insulitis	28	0	16
Number of ICI with hyperexpression of class I MHC	48	40	32
Number of ICI with hyperexpression of Class I MHC but no aberrant expression of class II MHC	4	14	14
Number of ICI with no hyperexpression of class I MHC but presence of aberrant expression of class II MHC	0	0	0
Number of ICI with insulitis and hyper-expression of class I MHC	16	39	2

islets. Double stains for hormones and class II MHC confirmed the previous observation that only B cells expressed this antigen (Figs 4.13, 4.14). When 2 adjacent serial sections were stained with AII and UUII, both polyclonal antisera to class II MHC, the results were identical (Fig 4.15). As with the first study using TAL-1B5, the staining for class II MHC was cytoplasmic.

Degree of class I MHC expression and insulin content of islets

A striking observation was the very marked degree of hyperexpression of class I MHC on insulin containing islets (4.16). In islets where this was present there was not only increased cytoplasmic staining but also considerable accentuation in the region of the plasma membrane of cells. Class I MHC hyperexpression was present in all 31 patients with residual insulin containing islets. Furthermore it was found in 2517 out of 2744 such islets (92%) but was only seen in 82 out of 6842 insulin deficient islets (1%). The pattern of staining when two adjacent serial sections were stained with the antisera directed against class I MHC heavy chain (AI) and beta-2-microglobulin respectively was identical (Fig 4.16). In contrast to the findings with class II MHC expression, all the endocrine cells in a given insulin containing islet appeared to hyperexpress class I MHC. This was confirmed by double stains for class I MHC and the respective hormones (Figs 4.17 and 4.18). Thus it appeared that when A and D cells were adjacent to B cells in an insulin containing islet in this disease they hyperexpressed class I MHC, but when they were physically divorced from B cells,

in an insulin deficient islet, they ceased to hyperexpress class I MHC. (The findings with PP cells were not formally assessed since all the insulin containing islets examined were in the glucagon rich lobe).

Occasional insulin containing islets in which there was no hyperexpression of class I MHC were observed in some cases. It was interesting to note that these islets tended to be grouped together in a non random manner, often within the same pancreatic lobule (Fig 4.19).

The relationship between class I MHC hyperexpression and aberrant expression of class II MHC

While class I MHC hyperexpression was seen in 92% of insulin containing islets, HLA-DR positive B cells were only seen in 14% (Table 4.2). By contrast, all islets in which there was aberrant expression of class II MHC hyper-expressed class I MHC. When whole islets were examined in the 40 serial sections of the 3 chosen cases this latter finding was confirmed (Table 4.4). Here, 120 islets hyperexpressed class I MHC but in only 88 of them was aberrant expression of class II MHC seen. In these three cases there were 10 insulin containing islets which did not hyperexpress class I MHC and aberrant HLA-DR expression was not observed in these islets.

The relationship between insulitis, class I hyperexpression and aberrant expression of class II MHC

All insulin containing islets affected by insulitis hyperexpressed class I MHC. Interestingly, such hyperexpression was not necessarily found in inflamed insulin deficient islets (Fig.4.20). However, many of the islets in which hyperexpression of class I MHC was observed

were not affected by insulitis (Table 4.2), and this was borne out in the serial section study of whole islets (Table 4.4).

As with the previous study, using TAL-IB5, the majority of islets in which aberrant HLA-DR expression was seen were not affected by insulitis. (Table 4.2). In the serial section study of whole islets, half the islets in which aberrant expression of HLA-DR was seen had no evidence of insulitis. From Table 4.2 it can be seen that while insulitis was observed in 716 insulin containing islets, aberrant expression of HLA-DR was present in only 157 of them. This suggested that aberrant HLA-DR expression may not always be present in inflamed insulin containing islets, an observation also made in Case 8 (Table 4.4).

It was suggested in chapter 2 that the destruction of B cells may proceed at a slower rate in polyendocrine diabetes than in cases where no other autoimmune phenomena were present. While these are too few cases to draw firm conclusions, the results in Table 4.3 would be in keeping with this, in that not only were fewer insulin containing islets inflamed, but only 2.8% of insulin containing islets had aberrant HLA-DR expression compared to 16% for the remainder of the recent-onset diabetics. Similarly, fewer insulin containing islets hyperexpressed class I MHC in the recent-onset polyendocrine diabetic patients.

Class I and II MHC expression in pancreatic diseases other than type 1 diabetes

Before giving these results a brief description of the pathology in the bone marrow grafted cases and those where death was due to Coxsackie B myocarditis will be given.

Nine pancreases were available from patients who had died following marrow grafting. In four of these patients the cause of death was given as graft-versus-host disease (GVH). Two patients with GVH and two patients without GVH had a mild chronic inflammatory cell infiltrate within the pancreas, which was maximal around ducts in 3 cases. There was no evidence of insulitis in any of these cases and no abnormality in the distribution of B cells was observed.

There were 12 pancreases from neonates who had died under the age of 2 weeks of culture proven Coxsackie B viral myocarditis. Extramedullary erythropoeisis was present in the pancreas in 11 of these but in 3 this could easily be differentiated from the presence of prominent insulitis with associated necrosis of endocrine cells (Fig 4.21). One additional case had apparent necrosis of some islets in the absence of either insulitis or autolysis of exocrine tissue (4.22). The inflammatory infiltrate consisted predominantly of eosinophils in 2 cases (Fig 4.21) and of lymphocytes with only a few eosinophils in the third case. (The infiltrate in the myocardium of the former two cases also had prominent eosinophils). In the cases with insulitis, the necrotic endocrine cells appeared to be B cells (Fig 4.21). In the 4th case, in which there was islet necrosis but no insulitis, A and D cells were also affected (Fig 4.22). There was no evidence of acinar cell necrosis or fat necrosis in any of the pancreases studied and at most there was only mild oedema and a few chronic inflammatory cells in the exocrine pancreas.

No evidence of aberrant expression of class II MHC on endocrine cells was seen in the pancreases of the 9 bone

marrow grafted patients, the five resections for chronic pancreatitis, the 10 cases of cystic fibrosis, the 10 cases of type 2 diabetes and the 12 cases with Coxsackie B viral infection. In pancreases where inflammation was present there was a low level of staining for class II MHC on the exocrine pancreas. Equally, in none of these cases did the degree of expression of class I MHC match that seen on insulin containing islets in type 1 diabetes. In inflamed pancreases there was considerable hyperexpression of class I MHC diffusely throughout the pancreas. This was most obvious in cases of cystic fibrosis. Usually, however, the degree of expression of class I MHC on islets was less than that observed in the exocrine tissue (Fig 4.23 and 4.24).

DISCUSSION

The principal reason for this study was to confirm the presence of aberrant HLA-DR expression on B cells in type 1 diabetes. The use of the antiserum, AII, which detected this complex in tissues fixed in all commonly used fixatives, allowed a greater number of cases and controls to be studied. The study could therefore confirm that class II MHC expression on B cells appeared to be unique to type 1 diabetes.

Graft-versus-host disease was chosen as a control because in this disease many cells are being 'rejected' by the graft. There was no evidence of insulitis in the cases studied so it is still not certain that aberrant expression of HLA-DR on B cells could not be the consequence of a direct immune attack. Another possible explanation for

Figure 4.12

Distribution of class I MHC on normal islet

Endothelial cells are positive but there is only minimal staining of endocrine cells. Acinar cells appear negative by this technique.

I.P. using antiserum AI x 350

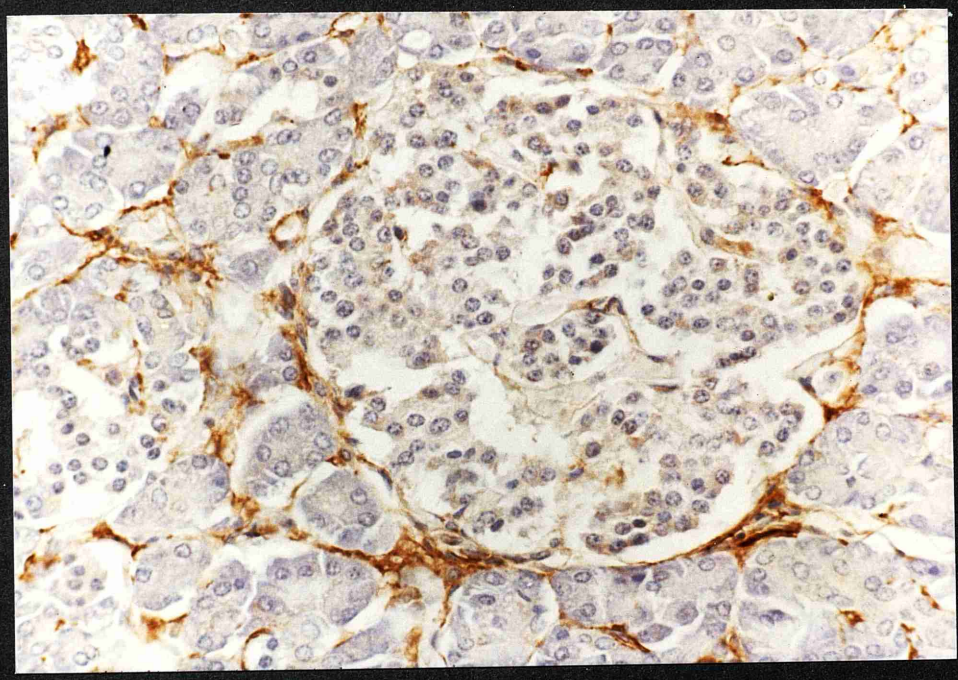


Figure 4.13

Class II MHC and insulin in type 1 diabetes

The section has been double stained for insulin (left) and class II MHC (right). Most of the endocrine cells staining positively for class II MHC contain demonstrable insulin.

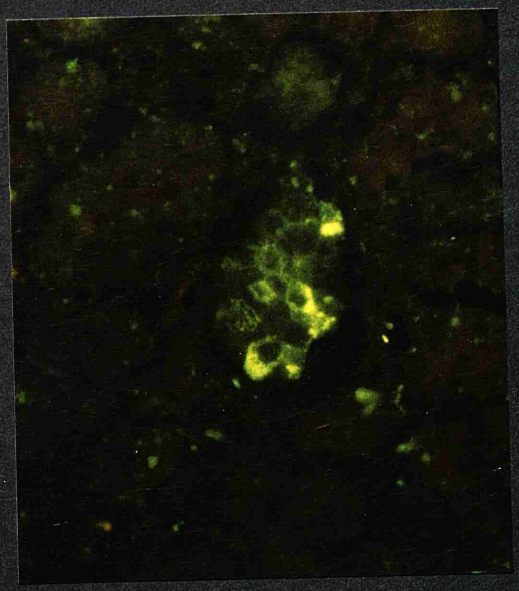
I.P. for class II MHC (antiserum A II) followed by I.F. for insulin x 340.

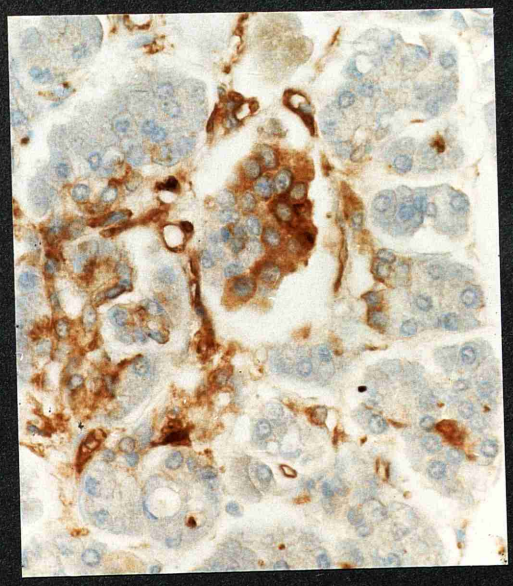
Figure 4.14

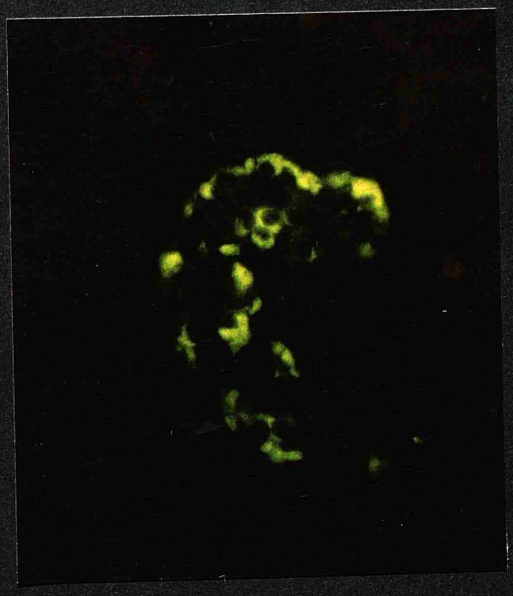
Class II MHC and glucagon in type 1 diabetes

This section has been double stained for glucagon (left) and class II MHC (right). The class II MHC positive endocrine cells do not contain glucagon.

I.P. for class II MHC (antiserum A II) followed by I.F. for glucagon x 280







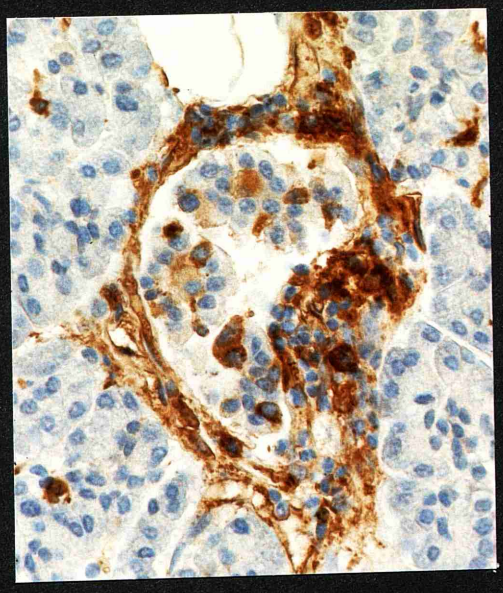
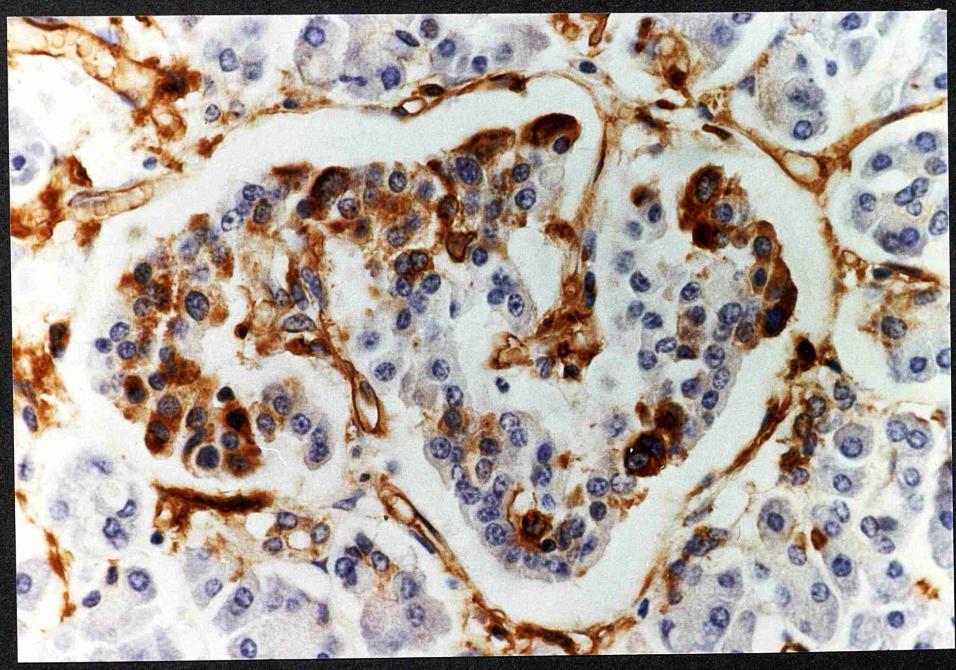


Figure 4.15

Aberrant expression of class II MHC in type 1 diabetes: Comparison of staining using two different antisera to class II MHC

Adjacent serial sections have been stained using indirect immunoperoxidase techniques with primary antisera A II (top picture) and UU II (bottom picture).

The results are virtually identical x 440



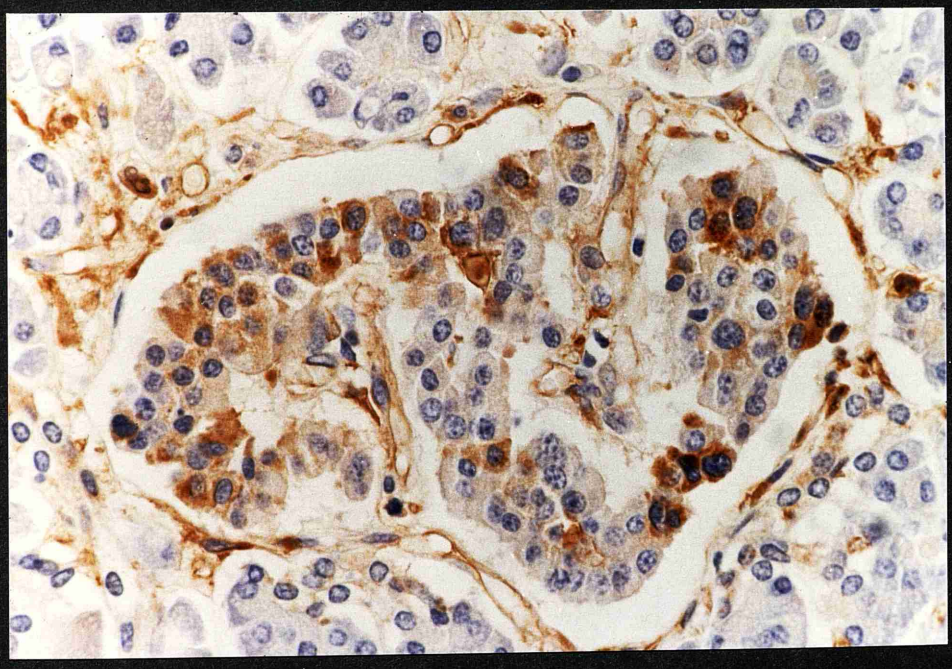


Figure 4.16

Class I MHC hyperexpression of insulin containing islets in type 1 diabetes

This section has been stained using an antiserum to class I MHC heavy chains. Note the marked hyperexpression on all endocrine cells in the large islet on the right compared to the small islet on the left.

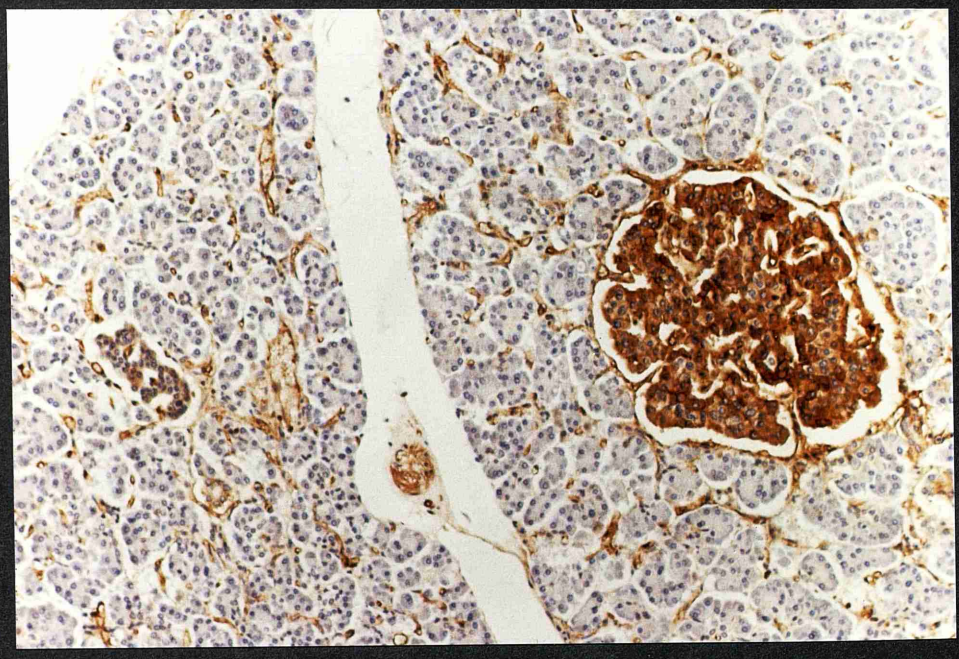
I.P. using antiserum AI.

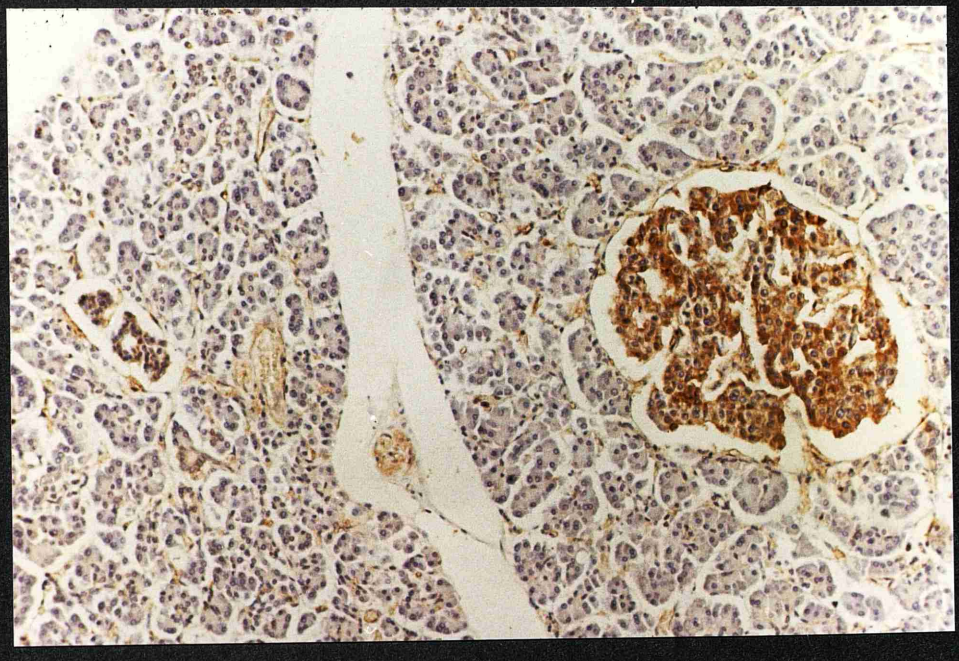
A similar pattern of staining is seen on this adjacent section stained by an I.P. technique for beta-2-microglobulin.

Only the islet on the right contains insulin.

I.P. for insulin.

All x 140.





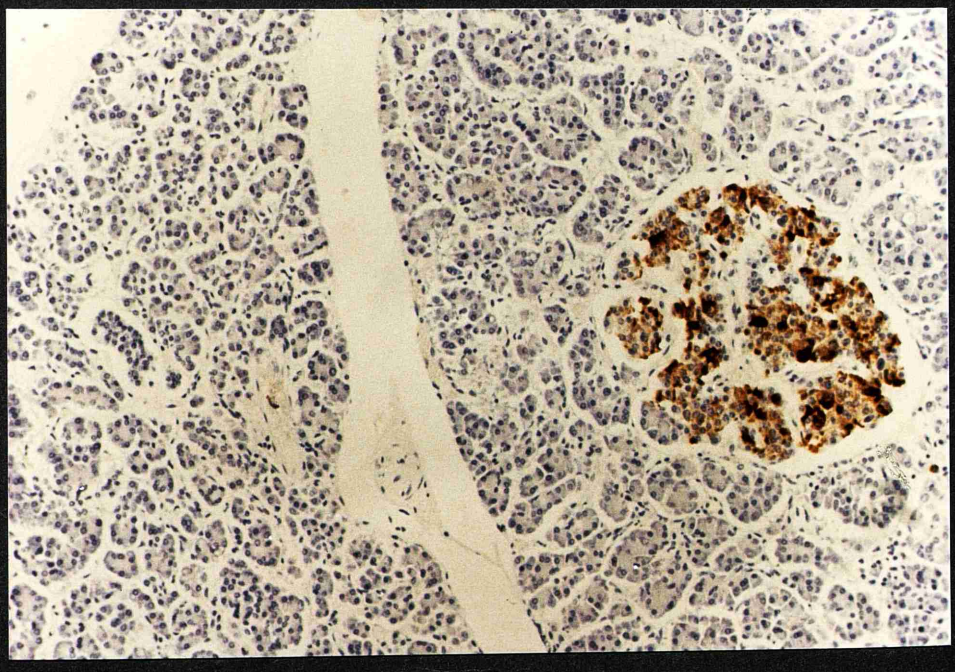


Figure 4.17

Class I MHC hyperexpression and B cells in type 1 diabetes

This section has been double stained for insulin (left) and class I MHC heavy chain (right). Note that many cells which hyperexpress class I MHC do not contain insulin.

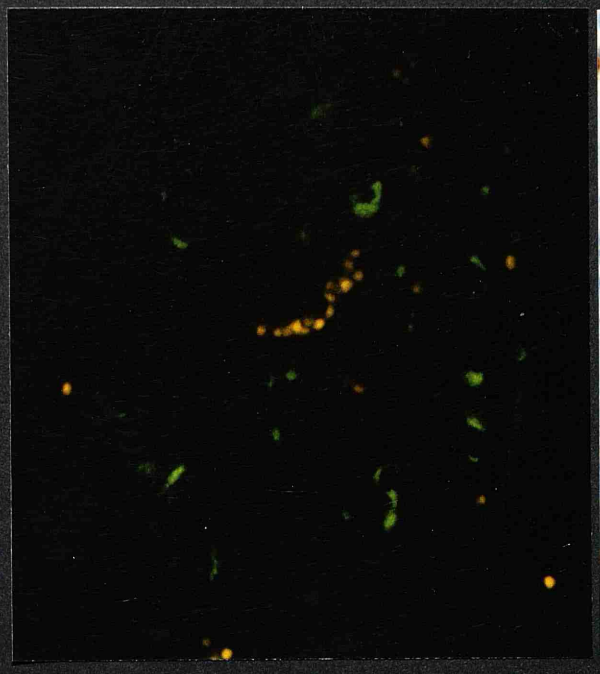
I.P. using AI followed by I.F. for insulin x 340.

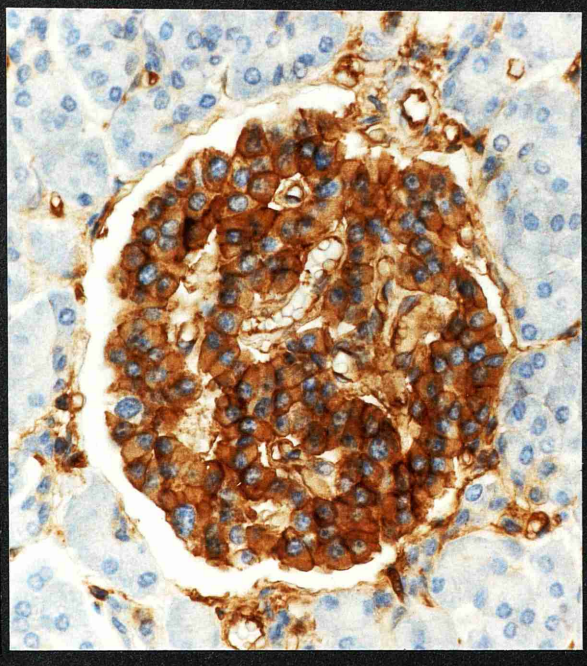
Figure 4.18

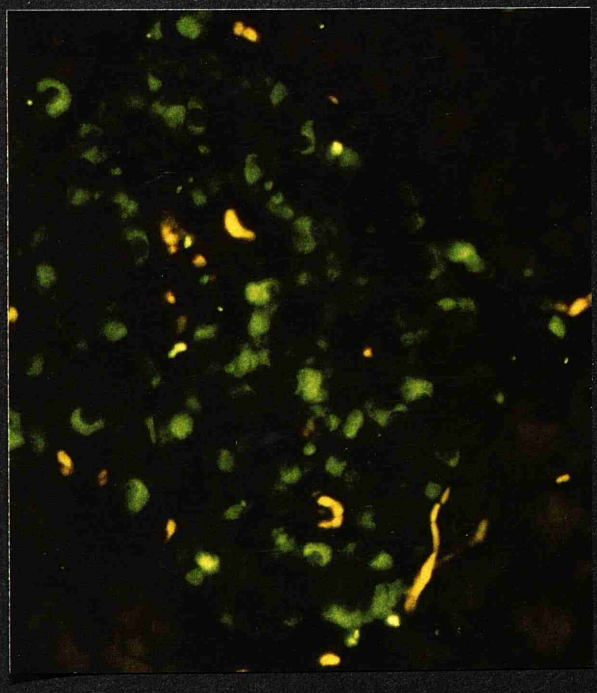
Class I MHC hyperexpression and A cells in type 1 diabetes

This section has been double stained for glucagon (left) and class I MHC heavy chain (right). A cells hyperexpress class I MHC.

I.P. using AI followed by I.F. for glucagon x 340







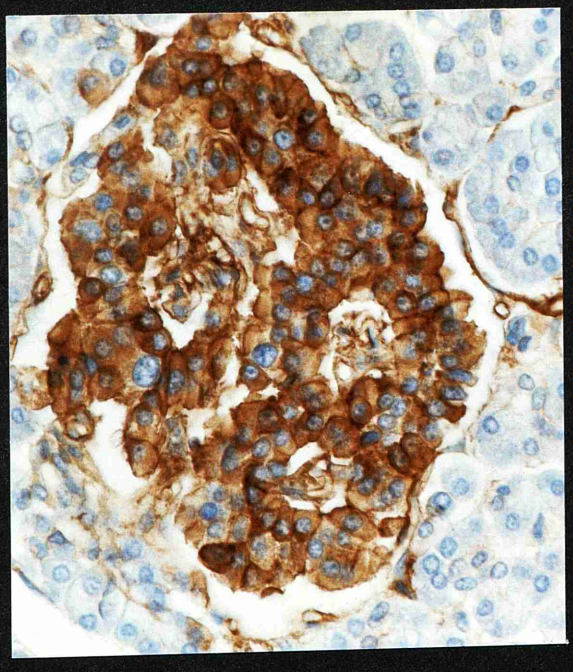


Figure 4.19

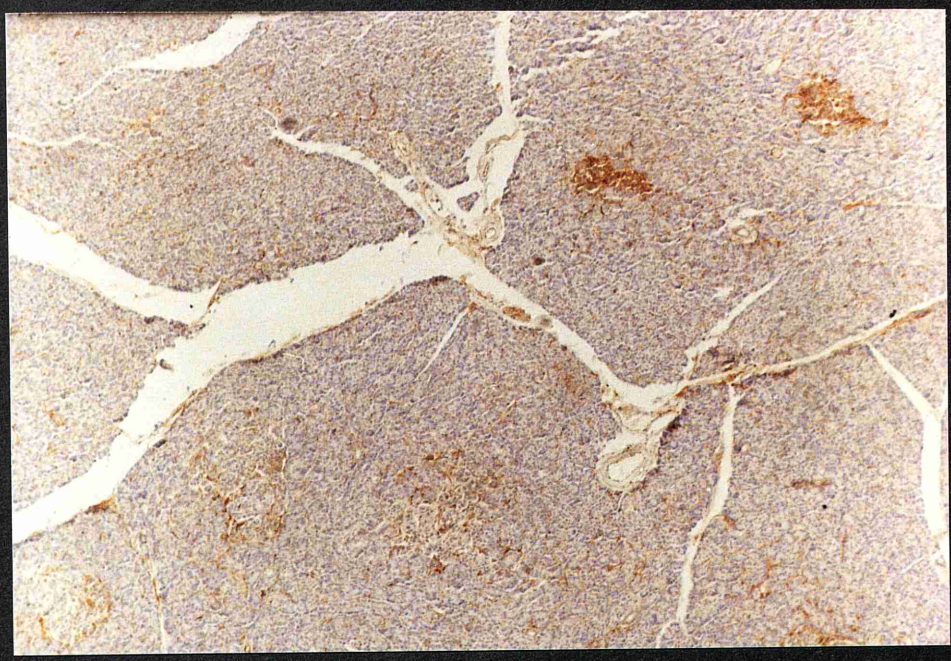
Insulin containing islets which do not hyperexpress class I MHC in type 1 diabetes

Two islets are seen in one lobule which hyperexpress class I MHC.

I.P. using AI x 55

While the two islets noted above contain insulin several other insulin containing islets are present in the adjacent pancreatic lobule which do not hyperexpress class I MHC.

I.P. for insulin x 55



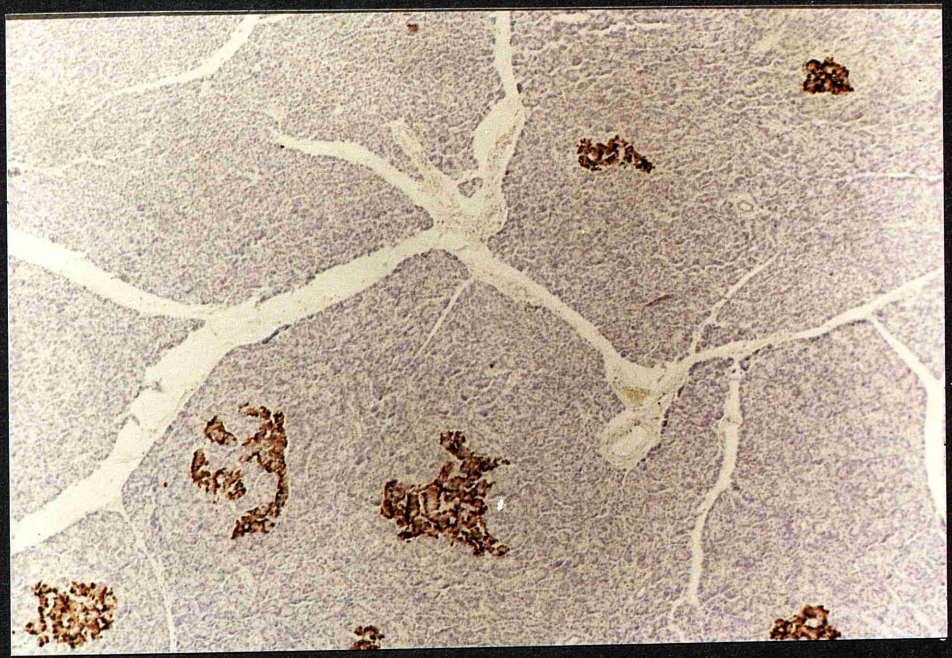


Figure 4.20

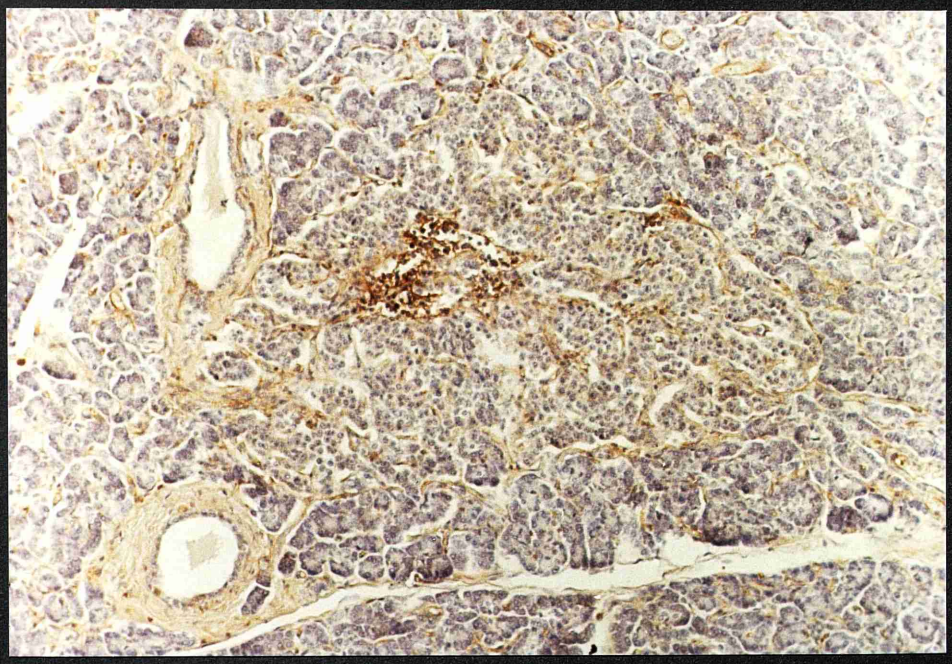
Class I MHC expression in an insulin deficient islet affected by insulitis

There is no hyperexpression of class I MHC on endocrine cells in this islet. The lymphocytes in the inflammatory infiltrate are strongly class I MHC positive.

I.P. using AI x 140

The islet is insulin deficient.

I.P. for insulin x 40



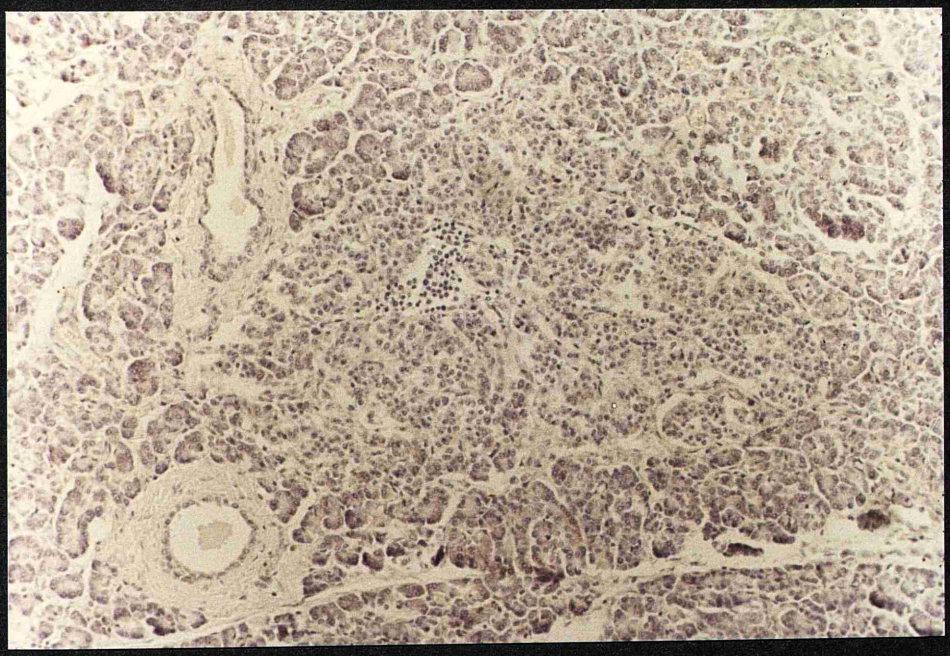


Figure 4.21

Insulitis in Coxsackie B viral infection of neonates

There is necrosis of some endocrine cells in the centre of this islet. Note the large numbers of eosinophils in the inflammatory infiltrate.

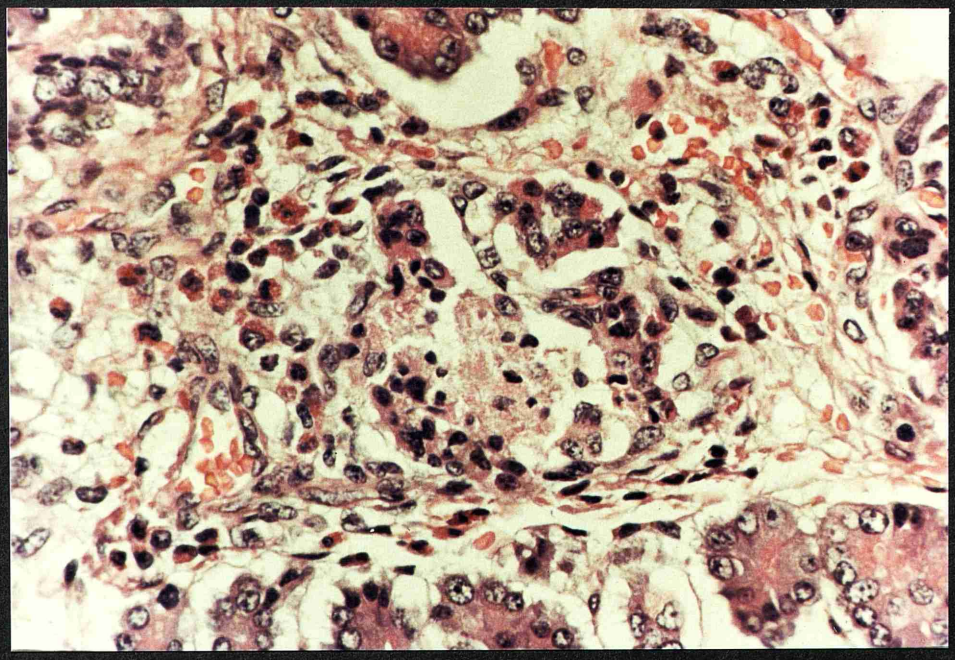
H & E x 440

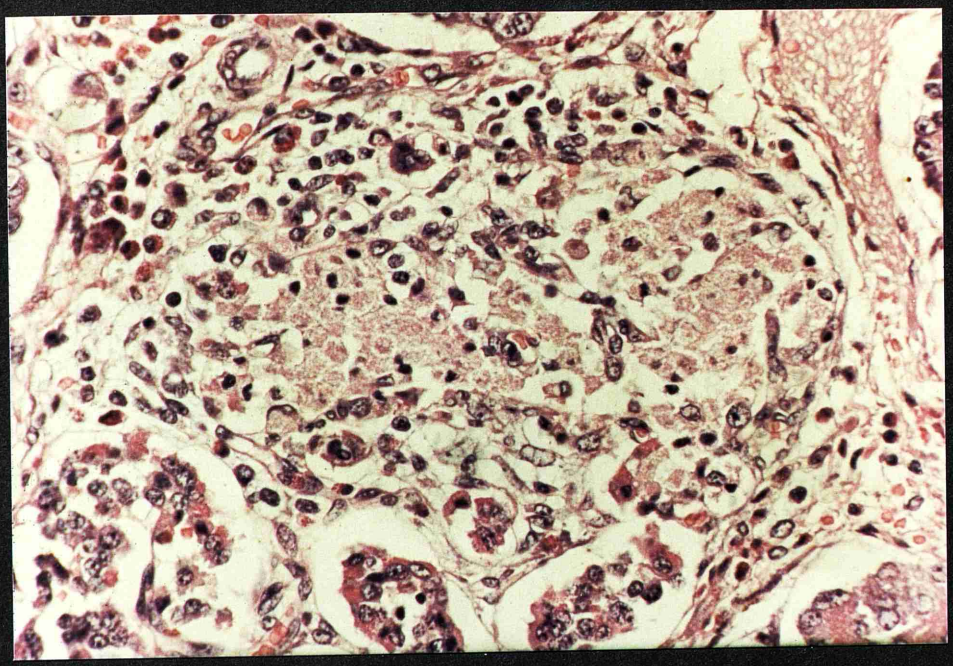
Large numbers of necrotic endocrine cells are present in this islet.

H & E x 340

The endocrine cells with pyknotic nuclei are B cells containing insulin.

I.P. for insulin x 340





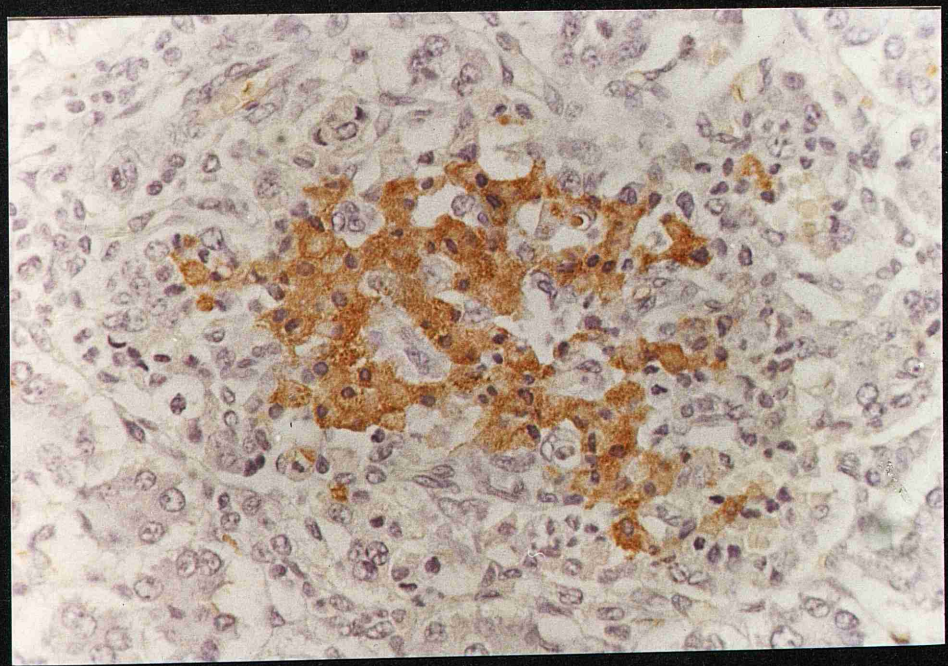


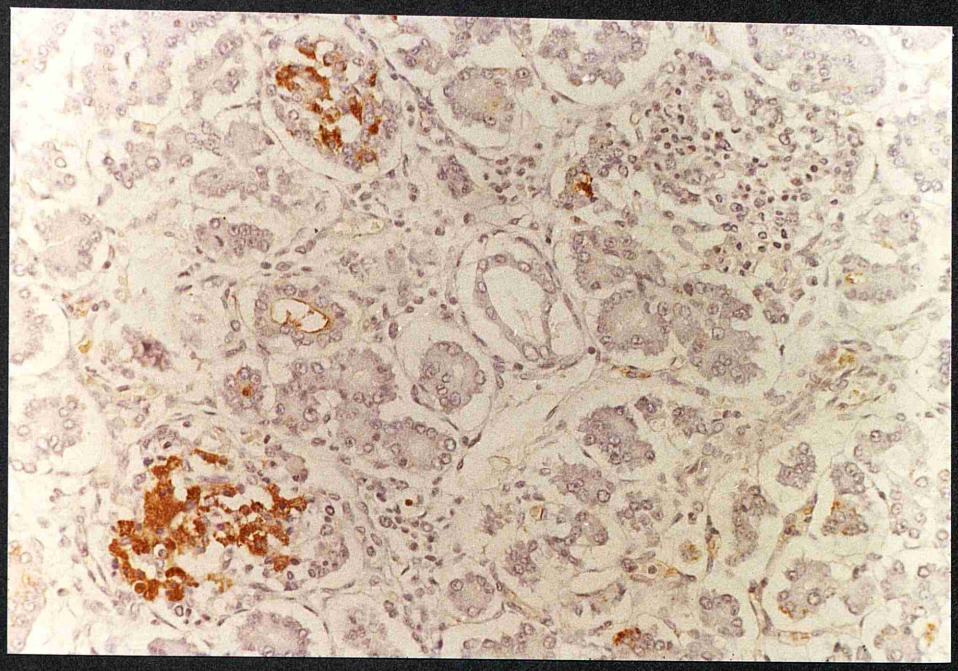
Figure 4.21 continued

Three islets are present; the one in the top right of the photograph is insulin deficient.

I.P. for insulin x 200

The insulin deficient islet has no loss of A cells.

I.P. for glucagon x 200



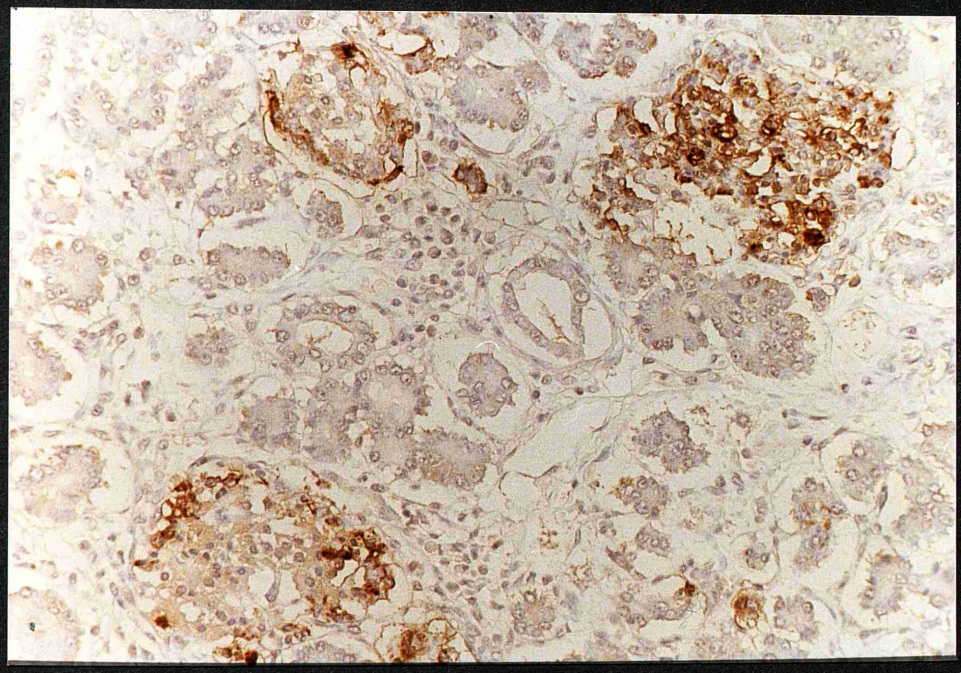


Figure 4.22

Islet cell necrosis with no inflammation in Coxsackie B viral infection of neonates

All the endocrine cells in these two islets appear necrotic. Note the lack of autolysis in the exocrine tissue.

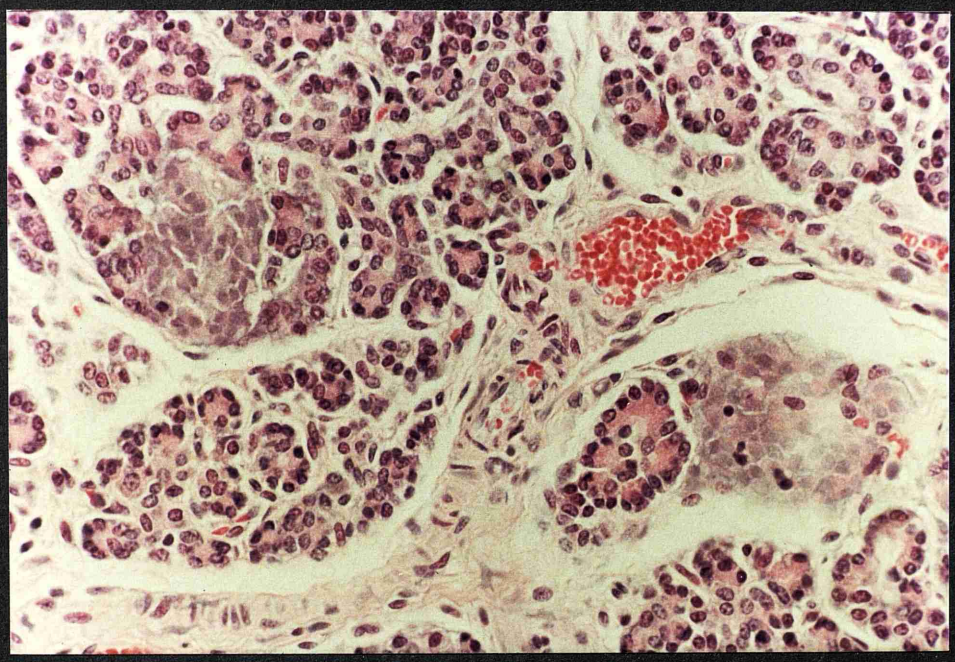
H & E x 300.

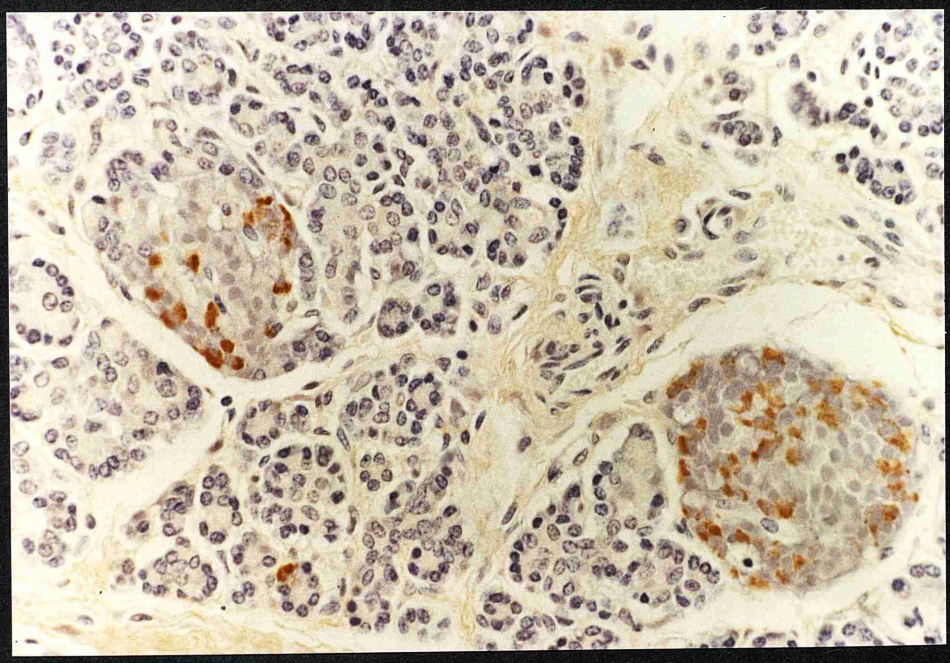
Necrotic B cells are present in these islets.

I.P. for insulin x 300

Necrotic D cells are also present in these islets.

I.P. for somatostatin x 300





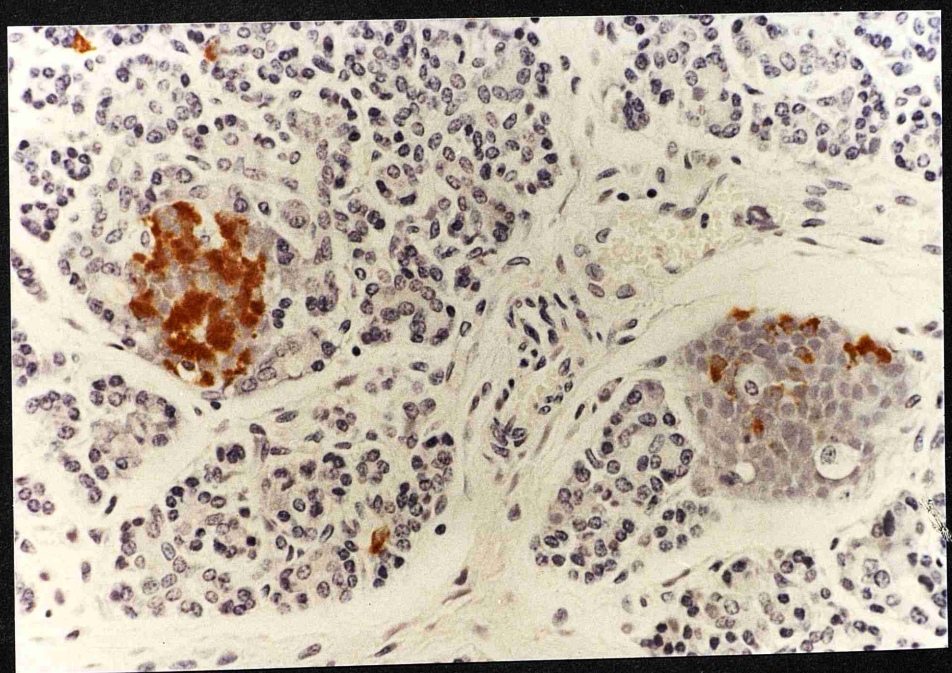


Figure 4.23

Class I MHC expression in chronic pancreatitis

There is marked hyperexpression of class I MHC on ductal epithelium. Islet endocrine cells do not hyperexpress class I MHC significantly.

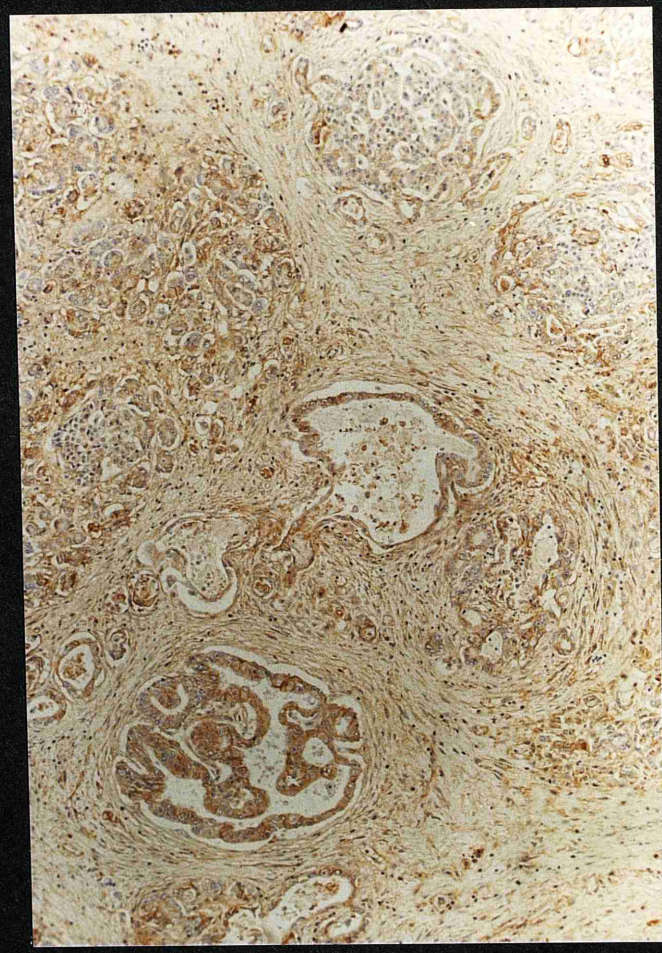
I.P. using AI x 130

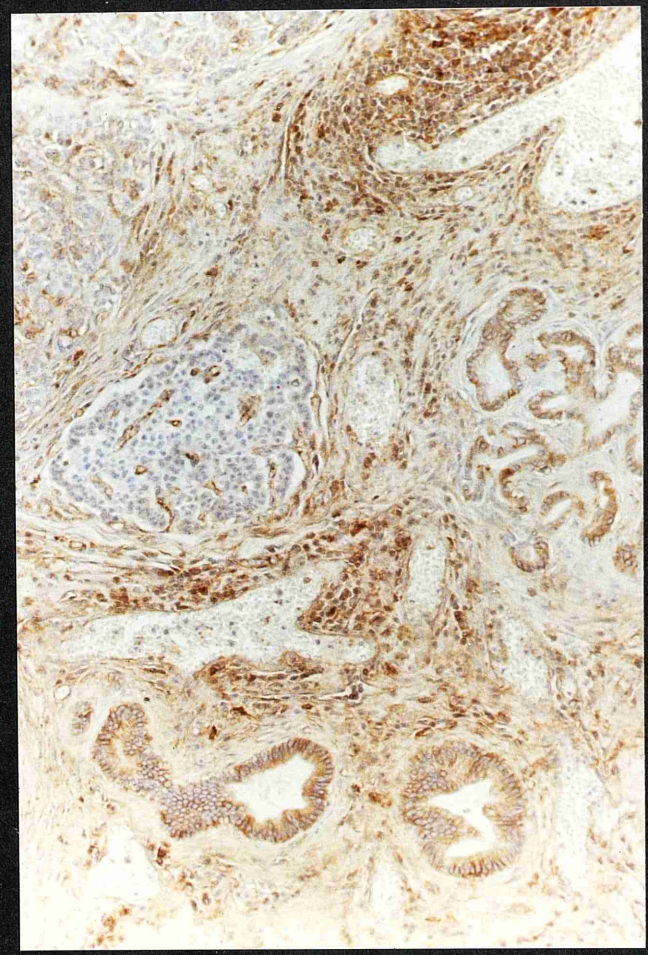
Figure 4.24

Class I MHC expression in the pancreas in graft-versus-host disease

While there is marked hyperexpression of class I MHC on all exocrine elements of the pancreas the islets only stain minimally more heavily than normal.

I.P. using AI x 70





HLA-DR expression on B cells in type 1 diabetes might be that this is a phenomenon exhibited by a dying cell. Four of the Coxsackie B virus associated cases had evidence of B cell necrosis but HLA-DR expression was not seen in them. Equally the presence of insulitis in 3 of these cases did not result in B cells showing class II MHC expression.

The second phenomenon to be studied in this section was the presence of class I MHC hyperexpression on insulin containing islets. This phenomenon also appeared to be unique to type 1 diabetes. In contrast to HLA-DR expression, however, all the endocrine cells in affected islets appeared to hyperexpress class I MHC equally.

It was argued in the previous section of this chapter, in the study using TAL-1B5, that the pancreas at clinical presentation of type 1 diabetes is likely to be qualitatively similar to the pancreas at the onset of B cell destruction. From this it was suggested that aberrant HLA-DR expression on B cells probably precedes insulitis within a given islet. The same arguments hold good for this study with the additional evidence that Coxsackie virus associated insulitis did not induce this aberrant expression. What is the position of class I MHC hyperexpression in the sequence of events that results in destruction of B cells ? Class I MHC hyperexpression was very common, affecting 97% of insulin containing islets in non-polyendocrine recent-onset diabetes. Indeed many of the islets, which on hormone staining were thought to be normal, turned out to have class I MHC hyperexpression. All islets in which there was aberrant HLA-DR expression hyperexpressed class I MHC, but many islets with the latter

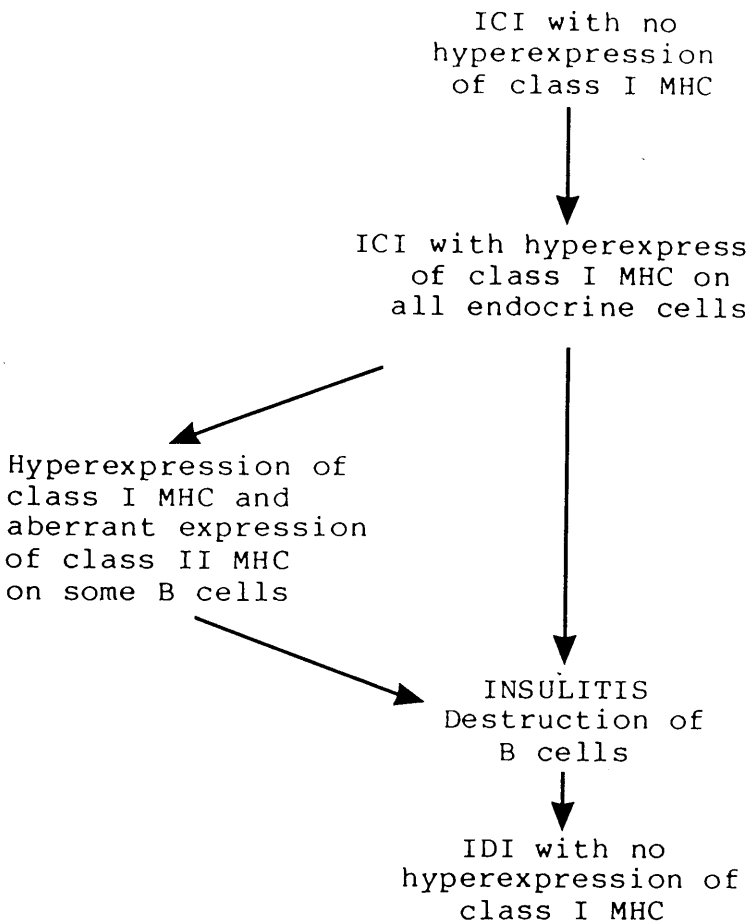
phenomenon did not have any evidence of aberrant HLA-DR expression (Tables 4.3, 4.4). Insulin containing B cells expressing class II MHC were never seen in islets devoid of class I MHC hyperexpression. There is therefore considerable evidence that within an individual islet hyperexpression of class I MHC on all endocrine cells precedes aberrant expression of HLA-DR on B cells.

Similar arguments can be put forward to show that class I hyperexpression precedes insulitis. In the vast majority of islets where this hyperexpression was seen there was no evidence of insulitis, but all inflammed insulin containing islets hyperexpressed class I MHC. Insulitis affecting insulin deficient islets was not necessarily associated with hyperexpression of class I MHC on A and D cells, and inflammatory diseases of the pancreas, while causing a generalised increase in expression of class I MHC on both exocrine and endocrine pancreas, were not associated with the same degree of hyperexpression as was observed in type 1 diabetes.

Thus the proposed sequence of events within an individual islet in type 1 diabetes is as follows (Fig 4.25). The earliest demonstrable abnormality is hyperexpression of class I MHC. This will affect all the islets in which B cell destruction will eventually take place. (Insulitis is the process by which B cells are destroyed and all inflamed insulin containing islets hyperexpress class I MHC). HLA-DR expression on B cells may not necessarily be a prerequisite for these cells to be destroyed since islets with insulitis were found where this phenomenon was absent. Thus hyperexpression of class I MHC

Fig. 4.25

Proposed sequence of events within individual islets in type 1 diabetes



ICI = insulin containing islet
IDI = insulin deficient islet.

on an islet may perhaps be followed either directly by insulitis, or by aberrant expression of HLA-DR on some B cells and then by insulitis. Insulin secreting B cells are killed in this inflammatory process leaving an islet consisting of A, D and PP cells which no longer hyperexpress class I MHC.

It was noted in chapter 2 that there was a marked lobular distribution of residual insulin containing islets in type 1 diabetes. Also, the presence of insulitis in one islet was frequently accompanied by insulitis affecting adjacent islets. In this connection it was of interest that insulin containing islets which did not hyperexpress class I MHC also appeared to be grouped together within lobules. This emphasises the suggestion that the events leading to B cell destruction in the pancreas are partially dictated in some unknown way by the lobular anatomy of the pancreas.

It has been shown that in many diabetics there is evidence of residual insulin secretion for several years after clinical presentation (Mustonen et al. 1984). Thirty of 39 patients in this report still had detectable C-peptide in their serum 2 years after diagnosis. In the present study, 97% of insulin containing islets in non-polyendocrine, recent-onset diabetics had hyperexpression of class I MHC at clinical presentation. Unless these 19 patients are radically different from those in the Finnish study quoted above, then it appears that islets may hyperexpress class I MHC for a long time, perhaps years, before the B cells in the islet are destroyed.

An intriguing aspect of the phenomenon of hyper-expression of class I MHC on insulin containing islets was that glucagon secreting A cells and somatostatin secreting D cells were also involved. Why should they hyperexpress class I MHC when adjacent to B cells, but not when they form insulin deficient islets ? It has been argued that the presence of insulitis is not the answer to this question, since hyperexpression on endocrine cells appears to precede insulitis, and inflamed insulin deficient islets may have no hyperexpression of class I MHC. It is unlikely to be due to local insulin secretion, since insulin containing islets could be found in which the phenomenon was not observed. Islet cell cytoplasmic anti bodies adhere to A and D cells on frozen sections of normal pancreas, but there is little to suggest that the anti genicity of these cells is different if they are present in an insulin deficient islet rather than an insulin containing one. One possible explanation is that the B cells in islets in which class I hyperexpression is observed are secreting some substance which has a paracrine effect on the adjacent A and D cells, causing them also to hyperexpress class I MHC. Candidates for this role might include alpha- and beta-interferon. A common stimulus for release of these substances from epithelial and connective tissue cells is viral infection. Both these interferons have been shown to cause hyperexpression of class I MHC on pancreatic endocrine cells <u>in vitro</u> (Pujol-Borrell et al. 1986).

It was argued in the second part of this chapter that aberrant expression of class II MHC on B cells may be the event which triggers the eventual autoimmune destruction of

these cells. Here it has been shown that within an individual islet hyperexpression of class I MHC probably precedes aberrant HLA-DR expression. These two suggestions are not necessarily mutually exclusive.

The study of pancreases from patients dying at clinical presentation of type 1 diabetes, when autoimmune destruction of B cells is already taking place, is at best a good reflection of the events involved in the initial induction of autoimmunity. It could be assumed that if insulin containing islets at clinical presentation appear to be undergoing a particular series of events, in which class I hyperexpression precedes aberrant HLA-DR expression on B cells, both of which precede insulitis, then other islets may have gone through a similar sequence of abnormal MHC expression in the prediabetic period, before any B cells were destroyed. If this were the case then the first of these abnormalities to be seen in the pancreas might have been hyperexpression of class I MHC on individual islets. This could conceivably have been present in the absence of any recognisable autoimmune phenomena, such as the presence of autoantibodies. Subsequent induction of HLA-DR expression on B cells could have initiated the proliferation of potentially autoreactive helper T lymphocytes which could, in the absence of appropriate suppression, have led to the production of autoantibodies and the generation of B cell specific cytotoxic T lymphocytes. Thus induction of autoimmunity in type 1 diabetes could be a "multistep" process in which the key event, but not necessarily the first event, is aberrant expression of HLA-DR on B cells.

CHAPTER 5

CHAPTER 5

The pathogenesis of type 1 diabetes - conclusions and indications of future research

The characteristic lesion of type 1 diabetes is the presence of insulitis (Gepts 1965). While it has been previously proposed that this lesion may be associated with destruction of B cells very little evidence for this has been forthcoming (Gepts & De Mey 1978). The first contribution that the present studies have made to the understanding of the pathogenesis of type 1 diabetes was the observation that insulitis was twenty times more likely to affect insulin containing islets than insulin deficient islets. This finding has provided the most substantial direct evidence yet that insulitis represents an immunologically mediated destruction of B cells.

There remains speculation on the role of viruses and autoimmunity in the aetiology of type 1 diabetes. Many patients with the disease present in late Autumn and mid-Winter and it was originally thought that if viruses were involved in its pathogenesis they would have a direct cytopathic effect on B cells, which might account for the seasonal variation. Of all the cases described in chapter 2 only the pancreas of the 22 year old Chinese male with acute onset diabetes showed a picture consistent with such a pathogenesis. Here all the islets appeared to have been affected simultaneously and at the time of death there was massive recent destruction of B cells. The mixed infiltrate of lymphocytes and eosinophils, found in this case, was also present in the pancreases of 3 neonates

who died of culture proven Coxsackie B viral myocarditis. (The histological findings in these Coxsackie cases confirmed the observations of previous authors that pancreatic endocrine cell necrosis and insulitis were present in a proportion of neonates with such an infection (Ujevich & Jaffe, 1980; Jenson, Rosenberg & Notkins. 1980)). Thus, from a histological point of view, the destruction of B cells in this Chinese patient would be consistent with a direct viral cytopathic effect, perhaps by a Coxsackie B virus. The contention that this man had a disease of a different aetiology from 'classical' type 1 diabetes was further supported by examination of class I and II MHC expression in his pancreas (results not given in chapter 4). Virtually all B cells were destroyed, so not surprisingly there was no evidence of aberrant expression of HLA-DR on islet endocrine cells. While there was some increase in the degree of expression of class I MHC on residual A and D cells, this was no more than that seen in inflammatory diseases of the exocrine pancreas and was markedly less than that seen in 'classical' diabetes. The exocrine pancreas also had mildy increased expression of both class I and II MHC, perhaps secondary to the diffuse inflammatory infiltrate. These findings of MHC expression were similar to those seen in the pancreases of the children with Coxsackie infection.

The histopathology of this case is unique in the world literature. The two previously reported diabetics with recent-onset disease from whom Coxsackie B virus was cultured at post mortem were said to have had more insulin containing islets than are normally found in 'classical'

diabetes (Gepts & LeCompte 1985). Thus, if the case reported here represents an acute cytopathic effect of a virus on B cells this must be a rare mechanism in the production of diabetes.

It has long been recognised that type 1 diabetes has a hereditary component to its aetiology. Initially, the genetic link in type 1 diabetes was thought to be with class I MHC type. Later it was shown that the relationship with class II MHC group was much stronger. While up to 98% of type 1 diabetics may possess either DR3 or DR4, it has to be emphasised that approximately 36% of the normal population also possess these antigens (Wolf et al 1983). Thus only about 1 person in 70 who possess DR3 or DR4 will develop diabetes. Since the concordance rate for type 1 diabetes between monozygotic twins is as high as 40% it is possible that a more direct genetic linkage than DR type may exist.

The hypothesis of Bottazzo et al (1983) that aberrant expression of class II MHC antigens on target cells may be a triggering event in the induction of autoimmunity was the stimulus for the studies reported in chapter 4. This hypothesis is attractive, partly because it is very simple, but principally because it encompasses the agent linked to the genetic predisposition to autoimmunity - DR type - with an event at the target cell - aberrant expression of HLA-DR. While it is recognised, as has been discussed above, that DR type probably does not explain the whole genetic predisposition to type 1 diabetes, it could be postulated that the DR gene may be in linkage disequilibrium with an as yet undiscovered gene. Such a

gene could control class II MHC expression, for example. Alternatively the linkage could be with DP or DQ genes.

It sometimes seems that the pancreas in type 1 diabetes was almost designed to test Bottazzo et al's hypothesis - the "islet organ" has been divided into a million parts and among these a range of appearances, from totally normal islets to islets where all the B cells have been destroyed, can be seen. Equally the B cells are surrounded by "control" cells - A, D and PP cells. Aberrant expression of HLA-DR on B cells appeared to be present in almost all cases of type 1 diabetes. This is all the more remarkable given the very small amount of tissue available for study in many instances. The phenomenon has been demonstrated using three different antibodies to class II MHC and was not seen in a wide variety of controls. It is therefore every bit as characteristic of type 1 diabetes as insulitis.

There is equally convincing evidence that hyperexpression of class I MHC affecting all endocrine cells in insulin containing islets typifies type 1 diabetes and may be an "earlier" event than aberrant expression of class II MHC on B cells.

While the aetiology of these two abnormalities of MHC expression is quite unknown one can tentatively suggest some possibilities. There are several reports of an increased incidence of IgM antibodies to Coxsackie B viruses in the sera of patients with recent-onset type 1 diabetes (Banatvala et al 1985, Eberhardt et al 1985). While this finding would be consistent with a recent infection by the virus it could also be evidence of a

persistent viral infection. There is one case report of a child with both islet cell cytoplasmic antibodies and raised titres to Coxsackie B4 present for 3 years before the onset of diabetes (Asplin et al, 1982). Recently, Coxsackie virus RNA has been demonstrated in heart biopsies of patients with chronic myocarditis, indicating that this virus can cause chronic as well as acute disease (Bowles et al, 1986).

It has been suggested that DR4 patients with recent-onset type 1 diabetes are more likely to have antibodies to Coxsackie B viruses than DR3 patients (Eberhardt et al, 1985). The former tended also to present with a more severe illness clinically. This suggests that DR4 individuals might handle the virus differently from persons with other DR groups. Using an in vitro system of killed virus, antigen presenting cells and T lymphocytes from a variety of donors of different DR types, some of whom were diabetic, it was shown that there was an increased frequency of peripheral blood T lymphocytes capable of reacting to Coxsackie B virus and Mumps virus in DR4 donors compared to others. DR3 donors on the other had had a decreased frequency of reactive T cells compared to the mean. These findings were present whether the donor was diabetic or not (Bruserud et al. 1985), Thus it is possible to envisage that insulin secreting B cells could become infected by a pancreotropic virus, such as Coxsackie B virus, and that a persistent infection of the cells, rather than an acute infection, could result. The outcome of such a non-cytopathic infection of B cells could be partly determined by the HLA-DR group of the individual.

There was no evidence of aberrant HLA-DR expression on B cells in the pancreases of the neonates with Coxsackie B viral infection. This does not prove, however, that such an infection could not induce HLA-DR expression in a genetically predisposed individual.

Attempts have been made to find agents capable of causing aberrant expression of class II MHC on B cells in vitro using islet cultures taken from pancreatic resections (Campbell & Harrison 1985, Pujol-Borrell et al. 1986). However it is significant that none of the islet donors in one of these studies were of DR3 or DR4 type (Harrison, personal communication). All equivalent studies may have a similar problem in that even islets from DR3 or DR4 individuals will be from people with odds of 70 to 1 against developing diabetes.

In summary, Coxsackie B viruses have been shown to infect B cells (Yoon et al. 1979). The fact that these viruses can cause both acute and chronic disease in the heart suggests that chronic infection of B cells may be a possibility. The susceptibility to a chronic infection in the pancreas could be genetically inherited and linked to HLA-DR type. Equally, whether or not a chronically infected B cell were induced to express class II MHC could be determined by DR type or by a gene in linkage disequilibrium with the HLA-DR gene. A persistent viral infection of B cells could cause them to release alpha- or beta-interferon. This could be one explanation for the presence of hyperexpression of class I MHC on pancreatic endocrine cells seen in diabetes. Thus it is possible that a persistent viral infection of B cells could initiate a

series of cellular events which could eventually result in an autoimmune reaction directed against B cells.

While this hypothesis remains purely speculative several studies are at present being started which should help establish whether viruses can cause autoimmunity in diabetes.

Firstly, an attempt will be made to demonstrate alpha- and beta interferon and tumour necrosis factor in fixed tissue, using polyclonal antisera and immunocytochemistry. If any of these substances were shown to be specifically present in the diabetic pancreases in islets where there was hyperexpression of class I MHC on endocrine cells it would be evidence that their release might be the cause of this hyperexpression. If any of them were demonstrated only in the B cells of such islets this would be suggestive (but not proof) of a viral infection of B cells.

Secondly, nucleic acid probes have been prepared for all common viruses. It has already proved possible to demonstrate the presence of several viruses in paraffin embedded formalin fixed tissue using in situ hybridisation techniques, although to date there have been no reports of detection of either Coxsackie B virus or mumps virus using such methods. A more simple way than in situ hybridisation of determining whether any of these viruses are present in the B cells of the type 1 diabetic pancreas might be to try to demonstrate viral coded protein using immunocyto-chemistry.

One criticism of the present approach is that only the pancreas at and after clinical presentation has been studied. Nobody has ever consciously seen a 'pre-diabetic' pancreas. How could one be obtained? While 'polyendocrine

diabetes' may be slightly different from other cases, the abnormalities of MHC expression and insulitis were still present. Approximately 10% of patients with autoimmune Addison's disease become diabetic and a study has been initiated looking for autopsy pancreas from patients who died of Addison's disease under the age of 40 years. One hundred death certificates have been identified of such patients in whom an autopsy was performed. The 'prediabetic' pancreas should be recognisable by the presence of any of the phenomena described - hyperexpression of class I MHC on islets, aberrant expression of class II MHC on B cells, insulitis or insulin deficient islets.

In the pancreas of a patient who has had type 1 diabetes for many years all the B cells are likely to have been destroyed. By studying this organ from patients with recent-onset diabetes, Gepts (1965) established the presence of insulitis as the characteristic lesion of the disease. The present studies have attempted to delve further back to abnormalitis which precede insulitis. These appear to include aberrant HLA-DR expression on B cells and hyperexpression of class I MHC antigens on all endocrine cells of insulin containing islets. A continuation of this approach may reach back even further into the origins of the disease, to agents which could effect these abnormalities of MHC expression.

Thus, study of the pancreas in diabetes, a hitherto neglected area of research, may eventually provide some of the answers to the aetiology and pathogenesis of the disease.

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