TRANSPLANTATION OF ORGAN CULTURED FOETAL ISLETS OF LANGERHANS IN MICE

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUREMENT OF THE DEGREE OF DOCTOR OF PHILOSOPHY, UNIVERSITY OF LEICESTER

JUNE 1997

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789 East Eisenhower Parkway
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Ann Arbor, MI 48106-1346

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Foetal islets are functionally immature but retain their capacity for proliferation if harvested and cultured in an appropriate manner. Graft function was shown to depend largely on the gestational age and conditions of organ culture prior to transplantation. The required period of organ culture for optimal graft function was investigated for foetal mouse pancreas of different gestational ages. The growth of the graft *in situ* also depended on the diabetic state of the host, and chronic hyperglycaemia appeared to impair graft function. Subsequent studies using NOD recipient mice as a model for IDDM showed that recurrent autoimmune disease was seen in foetal islet isografts but rapid rejection of allografts and foetal pig xenografts also occurred. The striking differences seen between the allo-, and xenograft response was the presence of many eosinophils that dominated the infiltrate at the xenograft site. However, HAR was not a problem in this discordant xenograft and Gal(α1-3)Gal expression, the major epitope for xenoreactive Ab, was not present on differentiated cells but was detectable on ductal cells. A brief treatment with a specific anti-CD4 MAb (GK1.5) had a profound effect in the survival of xenografts in NOD mice. There were consistent differences in xenograft survival and in the number of circulating T and B cells in other strains of mice, e.g. CBA, BALB/c, C57BL/6 compared to NOD mice. Prolongation of xenograft survival for up to 12 weeks was achieved with the use of peri-transplant and weekly treatment with anti-CD4 or anti-CD3 MAbs especially when the graft has been "immunomodulated" by using 90% O_2 in organ culture. Using this protocol foetal pig xenografts maturing under the kidney capsule of spontaneously diabetic NOD mice reversed hyperglycaemia and appeared also to secrete growth factor(s) that induced regeneration of β cells in the host pancreas.

ACKNOWLEDGEMENTS

I wish to extend my sincere gratitude to my supervisor, Dr. Roger James for his support. I also wish to thank Dr. Thomas Mandel for his guidance support, tolerance and enthusiasm throughout this project. I am especially indebted to Dr. Jiri Kovarik for his friendship, devotion and unceasing support during the writing period.

I am greatly indebted for the assistance provided by the staff of the numerous service departments of the Walter and Eliza Hall Institute: Steven Mihajlovic, and Adrian Warmington for the histological preparations; Robyn Muir and Frank Battye for flow cytometry; Oanh Tran from media preparation, Simon Olding, Drew Berry and Peter Maltezos for art and graphics and the numerous animal technicians who helped with the breeding and maintenance of the experimental animals used throughout these studies.

Finally, I extend my heartfelt gratitude to my parents Helen and John for their love, support and encouragement over the years.

I, Maria Koulmanda, hereby declare that; the work in my thesis was mainly done during the period of registration and most of the work was carried out by me unless stated in the acknowledgment.

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LIST OF ABBREVIATIONS

Ab	:	antibody
Ag	:	antigen
APCs	:	antigen presenting cells
BSA	:	bovine serum albumin
BW	:	body weight
CC	:	conventional condition culture (10% CO_2/90% air)
CSII	:	continuous subcutaneous insulin infusion
CTLs	:	cytotoxic T lymphocytes
DAF	:	decay accelerating factor (CD 55)
DBA	:	diaminobenzidine
DDW	:	deionized distilled water
DME	:	Dulbecco's modified Eagle's medium
FACS	:	fluorescence-activated cell sorter
FCS	:	foetal calf serum
GAF	:	Gomori aldehyde fuchsin
GHb	:	Glycosylated Haemoglobin
GTT	:	glucose tolerance test
H	:	histocompatibily
HAR	:	hyperacute rejection
H&E	:	haematoxylin and eosin
HiO_2	:	high oxygen culture (90% O_2/10% CO_2)
Ia	:	I region associated
ICA	:	islet cell antibodies
IDDM	:	insulin-dependent diabetes mellitus (Type 1 diabetes)
Ig	:	immunoglobulin
im	:	intramuscular

IMP	:	immunoperoxidase
ip	:	intraperitoneal
iv	:	intravenous
K	:	killer cell
KDS	:	balanced salt solution
LN	:	lymph node
MAbs	:	monoclonal antibodies
MCP	:	membrane associated cofactor (CD 46)
MHC	:	major histocompatibily complex
MIRL	:	membrane inhibitor of reactive lysis (CD 59)
MLC	:	mixed lymphocyte culture
MLR	:	mixed lymphocyte reaction
MNC	:	mononuclear cell
MST	:	mean survival time
MTPBS	:	mouse tonicity phosphate buffered saline
NAbs	:	natural antibodies
NIDDM	:	non-insulin dependent diabetes mellitus (Type 2 diabetes)
NK	:	natural killer cell
NOD	:	non-obese diabetic
NS	:	normal saline
NSS	:	normal swine serum
PB	:	peripheral blood
PI	:	propidium iodide
p/t	:	peri-transplant
RBC	:	red blood cells
RBG	:	random blood glucose
RIA	:	radioimmunoassay
sc	:	subcutaneously

SD	:	standard deviation
SEM	:	standard error of the mean
STZ	:	streptozotocin
Tc	:	cytotoxic T cell
TcR	:	T cell receptor
Th	:	helper T cell
Ts	:	suppressor T cell
UV	:	ultraviolet light
XNA	:	xeno-natural antibody
1^{o}	:	primary
2^{o}	:	secondary
7d CC	:	7 day culture under conventional contions
14d CC	:	14 day culture under conventional contions
21d CC	:	21 day culture under conventional contions

CHAPTER 1: A REVIEW OF THE LITERATURE

1.1 DIABETES MELLITUS

1.1.1 Pathogenesis

Diabetes mellitus is not a single disease but rather a group of disorders characterised by hyperglycaemia that can be due to either absolute or relative deficiency of insulin. In its various forms, diabetes in the twentieth century afflicts about 5% of the population in most Western societies (Diabetes statistics, 1995). The term "diabetes" (GK.= syphon), which means "to run through", was first reported by Aretaeus Cappadocia at the beginning of the second century AD. It was not until 1710 that William Cullen introduced the term "mellitus" (meli GK.=honey) because of the characteristic feature of glycosuria. It is possible that ancient Indian, Japanese and Chinese physicians noticed the sweet taste of urine in this disorder.

It has become apparent that diabetes mellitus is a heterogeneous disorder characterised by hyperglycaemia (Papaspyros, 1964) but there are two major forms: IDDM also known as Type 1 or juvenile-onset diabetes, and non-insulin dependent (NIDDM) also known as Type 2 or maturity-onset diabetes. IDDM usually begins in childhood or adolescence and mostly before the age of 25 years. The onset symptoms and signs of polyuria, polydipsia, polyphagia, hyperglycaemia, glycosuria, insulinopenia and weight loss have been recognised as the classical features for centuries. The primary pathogenic feature of IDDM is the destruction of the β cells of the pancreatic islets, leading to the reduction or absolute deficiency of pancreatic insulin secretion. Overt disease has an abrupt onset, the patient becomes dependent upon insulin injections and develops a proneness of ketoacidosis (Hagopian and Lernmark, 1992).

In contrast, NIDDM usually begins over the age of 40 years and affects females more often than males. Classically the patient is over-weight, and often the diagnosis is made on routine examination of the blood or urine from an asymptomatic person. Treatment is usually initially by dietary control and, if needed, weight reduction. Oral hypoglycaemic agents, may also be required in many patients. In some cases insulin is required to correct persisting hyperglycaemia when other treatments fail. In most cases NIDDM is found to be clearly associated with dietary abundance and in this situation dietary restriction and weight loss

reverses as many as 75% of the cases of hyperglycaemia at least transiently. This thesis will deal only with IDDM as it is towards this condition that pancreatic transplantation is aimed.

1.1.2 Aetiology of insulin dependent diabetes

The pathogenesis of both forms of diabetes is not fully understood. Destruction of insulin-secreting β cells of the pancreas is responsible for IDDM. A variety of factors including viruses, chemicals and immune reactions mediated by lymphoid cells or antibodies have been implicated as possible causes of this destruction, and over the past few years it has become clear that autoimmune abnormalities are associated with the majority of cases. In some cases diabetes is ascribable to a single factor such as pancreatectomy, pancreatic agenesis or exposure to certain β cell cytotoxins but these are not classical forms of IDDM and as usually returned to as secondary diabetes.

Viral infection has been thought to be implicated in the pathogenesis for over 60 years. Yoon and colleagues (1979) made the most impressive finding by isolating coxsackievirus B4 from a child who died from viral encephalitis and severe ketoacidosis soon after the onset of acute IDDM. When this virus was injected into mice they developed hyperglycaemia, insulitis and β cell necrosis. Other viral candidates have included mumps and rubella viruses (Craighead, 1981). About 20 percent of people born with congenital rubella develop IDDM later in life. Viruses have been hypothesised to induce IDDM by modifying β cell antigens, molecular mimicry, direct lysis of β cells and causing functional alterations in effector or regulator lymphocytes (Yoon, 1991).

Toxins, such as pyriminil, alloxan and streptozotocin are of particular interest because they are directly cytotoxic to the β cells and streptozotocin is frequently employed in inducing IDDM in experimental animals. A rat poison "Vacor" related to streptozotocin has also been responsible for diabetes in Korea and in USA.

In the last decade new technology has opened novel areas for scientists to identify the relationship of IDDM to the major histocompatibility complex (MHC) uncovering possible new molecular markers of the disease. The disease was first correlated with the expression of HLA-B8 and HLA-B15 haplotype, and in 1983 Cudworth and Wolf showed that 50% of IDDM patients had both HLA-DR3 and HLA-DR4 and 97% of patients expressed at least one of these alleles, although only a small proportion of genetically susceptible people become diabetic.

More recently, a still stronger association was made with the HLA-DQ locus, and in an exciting development it was found that alteration of only one amino acid residue at position 57 of the HLA-DQβ gene may determine susceptibility in Caucasians (Todd, 1987).

Susceptibility to development of type 1 diabetes is also influenced by immunoglobulin heavy chain haplotypes (Field *et al,* 1991) and either the insulin gene or a closely linked gene on chromosome 11 (Thomson *et al,* 1989). Julier and colleagues (1991) mapped this gene or genes affecting HLA-DR4 IDDM susceptibility to a 19-kilobase region spanned by a variable number of random repeat loci in the 5' region of the insulin gene and the third intron of the gene for insulin-like growth factor II.

The presence of autoantibodies in the serum of patients with autoimmune endocrine diseases has been recognised for some time. Cytoplasmic islet cell antibodies (ICA) in IDDM were first reported by Bottazzo *et al,* (1974) and MacCuish *et al,* (1974). It has been reported that 70% of patients with recent onset IDDM and a similar proportion in the pre-diabetes stage have ICA (Srikanta *et al,* 1985), compared to 0.5% -2.0% of non-diabetic control subjects (Drell and Notkins, 1987). Studies of first degree relatives of patients with IDDM have revealed that ICA are present for long periods before the onset of clinical disease (Gorsuch *et al,* 1981). Srikanta and colleagues (1983) investigated the presence in patients of ICA in initially discordant monozygotic twins and triplets and found that ICA were present up to 8 years before the onset of IDDM.

Baekkeskov and colleagues (1982) showed that 30 percent of people in the pre-diabetic phase, 80 percent of whom had ICA, became diabetic. Serum from >80% of newly diagnosed diabetic patients immuno-precipitated a 64-kilodalton (Kd) human islet cell protein (Baekkeskov *et al,* 1982). The 64Kd autoantibodies have been detected up to several years before the clinical onset of disease (Baekkeskov *et al,* 1987; Atkinson *et al,* 1990). In the last five years this 64Kd autoantigen has been identified as glutamic acid decarboxylase (GAD), an enzyme involved in the biosynthesis of the inhibitory neutrotransmitter GABA (Baekkeskov *et al,* 1990).

1.1.3 Autoimmunity

Autoimmunity is a pathological condition in which the regulation of the immune system is disturbed and self components may be destroyed. In healthy individuals the immune

system is non reactive or tolerant to self antigens. During foetal development and prenatally the cells of the immune system are highly susceptible to the induction of self tolerance (Roitt *et al.* 1987). Tolerance is induced in immature B- and T-lymphocytes and occurs by contact with the respective antigens in the thymus for T-lymphocytes and in the liver and later bone marrow for B-lymphocytes. Lymphocytes which are directed towards self-antigens are then clonally deleted or negatively selected by the denial of maturation and proliferation. Negative selection alone, however, cannot account for tolerance since even in healthy individuals autoreactive T- and B-lymphocytes are detectable (Rose, 1988), furthermore antigen presenting cells (APCs) are equally capable of presenting foreign antigens and self antigens to lymphocytes. Therefore clonal anergy and active suppression are two further mechanisms accounting for self tolerance (Snell, 1987).

The failure of tolerance leads to autoimmunity which can be either a systemic or an organ specific disease. Autoreactive effector cells or autoantibodies, as well an non specific elements of the immune system such as cytokines and complement, lead to tissue destruction and inflammation. The progressive destruction of the target tissue usually ends in the loss of its function. The onset of the autoimmune disease itself seems to be induced by multiple factors such as genetic predisposition, crossreactivity with bacterial and viral antigens and hormonal regulation. For example, there is a good corelation between the development of IDDM and the expression of MHC class II loci HLA-DR3 and HLA-DR4 (Thomson, 1988), also the susceptibility of this disease is believed to be linked to HLA-DQA1, HLA-DQB1 and HLA-DRB1 (Todd, 1990).

1.1.4 Insulitis

Lymphocytic infiltration of the islets was described early this century by Schmidt (1902) but for a long time was considered a rare occurrence with little bearing on the aetiology of diabetes. It was not until 1940 that von Meyenburg named these inflammatory lesions, which are present in the islets of Langerhans, "insulitis". Insulitis has been noted in autopsy specimens of the pancreas from some patients with recent onset IDDM. These lesions consist of mononuclear cell infiltration in and around the islets and provide histological evidence that the disorder affects the islets. Gepts (1965) observed insulitis in 13 out of 22 cases. Foulis and colleagues (1987) conducted an extensive study and observed insulitis in 47 of 60 patients

(78%) with known time of onset of IDDM. It has been estimated that at presentation patients with IDDM have already lost 90% of their β cells (Grepts, 1984). Foulis *et al,* (1987) noted that insulitis was more often found in islets containing β cells than in β cell depleted islets. Immunocytochemical staining of the pancreas of a child with recent onset of IDDM revealed that although $CD4^+$ cells were present, the majority of cells infiltrating the islet were $CD8^+$ and about 90% of the invading lymphocytes expressed HLA-DR (Bottazo *et al,* 1983).

The first experimentally induced lesion of the islets of Langerhans (insulitis) in animals was observed by Lacy in 1965. He injected guinea pig antiserum against bovine insulin into rats to produce a diabetic state by neutralisation of the endogenous insulin. Histology of the pancreas showed mononuclear and eosinophilic leucocytes within some islets in a few animals with mainly an interstitial inflammation of the exocrine pancreas. Like and Rossini (1976) reported that injections of multiple low doses of streptozotocin into some strains of mice resulted in mononuclear cell infiltration of the islets and in the delayed development of IDDM.

Much has been done on experimentally induced insulitis but it was in 1977 that the first spontaneous autoimmune model of diabetes was reported by Nakhooda in the BB-Wistar rat. Three years later, Makino *et al,* (1980) described the non-obese diabetic (NOD) mouse. Both animal models develop an acute form of spontaneous diabetes mellitus characterised by hyperglycaemia, low serum insulin levels and weight loss.

This thesis will deal mostly with the NOD mouse as an experimental model for autoimmune diabetes of IDDM.

1.1.5 Complications of diabetes

Since the discovery of insulin in 1921 the prolongation of life in insulin-treated IDDM patients has revealed that diabetes progresses to cause complications affecting a range of systems. Most of the complications occur due to changes in the blood vessels and diabetics are prone to two types of vascular disease, macroangiopathy and microangiopathy.

Macroangiopathy causes hardening and degeneration of the wall of the medium and large arteries, leading to premature coronary artery disease causing angina pectoris and myocardial infarction, cerebral ischaemia progressing to stroke, and peripheral vascular disease leading to distal gangrene requiring amputation.

Microangiopathy affects small blood vessels and typically involves thickening of the basement membrane, especially affecting the eyes, kidneys and the peripheral nervous system. Diabetic retinopathy is the most common cause of blindness in the Western world. Vascular degeneration within the retina can cause microaneurysms, retinal haemorrhage, capillary closure and retinal ischaemia. Eventually blindness may result from haemorrhage into the vitreous and retinal detachment. Small vessel changes in the kidney may result in glomerulosclerosis. Microangiopathy causes damage to capillaries in the glomerulus, which causes proteinuria and can progress to chronic renal failure. Progression of these complications is often slow occurring over 10 to 15 years. Diabetic retinopathy is now often controllable by laser therapy, if detected early.

Neuropathy may also be caused by vascular insufficiency, but vitamin B deficiency and high blood glucose levels also are implicated in the metabolic destruction of the neuron itself. The neuropathy may affect the peripheral nerves causing loss of sensation or pain in the hands and feet, in cranial nerves causing double vision, for example, or in the autonomic nerves causing gastric paresis, diarrhoea, or postural hypotension. Thus, the primary disorder of blood glucose control in diabetes may affect many systems and produce a range of disabilities.

1.2 THE NON-OBESE DIABETIC MOUSE AS A MODEL OF IDDM

The NOD mouse is a rodent model of human IDDM with features similar to those observed in human diabetics including polyuria, polydipsia, hyperglycaemia, glycosuria and hypercholesterolaemia (Makino *et al,* 1980). The founder mouse was discovered fortuitously by Susumu Makino in 1974 while he was attempting to develop a mouse model of cataract. The offspring of the founder mouse were mated and selectively bred to establish the diabetes prone (NOD) and diabetes resistant, non-obese non-diabetic (NON) strains (Makino *et al,* 1980). The incidence of diabetes in the NOD strain is much greater in females with ~ 80% but only 20% of males developing IDDM by 30 weeks of age. However, this varies from colony to colony.

Despite the fact that not all mice develop overt diabetes, virtually all mice exhibit insulitis (Makino *et al,* 1980). Just as in human diabetic patients, NOD mice exhibit a genetic predisposition, abnormalities in cellular and humoral immunity and disease recurrence in

pancreas grafts. Much of the data are in agreement with the pathogenesis of disease being autoimmune in nature.

1.2.1 Genetic susceptibility

Serological analysis of class I MHC alleles demonstrated that the NOD mouse carries the H-2K^d and H-2D^b haplotype (Hattori *et al,* 1986). Treatment of NOD mice with monoclonal antibody directed against the K^d molecule prevented both spontaneous and cyclophosphamide-induced diabetes, whereas administration of anti-D^b antibody proved ineffective (Taki *et al,* 1991) indicating that class I K^d-restricted T lymphocytes play a role in β cell destruction.

Analysis of the class II alleles revealed that there was no surface expression of I-E due to an absence of mRNA for I-Eα- and a unique I-A molecule was present (IA^{g7}) (Hattori *et al,* 1986). Further investigation showed that I-Aα and the 3' region of I-Aβ were identical to the H-2^d haplotype sequence and that the uniqueness was attributed to differences in 5' region of the I-Aβ chain molecule (Acha-Orbea and McDevitt, 1987). Of particular interest were 5 unique nucleic acid changes between residues 248 and 252, resulting in a proline-aspartic acid to histidine-serine substitution at positions 56 and 57. Much interest has focussed on the role of amino acid 57 of HLA-DQ in conferring susceptibility to autoimmune diseases, including IDDM in humans (Todd *et al,* 1987).

1.2.2 Environmental factors

The important role of diet in modulating the penetrance of diabetogenic genes has been demonstrated. Casein (Elliott *et al,* 1988), and brewer's yeast (Coleman *et al,* 1990) appear to increase the incidence of diabetes while semi-purified or elemental diets reduce it. The diabetogenic factor appears to be soluble in lipid (Coleman *et al,* 1990).

Ambient temperature also affects the incidence of diabetes; housing NOD mice at a higher temperature (23.7+1.7^{o}C versus 21.0+1.8^{o}C) reduces the incidence of diabetes. It has been postulated that lower temperature results in greater food intake, increasing exposure to a diabetogenic element in the food (Williams *et al,* 1990).

Immune modulation by viruses has been proposed as a mechanism to both decrease (Leiter and Hamaguchi, 1990) and increase (Hermitte *et al,* 1990) the incidence of autoimmune diabetes. Chronic infection of NOD mice with Murine Hepatitis Virus resulted in a lowered

incidence of diabetes (Wilberz *et al,* 1991). Infection by viruses such as LCMV (Oldstone, 1987) which primarily affects CD4+ T lymphocytes, and lactate dehydrogenase virus which preferentially affects macrophage function, (Takei *et al,* 1992) both prevent the development of IDDM in NOD mice.

1.2.3 Insulitis in the NOD mouse

In contrast to the human lesion, insulitis in the NOD mouse has been well studied. There is a general consensus in the literature that insulitis appears spontaneously at about four weeks of age. Initially, collections of lymphocytes are found on the side of an islet adjacent to ducts, blood vessels and lymphatics. The mass of lymphocytes is separated from the islet by a thin connective tissue sheath which is destroyed as the lymphocytes invade the islet. With time, the islet is eroded by lymphocytes and it is only the β cells which are destroyed. The final stage of insulitis is a small, shrunken islet with no β cells or lymphocytes and only glucagon and somatostatin cells remaining (Shimizu *et al,* 1987; Kanazawa *et al,* 1984; Fujino-Kurihara *et al,* 1985; Miyazaki *et al,* 1985).

1.2.4 Disease recurrence in pancreatic islet grafts

Studies from different centres have given contradictory results as to whether or not disease recurrence is MHC restricted in the NOD mouse. Serreze and colleagues (1990) found that NOD mice rejected cultured BALB/c islets but not cultured pituitary grafts. Lafferty and collaborators (1983) showed similar findings, however when the recipient NOD mice were immunised with BALB/c splenocytes the pituitary grafts were rejected, and they concluded that the disease was tissue specific and MHC-unrestricted. Analogous findings were obtained when completely MHC-mismatched islet allografts were used (Leijon *et al,* 1995).

In contrast to Laffety's and Nomikos finding, Terada and colleagues (1988) transplanted newborn pancreas from various mouse strains into diabetic NOD mice treated with a dose of CsA sufficient to prevent skin allograft rejection. Assessment of the pancreas grafts 10 days after transplantation revealed that totally MHC-mismatched grafts had the least infiltration whereas allografts sharing the MHC class I K^d allele and NOD isografts displayed the greatest graft destruction, indicating the disease recurrence was MHC-restricted.

Anti-CD4 MAb therapy also has been shown to delay disease recurrence in islet allografts (Hao *et al,* 1987), however this effect could not be prolonged by treatment with CsA

(Wang *et al,* 1988). A higher dose of CsA and anti-CD8 MAb therapy were also ineffective in maintaining survival of cultured islet allografts (Wang *et al,* 1988).

Wang *et al,* (1991) obtained similar results with disease recurrence in islet isografts. Anti-CD4 MAb prolonged graft survival whereas anti-CD8 MAb therapy failed to prevent β cell destruction. These investigators concluded that islet damage was mediated by a CD4+ T cell-dependent response.

1.3 CURRENT TREATMENT OF INSULIN DEPENDENT DIABETES

1.3.1 Intermittent parenteral insulin

The aim of insulin treatment is to control blood glucose levels and prevent acute metabolic death. Insulin injections may control the disease reasonably well but do not, in the long-term, prevent the development of the complications facing diabetic patients; renal failure, blindness, neuropathy, and macrovascular disease being the major causes of early morbidity and premature death (Marble, 1976). The insulin regimen used by most IDDM patients is the combination of short and intermediate acting insulin injections twice daily (MacPherson and Feely, 1990). Although effective for most IDDM patients, this cannot produce normoglycaemia at all times. In 1995 The Diabetes Control and Complication Trial (DCCT) has shown that intensive treatment of patients with IDDM can substantially reduce the incidence of long-term complications eg retinopathy, nephropathy and neuropathy (Crofford, 1995).

1.3.2 Continuous subcutaneous insulin infusion.

Over the past decade a number of sophisticated continuous subcutaneous insulin infusion systems (CSII) have been developed. The ultimate goal of CSII therapy is to deliver insulin at a more precisely controlled rate than is possible by bolus subcutaneous injections. The infusion can be delivered without multiple daily insulin injections. In an attempt to further optimise infusion delivery, intraperitoneal infusion has also been tested in an attempt to deliver insulin intraportally. However, research has shown that insulin absorption from the peritoneal space is not rapid, rather it is variable and most importantly, it does not necessarily occur through the portal vein. There is evidence that although CSII achieves a more physiological control of blood glucose, it still does not prevent long-term complications. In fact, CSII is rarely used now as there is a greater incidence of hypoglycaemia in these patients.

1.3.3 Approaches to pancreas and islet cell transplantation.

The objective of pancreas transplantation is to establish a normoglycaemic, exogenous insulin-independent state in an IDDM patient and prevent or halt the progression of the complications that affect over 50% of patients with long-term diabetes.

Transplantation using either whole pancreas or segmental pancreas grafts can result in prolonged normalisation of glycemia in diabetic patients. Bilous *et al,* (1989) showed that successful pancreas transplantation is associated with significantly less severe diabetic glomerulopathy in kidneys previously transplanted into diabetic patients. This also supports the hypothesis that achievement of normoglycaemia prevents the progression of diabetic glomerulopathy in humans.

Transplantation of vascularized pancreas in diabetic patients is showing steadily improving results but the immediate operative risk and life-long immunosuppressive medication required represent considerable disadvantages. Efforts are being made to develop simpler and safer methods of transplantation using pancreatic islets as free grafts, eg as isolated islets, foetal pancreas, or dispersed adult pancreas (Hellerstrom *et al* , 1988). The pancreas is unique as a transplant organ in having dual exocrine and endocrine components: the islets of Langerhans constitute only 1-2% of the pancreas. Therefore, most of the tissue transplanted in a pancreas graft is unnecessary for providing functioning β cells.

However, the main difficulties which prevent the successful function of islet transplants in humans are: (1) the immunological rejection of the grafts, (2) obtaining adequate amounts of suitable tissue, and (3) the recurrence of the autoimmune disease. The problem in treating IDDM patients by transplantation includes the large number of patients and the difficulty in obtaining an adequate source of tissue for transplantation. The foetal pancreas may be a suitable source due to the ability of foetal islet cells to proliferate after transplantation, which results in a large islet cell mass and produces fully functional grafts, at least in experimental animals (Mandel and Koulmanda 1992). But there are major problems in obtaining adequate amounts of suitable foetal tissue. Islet transplantation may cure diabetes if rejection and recurrence of the disease in the graft can be prevented. Recurrence of a specific autoimmune response towards the β cell is a potential mechanism for destruction of transplanted islet tissue.

The problem of rejection is being looked at in three different areas; (1) to modify the graft to reduce its immunogenicity various forms of immunomodulation by pretreatment of grafts *in vitro* have been employed in the animal models of diabetes, but none of these have been successful in long-term treatment in humans; (2) to treat the recipient by suppressing its immune system; and (3), to produce a "bio-artificial pancreas" by isolating the islets of Langerhans within a semi-permeable diffusion membrane surrounding the endocrine tissue. However, pilot studies using such systems have shown that isolated islets of Langerhans, fragments of insulinoma, or foetal pancreas retain function for only days to weeks, as judged by the inability to maintain euglycaemia in drug-induced diabetic rats (Friedman, 1989).

Auto-, iso-, allo-, and xenotransplantation of pancreatic islet preparations have been shown to reverse diabetes in animals with drug-induced diabetes and auto- and allografts have shown some success in humans (Scharp *et al*, 1990; Sibley and Sutherland, 1987). (1) Islet auto-transplantation following total pancreatectomy for benign disease has been successful in many cases (Wahoff *et al*, 1995). Cutfield (1985) showed that dogs transplanted with segmental autografts had functional grafts for longer than four years. (2) There is good evidence that iso-transplantation with whole pancreas or isolated islets of Langerhans has been highly successful in preventing or reversing glomerular lesions in animals with drug induced experimental diabetes, but not in the BB rat or NOD mouse where recurrence of the disease in the graft occurs (Mandel and Koulmanda, 1995). (3) Islet allografts in humans have several advantages as a transplant source for the treatment of IDDM. A number of centres have undertaken studies in this area. Optimal results have occurred when pooled tissue from 6 to 20 donors was implanted. Peterson *et al*, (1989) reported insulin secretion for up to 1 year and the absence of an anticytoplasmic islet cell antibody response in the recipients. Nevertheless, insulin independence was not produced and the graft did not secrete insulin in response to a glucose challenge. The large amount of donor tissue required to reverse IDDM makes human foetal pancreatic transplantation an experimental approach. (4) Islet xenografts are probably the most likely solution to the shortage of donor tissue but many problems need to be solved before this approach to the treatment of IDDM by islet replacement becomes a realistic option. The choice of a suitable donor species will depend on both potential availability and ethical concerns, as well as physiologic suitability. Domestic animals such as pigs, already being

widely bred for human use, are a potentially limitless source of islets. The pig may well be the most appropriate species for human xenotransplantation for a number of reasons (Cooper *et al,* 1991). (a) pigs are a commonly used domestic species for human consumption. (b), pig insulin is almost identical with human insulin differing by only one amino acid in some 50, and has been widely used for decades. (c), pig metabolism is similar to human metabolism and a successful islet xenograft of porcine tissue should both respond to and regulate BG levels appropriately. (d), pigs are easy and cheap to breed and reproduce well with large litters and rapid maturation.

1.4 THE MODEL OF MOUSE FOETAL ISLET TRANSPLANTATION

A variety of pancreas and islet transplantation experiments have been performed in animals with both drug-induced and autoimmune models of diabetes, in the attempt to answer questions raised regarding replacement of endocrine tissue in human IDDM.

1.4.1 Foetal pancreas iso-transplantation

1.4.1.1 The amount of tissue required to reverse and maintain euglycaemia.

The amount of isogeneic transplanted tissue required to reverse hyperglycaemia and maintain euglycaemia in an animal model of drug-induced diabetes depends on, (a) the gestational age of the donor tissue, (b) *in vitro* treatment of the graft, (c) the severity and chronicity of diabetes in the recipient.

1.4.1.2 The gestational age and culture time of foetal pancreas grafts

McEvoy *et al,* (1976) reported that organ culture of foetal pancreas results in a selective loss of acinar tissue, probably due to a lack of corticosteroids in the medium. Mandel *et al,* (1982a) compared the effect of gestational age and culture time of the donor tissue used for transplantation in drug-induced diabetic mice. Either 12 or 17 day foetal pancreas was used and the tissue was either uncultured or cultured for 14 days. Uncultured 12 and 17 day foetal pancreas produced poorly developed grafts with small islets; in contrast, if cultured for 14 days both 12 and 17 day grafts resulted in large grafts with well developed islet cells. Cultured 17 day foetal pancreas grafts were the most effective and always ameliorated diabetes, whereas 12 day foetal pancreas were only partially effective (Mandel *et al,* 1982a).

Simeonovic *et al,* (1990a) studied the development and function of "proislet" isografts transplanted into diabetic CBA/H mice. The proislets were isolated from foetal mouse pancreases (17 days gestation) by collagenase digestion and cultured for 4 days. Simeonovic and colleagues used four or eight donor equivalent isografts per recipient to reverse drug induced diabetes; reversal occurred 30±7 and 74±26 days after transplantation, respectively. One of the disadvantages of this method of islet preparation is that islet tissue is lost or damaged during the digestion. This could explain the need to use four to eight donor equivalents per recipient to reverse diabetes.

Georgiou and Mandel (1984), studied the islet cell subpopulations in cultured mouse foetal pancreas and pancreatic isografts using the immunoperoxidase technique, and studied the structure and function of cultured foetal pancreas isograft tissue. They found that proliferation of endocrine β cells was highest during the first three weeks of culture and that these cells rapidly declined in number thereafter as the δ cell mass increased. Morphologically the optimal culture time, based on β cell mass, was approximately 10 to 14 days. Immunoperoxidase (IMP) staining of long-term isografts demonstrated the presence of large islets with the typical morphologic structure of normal adult pancreatic islets.

Chapter 3 will address the effect of gestational age and organ culture time in foetal and neonatal mouse pancreas.

1.4.1.3 Effect of severity and duration of diabetes on graft function

In most animal models diabetes has been of short duration (4-6 weeks pretransplant) in contrast to the prolonged disease present before islet or whole pancreas transplant is generally attempted in humans ie., transplants in humans are almost always done when generalised advanced microvascular disease is already present in the recipient typically many years (10 -30) after onset of disease. Mandel *et al.* (1987), examined the effects on foetal islet isograft function of (a) diabetes of prolonged duration, (b) diabetic control by continuous insulin infusion, and (c) the graft recipient's age. The data showed that duration of diabetes and good peritransplant control, but not recipient age, influenced graft function.

Cuthbertson *et al,* (1988) studied the effect of chronic diabetes on growth and function of foetal islet isografts in mice. One group of animals received continuous intraperitoneal infusion of regular insulin via an Alzet 2002 Osmotic pump at the rate 0.5 U/day for 14 days

prior to grafting. The chronically diabetic animals which received diluent alone took 11 weeks to reach euglycaemia, compared to 7 weeks from their littermates that had received insulin. Graft insulin content was decreased from 16,000 ng in the acutely diabetic animals to 9,600 ng in the chronically diabetic non-insulin treated group. These studies suggest that there is a beneficial effect of pretransplant insulin therapy.

Chapter 4 will address the effect of severity and duration of diabetes on the graft function in foetal mouse pancreas.

1.4.1.4 The site of foetal transplants: Portal versus systemic venous drainage.

Studies from this laboratory have clearly shown that it is possible to reverse streptozotocin-induced diabetes in mice with either isografts (Mandel *et al,* 1984b) or allografts (Georgiou and Mandel, 1985a and 1985b) of organ cultured foetal pancreas. Kidney subcapsular grafts drain insulin into the systemic venous circulation thus by-passing the physiological first-pass of the hormone through the liver (Felig, 1975). However, Reece-Smith *et al,* (1982) and other have suggested that portal venous drainage is more effective in controlling diabetes than insulin secreted into the systemic circulation. Three years later Kruszynska *et al,* (1985a and 1985b) reported that systemically draining islet grafts had the ability to achieve consistent euglycaemia. Cuthbertson and Mandel, (1986) performed a study to assess the relative efficacy of transplanting a known amount of cultured foetal pancreas into either the systemic or portal drainage sites. The degree of glycaemic control was assessed by regular estimations of (a) body weight, (b) random blood glucose, (c) glycosylated haemoglobin, (d) by challenging the recipients with arginine and (e) insulin content of the grafts. They concluded that the data showed that portally draining grafts are more effective in controlling the response to a metabolic challenge than systemically draining grafts.

1.5 ALLOGRAFT REJECTION

1.5.1 Modification of graft immunogenicity by organ culture and oxygen concentration

Gorer *et al,* (1936 and 1937) made one of the key discoveries in the field of transplantation showing that the tissue and cells of genetically different individuals contain histocompatibility antigens which play a major role in the rejection of tumours. Organ culture prior to transplantation has been shown to reduce islet graft immunogenicity (Collier and

Mandel, 1983; Lacy *et al,* 1982) in inbred mouse models. Cultured grafts appear to lack cells containing class II MHC antigens, yet allografts containing only class I MHC are rapidly rejected if uncultured (La Rosa and Talmage, 1983). These findings indicate that class II antigens are not always necessary for the induction of graft rejection.

T cell interactions involved in the rejection of islet allografts remain controversial. Bowen *et al,* (1980) and Faustman *et. al,* (1982) showed that the prolongation of allograft survival after culture in high oxygen concentration is due to the selective destruction of "passenger leucocytes"; ie macrophages and dendritic cells and endothelial cells capable of activating recipient lymphocytes. Furthermore, they showed that there was a important role for CD4+ T cells in islet allograft rejection by finding that naive CD4+ T cells were not sufficient to cause islet allograft rejection, whereas activated CD8+ T cells were able to mediate such a rejection. Experiments using endocrine allografts depleted of MHC class II expressing cells by culture in a high concentration of oxygen support a role for CD8+ cells in allograft rejection but suggest that a co-operation between the two types of T cells is required. Prowse *et al* , (1983) found that the injection of specifically activated CD8+ cells led to the prompt rejection of cultured pancreatic islet allografts. Shizuru *et al* , (1987) and Hao *et al,* (1987) attempted to show that depletion of CD4+ *in vivo* decreased the capacity to reject pancreatic islet allograft in mice. However, it is not clear if CD4+ T cells are sufficient to reject the islet grafts or whether they require CD8+ T lymphocytes to mediate these responses.

1.5.2 Mechanism of allograft rejection

The major problem in the transplantation of allogeneic tissues has been graft rejection. The allografts express MHC class I and class II molecules which are recognised as foreign by CD8+ and CD4+ T cells respectively of the recipient. MHC-incompatible tissue can induce strong primary responses leading to allograft rejection and graft-versus-host disease. Fully MHC-incompatible allografts usually induce much stronger resposes than semi-allogeneic tissue (Lechler and Batchelor, 1982). MHC class I and class II antigens are prime targets for host immunoreactivity, class I antigens are expressed by most somatic cells, these interact with and activate CD8+ cytotoxic T cells. Class II antigens are constitutively present on dendritic cells, Langerhans cells in the skin, vascular endothelium and circulating B-lymphocytes and monocytes, they interact with and activate CD4+ T cells.

The immune system recognises molecules invading the host by availing itself of three different types of recognition structures; immunoglobulin (Ig) receptor, T-lymphocyte cell receptors (TCRs), and cell-surface products of class I or class II MHC genes (Paul, 1989).

The initiation of a cellular response to allografts does not necessary need to depend on direct recognition of alloantigen but can also occur in response to peptides of donor antigens processed and presented in association with host MHC molecules on recipient APCs (Wecker and Auchincloss, 1992). MHC class I and class II molecules are associated with peptides on the cell surface (Monaco, 1992; Neefjes and Ploegh, 1992), in general class I molecules present peptides of endogenous antigens while class II molecules present peptides of exogenous antigen. However, studies from Grant and Rock (1992) and Michalek *et al,* (1992) suggest that in some cases class II molecules can present endogenous peptides and class I molecules can present peptides from exogenous antigens.

Whether recognition by the indirect pathway actually contributes to graft rejection was addressed by different authors. Firstly Priestley *et al,* (1992) used antibodies specific for recipient MHC class II antigens to block rejection of rat kidney expressing allogeneic MHC and concluded that the indirect pathway was entirely responsible in this allogeneic situation. Muluk *et al,* (1992) examined the ability of T cells from immunosuppressed recipients of kidney grafts to generate responses to mitogens, alloantigens presented by allogeneic APCs and foreign antigens presented by self APCs. They concluded that suppression of rejection activity in the late stages after transplantation correlated with suppression of the indirect pathway but did not require suppression of the direct recognition of alloantigens.

Wecker and Auchincloss in their review in 1992, concluded that the direct pathway is responsible for the strength of early allograft rejection when the donor and recipient are not matched at the MHC level but that over time, as donor APCs are replaced by recipient cells, futher rejection activity depends progressively on the indirect pathway.

Finally, the allograft response is not only dictated by the genetic differences between donor and recipient, the type of tissue is another determinant of immunogenicity. A rough vulnerability of different tissues to graft rejection has been constructed, from most to least vulnerable; starting from bone marrow, skin, pancreatic islets, gut, lung, heart, kidney to liver (Warrens *et al,* 1994).

Despite the vast amount of work in this area, the nature of this mechanism still remains uncertain.

1.5.2.1 Acute allograft rejection

In general acute cellular graft rejection is always preceded by a dense infiltration of mononuclear cells at the graft site. The number of lymphocytes in the graft area exceeds by far the number needed to destroy the graft. The infiltrate usually contains both CD4+ and CD8+ cells. The infiltration followed by destruction of the allograft is variable depending on the mouse strain combination used and the tissue grafted and is also related to minor antigenic differences. Hutchinson (1991) reported that in allografts when allogeneic MHC class I and II antigens are present, the entire T cell network seems to be triggered and both CD4+ve and CD8+ve T cells are activated and become involved in graft damage. Activated CD4+ve T cells also act as helper cells for B cell maturation. The intense inflammatory response that is produced at a graft site results in cytokine release, macrophage activation, natural killer cells and a cascade of increasing inflammation. The release of a variety of cytokines by activated cells in the graft site also results in 'upregulation' of MHC, particularly class I molecules and other antigens on the graft cells, making them better targets for direct T cell mediated killing. These process include cytokines like interleukin-1 produced by activated macrophages, as well as a number of lymphokines produced by actvated lymphoid cells (Dallman and Clark, 1991).

Until recently the highly specific and rapid destruction seen in a graft was assumed to be caused by cytolytic T-lymphocytes (CTLs), as shown *in vitro* by their ability to lyse donor target cells directly without antibody (Hayry and Defendi, 1970) and *in vivo* by the inability of T cell depleted animals to reject allografted tissue (Clason *et al,* 1982). However, CTLs require help from CD4+ cells and macrophages, particularly in acute rejection. CTLs are usually CD8+, whereas cells responsible for delayed-type hypersensitivity are CD4+ cells. However, both CD4 and CD8 +ve cells appear to be intimately involved in graft rejection. The role of CD8+ T cells has recently been called into question (Walsh *et al,* 1996). Despite this, most allografts are retained and the intense immune response can be contained with immunosuppressive agents available.

1.5.2.2 Chronic allograft rejection

Traditionally, host humoral activity had been considered responsible for the chronic rejection process. The chronic rejection response is characterised by infiltration, fibrosis, vasculitis and vascular obliteration. The relationship of cytotoxic antibodies to endothelial injury and arteritis has long been documented, also circulating antibodies directed against HLA and other graft antigens can cause rejection of organ allografts. In long functioning human renal allografts, areas of luminal obliteration have been associated with IgG deposition on vascular endothelium. Antibodies eluted from chronically rejecting renal grafts show different antidonor specificities as well as non-specific HLA activity and react against endothelial cells and kidney cell antigens (Ende *et al*, 1979).

Interestingly, few qualitative differences have been found between cell populations infiltrating acutely rejecting, chronically rejecting, or well-functioning allografts in an unresponsive host. These cells, regardless of length and state of graft function, consist primarily of T-lymphocytes and monocytes/macrophages (Roberts, Hayry, 1976). Cytokines have also been implicated in chronic rejection, eg IL-1, TNF-α, TNF-β and IFN-γ.

1.6 XENOGRAFT REJECTION

Transplantation of solid organs (heart, lung, liver and kidney) into discordant recipients result in hyperacute rejection (HAR) within minutes to hours. This is due to the presence of preformed natural antibody directed against graft epitopes in recipients never before exposed to the xenoantigens. Antibody-mediated HAR remains the primary biological impediment to xenotransplantation of whole organs (Platt and Bach, 1991).

1.6.1 Hyperacute rejection

HAR occurs mostly when transplants are performed between phylogenetically unrelated species, eg between rabbits as recipients of pig organs. Similar discordance applies in humans receiving pig organs. Calne (1970) was the first to propose a system of nomenclature to categorise the rejection process in xenotransplantation. Species combinations where an HAR response was observed were referred to as "discordant", whereas those with a rejection process more analogous to allograft rejection were termed "concordant". Concordant xenografts are generally between phylogenetically closely related species eg, mice and rats, or higher apes and

humans. Discordant xenografts are between phylogenetically distant species such as guinea-pig to rat or pig to human (Auchincloss, 1988). In discordant xenogenic transplant situations rejection is rapid, occurring often within minutes. In such situations rejection is mediated by preformed natural antibodies (NAbs) that react primarily with the carbohydrate terminal sugar Gal(α1-3)Gal (Galili *et al,* 1988; Sandrin *et al,* 1993) on endothelial cells of the graft vascular bed. This reaction converts a normally anticoagulant surface into a procoagulant one and results in endothelial cell activation, blood stasis, platelet accumulation and thrombosis with rapid graft infarction (Platt and Bach 1991; Bach *et al,* 1994). Control of HAR is an active area of research and ways of permanently depleting a potential recipient of damaging NAbs are actively being sought. Such methods include plasmapheresis to reduce levels of circulating Abs (Kimikawa *et al,* 1992) combined with treatments that will prevent or retard their reappearance such as splenectomy and immunosuppression (Carobbi *et al,* 1992).

Rejection of discordant xenografts is similar to the HAR occuring in human kidney allografts in recipients with induced pre-existing antibodies against either donor HLA antigen or against incompatible ABO blood group oligosaccharides on the allograft endothelium. Discordant xenograft rejection is mediated by activation of the complement system either by via the alternate or the classical pathways, depending on the particular species combination of the xenograft. The critical regulatory component of complement is the serum protein C3. After proleolytic cleavage, C3 is activated and initiates the destructive effector mechanism of complement (Atkinson and Farries, 1987; Frank and Fries, 1989).

Porcine xenografts placed into primates are destroyed by antibody dependent complement activation, ie classical pathway complement activation There are a number of reports that support the hypothesis that primate antibodies recognise the endothelium of the pig xenograft and trigger rapid rejection by activating complement via the classical pathway. Such antibodies are present in all humans and old world primates, even if individuals are not exposed to pig antigen (Platt and Bach, 1991). These NAbs bind to pig endothelium and activate C1, C2 and C4, which activate complement components and form the classical pathway and convert C3 to C3b. C3b may covalently bind to the C3 convertase to form a C5 convertase which cleaves C5 into C5b and initiates formation of the membrane attack complex. C3b may also react with inhibitors to be inactivated, or C3b may covalently bind to cell surface and interact with

alternative pathway components factor B and D to form the alternative pathway C3 convertase (Fig 1.1a). Streilein (1991) and Geller *et al,* (1992) in their studies have indicated that NAbs inducing the classical complement pathway rather than the alternative pathway are responsible for the acute rejection of pig organs by primates.

In contrast, activation via the alternative pathway occurs because C3 has an unstable thioester such that a small amount of C3 is continuously activated to C3b (C3 tickover) (Lu *et al,* 1994). This C3b binds to repetitive hydroxyl and amine groups on cell surfaces. The cell surface C3b forms a C3 convertase (C3bBbP) by binding factors B, D and properdin (P) (Calne, 1989). The C3 convertase activates more C3 in a positive amplification loop, activates C5 to form C5a anaphylatoxin (Fig 1.1 a), which recruits and activates inflammatory cells, and initiates construction of the membrane attack complex (C5b678(9))n, which lyses the target cell (Lu *et al,* 1994).

Therapeutic strategies to prevent natural immunity of primates against pig solid organs include genetically engineered pigs that have human inhibitors of complement on their cell surfaces. There are three different membrane bound inhibitors that may be considered: 1) CD46, membrane cofactor protein (MCP), 2) CD55, decay accelerating factor (DAF) and 3) CD59, membrane inhibitor of reactive lysis (MIRL) that inhibits formation of the membrane attack complex (C5-9) that lyses target cells. CD46 and CD55 inhibit C3b, a central component in both the classical and alternative pathway. Recently, transgenic pigs with genes for human inhibitors of complement have been produced (White and Wallwork, 1993) (Fig 1.1 b). Hearts from the human DAF transgenic pig have survived in primates with triple immunosuppressive therapy for more than 60 days (White and colleagues, unpublished) indicating that HAR in discordant xenografts can be overcome.

In contrast to solid organs, if free grafts of cells are used, as would be the case in islet cell transplantation in IDDM or in central nervous system tissue transplantation in Parkinson's disease, if the transplanted cells are not rejected they become vascularized by an ingrowth of host vessels and should not be targets for NAbs. However, rejection is still seen in non-immunosuppressed recipients and even with immunosuppression it is difficult or even impossible to control (Auchincloss, 1988). In the absence of HAR, xenograft rejection appears to follow the same general rules as the response to primary allografts; ie a T cell mediated

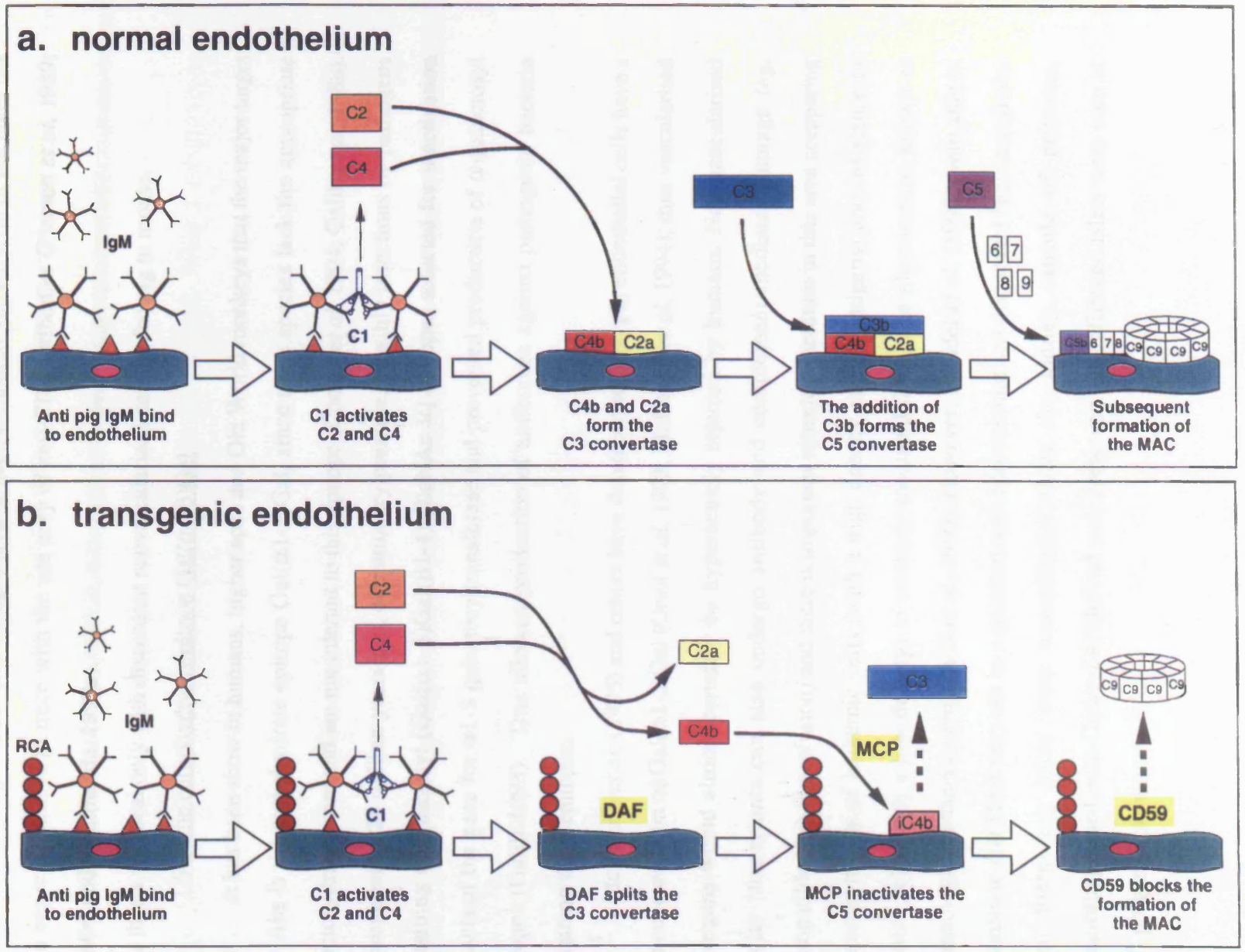


Fig 1.1 (a) Activation of complement by natural antibodies via the classical and alternative pathway on normal endothelium.

(b) The prevention of complement activation by transgenic endothelium expressing DAF, MCP and CD59.

response is generated that appears to be different from the allograft response mainly because of its apparently greater dependence on CD4+ve T cells, perhaps to a much greater degree to that in an allograft response (Simeonovic *et al,* 1990b) at least in mice. In the relatively few published studies where allo- and xenografts have been compared, the survival of the latter has been easier to achieve in mice with the use only of anti-CD4 antibodies (Pierson *et al,* 1989; Mandel and Koulmanda, 1992a).

This thesis will deal only with discordant neovascularised xenografts; pig to mouse.

1.6.2 The carbohydrate epitope Gal(α1-3)Gal

It has been show in humans, higher apes and Old World monkeys that the major target of NAbs is the carbohydrate epitope Gal(α1-3)Gal, since these species lack the appropriate galactosyltransferase and are not tolerant to this epitope (Cooper *et al,* 1994; Galili *et al,* 1988). Approximately 28 million years ago, evolutionary pressure, possibly infections by pathogens containing α-galactosyl [Gal(α1-3)Gal(β1-4) GlcNAc-R] epitopes selected for inactivation mutants of the gene for α1-3 galactosyltransferase and prevented production of α-galactosyl epitopes (Galili 1993). This allowed production of antibodies against pathogenic bacteria containing these epitopes.

McKenzie *et al,* (1995) and others have demonstrated that pig endothelial cells have a high concentration of Gal(α1-3)Gal (Oriol *et al,* 1993; McKenzie *et al,* 1994); thus vascularised pig organs would almost certainly be hyperacutely rejected by humans. Non-vascularised cellular transplants can also undergo antibody and complement mediated damage (eg, incompatible blood transfusion) and there is important historical literature in this area comparing the susceptibility of leukemic cells (with a high density of H-2 antigens) and resistance of sarcoma cells (with a low density) to antibody mediated damage, a phenomenon known as immunological enhancement (Snell *et al,* 1960). However, Mandel *et al,* (1995) were able to demonstrate that HAR may not be a problem with non-vascularised discordant islet xenografts. When foetal pig islets were transplanted under the kidney capsule of primates, immunosuppressed with CsA/AZA/MePred well preserved non-infiltrated islets were seen at day 7 post-transplantation.

1.6.3 Cellular xenograft rejection

Specific cellular immune responses to xenoantigens are similar to those of alloantigens in that they are mediated by CD4+ T-lymphocytes responding to xenogeneic class II MHC molecules. Kirk *et al,* (1993) reported that binding is mediated via the T cell receptor (TCR) since a distinct repertoire of Vβ expressing T cells develops. However, direct interaction between the host TCR and xenogeneic class II molecules may not be strong enough to produce T cell activation. Xenoantigens may need to be processed and presented to T-lymphocytes by host antigen-presenting cells (APC) (indirect presentation). However, Wolf and Gill (1993) demonstrated that *in vitro* generated xenogeneic T cell lines could trigger the *in vivo* specific destruction of pancreatic islets when transplantated in adjacent sites in severe combined immunodeficiency (SCID) mice, indicating direct xenograft recognition could cause graft destruction.

Whether direct antigen presentation plays any role in xenotransplantation remains controversial.

1.6.4 Clinical pancreas xenografts

The first recorded clinical pancreas xenograft was in 1894 when pieces of sheep pancreas were used as a subcutaneous graft in a patient with terminal diabetes (Williams, 1894)). There followed a period of inactivity and in the last twenty years the emphasis has been on pancreas and islet allografts. In the last ten years there have been a number of studies where xenografted islets have been tested in patients but the majority of these studies are poorly documented and largely anecdotal so that little if any reliance can be placed upon them. However, there was recently a careful study in Sweden where 10 patients had foetal pig islets implanted either intraportally or, in a few patients, under the renal capsule of an allografted kidney. In these patients graft survival was monitored either by porcine C-peptide production (pig C-peptide does not cross-react with human C-peptide), or by biopsy of the graft site. There is well documented evidence that graft survival has been achieved in at least some patients but without functional benefit (Groth *et al,* 1994).

1.7 MONOCLONAL ANTIBODIES

Anti-T cell MAbs can mediate either specific or non-selective immunosuppression *in vivo*. Specific immunosuppression may induce tolerance towards autoantigens or alloantigens, whereas non-selective immunosuppression is induced by the administration of MAb targeting key molecules expressed by defined T cell populations.

Monoclonal antibodies (eg OKT3) against T cell surface antigens of the TCR have been used successfully to treat clinical allograft rejection in humans. In addition, they have been used in animal models to study the role of T cell subsets in autoimmune diseases, humoral responses, allograft and xenograft rejection.

1.7.1 Anti-CD3 monoclonal antibodies

MAbs to CD3 and other components of the T cell receptor (TCR) are well suited to selective immunosuppression because of their direct interaction with monomorphic determinants of the TCR complex involved in antigen recognition and signal transduction. The anti-CD3 hamster MAb (145 2C11) is raised in mice and is specifically directed at the murine CD3 epsilon chain. Its effects *in vitro* and *in vivo* have been extensively studied by several groups (Wissing *et al,* 1991; Hirsch *et al,* 1989; Alegre *et al,* 1990; Ferran *et al,* 1990a; Chatenoud *et al,* 1994). A single high dose of 400 μg/animal of 145 2C11 MAb given within 24-48 hr after transplantation significantly prolongs the survival of completely mismatched skin allografts (Hirsch *et al,* 1988). Hayward and Shreiber (1992) showed that 145 2C11 can prevent and treat type 1 diabetes in the NOD mouse model. There have also been reports of 145 2C11 treatment in murine allotransplantation and some suggest that it is effective but, as in humans, side effects are common and may be lethal (Hirsch *et al,* 1988; Mackie *et al,* 1990). It has been shown that 145 2C11 MAb induces a strong cytokine release and morbidity whereas rat anti-CD3 MAbs 17A2 and KT3 did not (Vossen *et al,* 1994). KT3 (IgG2a, λ) has been established from cells of Sprague-Dawley rat hyperimmunised with CBA T6 thymocytes, described by Tomonari (1988).

Mandel and Koulmanda (1992b) reported that prolonged xenograft survival was seen in NOD mice treated with KT3 MAb when large amounts were used (2 mg/dose), however, decreasing the amount of KT3 to 1mg/dose resulted in graft rejection. When both anti-CD3 (KT3, 1mg/dose) and anti-CD4 (GK1.5, 0.3mg/dose) were given for 6 weeks followed by

weekly anti-CD4 only, excellent xenograft survival in NOD mice for up to 20 weeks was seen with no evidence of rejection (Mandel and Koulmanda, 1994).

In this thesis I have exclusively used KT3 MAb which seems to be safe in contrast to 145 2C11, because no mice died as a result of treatment.

1.7.2 Clinical studies using anti-CD3 antibodies

Anti-CD3 (OKT3) is a mouse MAb of the IgG_{2a} subclass, specifically directed against the glycoprotein epsilon portion of the CD3 complex on the human T cell surface (Kung *et al,* 1979; Van den *et al,* 1984). OKT3 was first reported by Cosimi *et al,* (1981) to be an effective agent in to reversing renal allograft rejection. OKT3 has been successfully used in the treatment of acute primary renal allograft rejection, renal allograft rejection unresponsive to conventional immunosuppression (Ortho Multicenter Transplant Study Group, 1985; Monaco *et al,* 1987; Fung *et al,* 1987) and primary hepatic allograft rejection (Cosimi *et al,* 1987; Fung *et al,* 1987). Subsequent multi-institutional randomized studies showed that OKT3 reversed over 90% of rejection episodes, in contrast to a reversal rate of approximately 70% in steroid treated renal allograft recipients (Ortho multicenter transplant study group, 1985). The beneficial effects of OKT3, compared to ALG, include reduced acute rejection episodes, improved allograft function and a lower incidence of cytomegalovirus infection (Goldstein *et al,* 1986; Light *et al,* 1989).

However, severe immunodeficiency from such MAb treatment can cause an increased incidence of infections and tumours. A major drawback to the use of OKT3 as an immunosuppressive agent is the associated cytokine release syndrome (Herbelin *et al,* 1995; Gaston, 1994).

The remarkable immunosuppressive effect of OKT3 has stimulated evaluation of other MAbs directed against the alpha and beta chain of the TCR; T10B9.1A-31 (T10B9) is an IgM murine Ab and should be less mitogenic in humans than the IgG_{2a} molecule of OKT3 and therefore less likely to result in cytokine release (Cosimi, 1995). BMA031 is another murine IgG_{2b} Ab and would be expected to be less mitogenic and is reported to reduce cytokine induction.

1.7.3 Anti-CD4 monoclonal antibodies

Anti-CD4 MAbs react with a more restricted subset of lymphocytes and therefore provide a more selective suppression of the immune response. The rationale for targeting the CD4 molecule is based upon its involvement with the TCR in the recognition of class II MHC antigens and in the signaling events which result from this recognition (Cosimi, 1995). Anti-CD4 MAbs have been extensively used in mice. Most studies were performed using the antibody GK1.5 which is a cell-depleting rat IgG2b MAb (Dialynas *et al,* 1983). GK1.5 induces a profound CD4-selective lymphocyte depletion in blood, spleen and lymph nodes (but not thymus) after a single injection of 0.2 - 5mg. The depletion is rapid and complete (>90%) and persists for approximately 10 days, recovery starting thereafter and reaching completion after some 4-6 weeks (Emmrich and Bach, 1993). Some researchers stress that greater than 95% depletion of CD4+ cells is needed to demonstrate an effect of the therapy (Shizuru *et al,*1987; Herbert and Roser, 1988; Shizuru *et al,* 1990), while others have demonstrated that depletion is not essential and suggest that it is the epitope recognised that is critical to the graft prolongation effect of the anti-CD4 MAb (Cobbold *et al,* 1984; Charlton *et al,* 1988a). Until more is known about the epitope of CD4 that interacts with the class II MHC molecule, it will be difficult to decide whether or not blocking of this site is critical in preventing the induction of the immune response.

Anti-CD4 MAbs, and particularly GK1.5, inhibit the onset of autoimmune diseases. NOD mice treated before onset of insulitis at a young age (2 weeks) by giving a high dose of GK1.5 twice weekly for 12 weeks, do not develop insulitis and diabetes (Koike *et al,* 1987). Injecting a single dose of GK1.5 in young NOD mice at various ages (2, 4, 5 and 6 weeks), Hayward *et al,* (1988) observed the prevention of insulitis when the treatment was given at week 5 (with partial effect at weeks 2-4) and if given together with an anti-CD8 MAb, suggesting that at this early stage either T cell subset could induce insulitis. GK 1.5 MAb significantly reduced established insulitis when given at the time of disease onset. Anti-CD4 MAb may also affect suppressor cells as exemplified by the induction of sensitivity to diabetes transfer afforded by anti-CD4 in adult thymectomized NOD mice (Boitard *et al,* 1989).

Anti-CD4 MAb has also been shown to induce tolerance or donor specific unresponsiveness to cardiac and pancreatic islet allografts when given before transplantation

(Madsen *et al,* 1987; Thompson and Mandel, 1990a). In low responder strains of rats, long-term acceptance of MHC mismatched heart grafts can be induced with two weeks of therapy (Hall, 1989) and in high responder strains significant delays in MHC-incompatible heart graft rejection can be induced by anti-CD4 MAb therapy; additionally a small proportion of rats developed tolerance (Herbert *et al,* 1988).

Simeonovic *et al,* (1990a), reported that xenografts can survive, and even tolerance can be achieved, in CBA mice that have been immunosuppressed with large doses of anti-CD4 MAb. However, this has not been achieved in NOD mice with the same treatment. Mandel and Koulmanda (1992a), reported excellent xenograft survival in CBA males and BALB/c females after a peri-transplant (Days -1, 0, +1) treatment with anti-CD4 MAb, but xenografts in NOD females failed to survive with the same treatment.

1.7.4 Clinical studies using anti-CD4 antibodies

The first report on anti-CD4 MAb treatment dates back to 1987 (Herzog *et al,* 1987) and describes clinical improvement in patients with rheumatoid arthritis who received anti-CD4 (VIT4 and M-T151) for 7 consecutive days (10 mg/day). A more detailed report of the same study (Herzog *et al,* 1989; Walker *et al,* 1989) demonstrated reduction of the number of swollen joints and the Ritchie's articular pain index within 7 days of treatment. Little is known about the anti-CD4 effects on cells other than T-lymphocytes. A moderate depletion of circulating monocytes has been observed after treatment (Herzog *et al,* 1989; Horneff *et al,* 1991a) and in a few cases monocyte-derived cytokines were elevated immediately after injection of the antibody but only for a few hours (Horneff *et al,* 1991b). It is unknown whether antibody binding to $CD4^+$ monocytes has a major influence on the therapeutic response.

Cosimi *et al,* (1990) in pre-clinical studies, demonstrated delayed allograft rejection up to 8 weeks following a single high dose of OKT4A in primate recipients. Clinical studies using OKT4A (12 days) together with a "triple drug therapy" in renal allograft recipients have shown rejection episodes occuring in 26% of the treated patients during the first 90 days (Delmonico, unpublished observation). Further studies with OKT4A have been suspended and other anti-CD4 MAbs like Max 16H5, BL4 and MT-151 are now being evaluated for clinical trails. In contrast to the data obtained from rodent recipients, no tolerance could be induced in the primates or humans with anti-CD4 MAb therapy.

1.8 AIMS OF THE STUDY

The purpose of this thesis is to focus on ways of making islet transplantation, especially xenografts, an option for the treatment of IDDM.

1. To develop a murine model suitable for pancreatic islet transplantation.
2. To study approaches aimed at reducing graft rejection, ie. organ culture and HiO_2.
3. To determine the baseline characteristics of rejection in each graft (iso-, allo- and xenografts) before any manipulation of the graft or the recipient was performed.
4. To characterise attributes that might contribute to the immunogenicity of the grafts.
5. To relate these attributes to the observed rejection rates, to gain a better understanding of what determines recurrence of the autoimmune disease or graft rejection.

CHAPTER 2: MATERIALS AND METHODS

2.1 MICE

2.1.1 Source of mice

Strains of mice used in these studies were:

Strain	H-2 Haplotype
C3H/HeJGifWehi	$K^kI\text{-}A^kI\text{-}E^k\ D^k$
CBA/CaHWehi	$K^kI\text{-}A^kI\text{-}E^k\ D^k$
BALB/cAnBradleyWehi	$K^dI\text{-}A^dI\text{-}E^d\ D^d$
C57BL/6	$K^b\ I\text{-}A^b\ I\text{-}E^b\ D^b$
NOD/Lt	$K^dI\text{-}A^{g7}I\text{-}E^{g7}\ D^b$
NOD/Wehi	$K^dI\text{-}A^{nod}I\text{-}E^b\ D^b$

The mice were bred and kept under specific pathogen free conditions at the Walter and Eliza Hall Institute's (WEHI) animal breeding facility (Clive and Vera Ramaciotti Research Laboratories, Kew, Vic. Aus.). At about 5-6 weeks of age they were transferred to the Institute (Parkville, Vic. Aus.) and thereafter maintained in communal mouse rooms operating on a 12 hour day-night cycle with room temperature controlled at 21 - 23^oC. All mice were maintained in conventional plastic boxes measuring 15 cm wide x 30 cm long x 12 cm high and containing sawdust and cotton wool. Animals awaiting experimentation were kept in the stock mouse room in groups of 8 per box and this was reduced to 5 per box at the commencement of each experiment. The mice were given standard food (Barastoc) and acid water (0.022% HCl, pH2.5) *ad libitum* and checked daily.

Pancreatic islets for *in vitro* and transplantation experiments were obtained from 13, 14, 15, 17 and 18-day foetal and neonatal (less than 24 hours old) pancreata. Mating was detected by the presence of a vaginal plug this was regarded as day zero of gestation.

2.1.2 Induction of diabetes with streptozotocin.

Unless otherwise stated six to eight week old females were used for all transplantation experiments. Streptozotocin (STZ) (Sigma, St Louis, MO, USA.) was given on a body weight basis with the dose depending on the mouse strain. The appropriate dose of STZ for the induction of diabetes has been determined previously by a series of dose-response experiments

performed in the Transplantation Unit. BALB/c mice were given 250 mg/kg body weight and C3H and CBA mice 275 mg/kg intravenously via the tail vein.

2.1.2.1 Insulin treatment

Ultralente porcine insulin 100 U/ml (CSL, Parkville, Vic. Aus.) was diluted with Hemaccel to a final dilution of 10 U/ml. Treatment was commenced once diabetes was diagnosed on the basis of decreasing body weight, increased cage wetting and changes in the coat appearance and confirmed by blood glucose measurement. The dose varied from 1 to 4 units per day given subcutaneously (sc), additionally mice received 200 to 300 µl normal saline per day intraperitoneally (ip) to maintain body fluids.

2.1.3 Tests

2.1.3.1 Arginine and glucose tolerance test.

For both the arginine and glucose test, the animals were fasted for 3 hours and blood samples taken from the orbital venous plexus for blood glucose estimations.

For the arginine tolerance test, the animals were given 2 mg/g body weight arginine in a volume of 0.2 ml ip (Calbiochem, Behring, CA, USA.) and blood samples were taken at 0, 5, and 30 minutes after challenge (0 time being the basal measurement).

For the glucose tolerance test (GTT), the animals were given 20% glucose (Ajax chemicals Pty. Ltd. Auburn, NSW. Aus.), intravenously (iv) in a dose of 1 mg/g body weight (0.1 ml iv), and blood samples were taken at 0, 5, 30 and 60 minutes after challenge.

2.1.3.2 Glycosylated haemoglobin.

Glycosylated haemoglobin (GHb) was measured using a "Glyctrac" kit (Corning Medical and Scientific, Palo Alto, CA, USA.) and, although designed for human use, with minimal modification it could be used for mouse blood analysis.

The method was as follows:

1. The agar electrophoresis film was peeled from its hard cover.
2. 1.0 µl of control or sample was added to the sample well of the agar film.
3. The agar film was inserted into the cassette holder of the Electrophoresis cell cover, matching the cathode (-) side of the agar film with the anode (+) side of the cell cover.
4. The power supply was turned on and the voltage set at 60 V, for 40 min.

5. The cell cover was removed and the excess buffer drained, the agar film was placed on a drying shelf in an incubator.

6. The gel was read at 420 nm using a scanning densitometer.

2.1.3.3 Random blood glucose and body weight

Random blood glucose (RBG) and body weight (BW) were measured in non-fasting animals (unless otherwise stated) and with blood samples taken at approximately the same time each day to minimise diurnal variation.

Body weight was measured on a Mettler PB300 top-loading electronic balance, to the nearest 0.1 gm.

Blood glucose was measured from a 10 µl serum sample taken from the orbital venous plexus, on a Beckman Glucose analyser II. This instrument determines glucose by means of the oxygen rate method employing an oxygen electrode. A 10 µl samples is manually pipetted into the enzyme reagent in a cup containing an electrode that responds to oxygen concentration. Solid-state electronic circuitry determines the rate of oxygen consumption which is directly proportional to the concentration of glucose in the sample. A range of glucose standards was analysed and repeated after every 20 samples.

2.2. FOETAL AND NEONATAL PANCREAS CULTURES

2.2.1 Sterile techniques

All tissue culture media, buffers and salt solutions were prepared by the Institute's Media Department. Buffers and salt solutions were sterilised by autoclaving (121^{o}C for 25 minutes) while tissue culture media were sterilised by filtration through a 0.22 µm pore size Millipore filter membrane (Millipore, Bedford, MA. USA.). All glassware was sterilised by dry heat at 180^{o}C for 3 hours, and microtiter pipettes (Finnipette, Helsinki, Finland) and dissecting instruments were sterilised by autoclaving. Millipore filter membrane (HV type 0.45 µm pore size) used for organ culture was cut into strips, boiled three times for 5 minutes in double distilled water (DDW) to remove detergent residues and finally autoclaved. All sterile work was carried out in an Oliphant UV HLF 4/30L laminar flow cabinet.

2.2.2 Culture media and solutions

For general work such as dissection of tissue and cell suspension preparation, mouse tonicity phosphate buffer saline (MTPBS) or mouse tonicity Hepes-buffered balanced salt solution (KDS) (Shortman *et al,* 1972) were used (see Appendix 1 for method), both were supplemented with 5% foetal calf serum (FCS) (Flow Laboratories, North Ryde, NSW. Aus.). The FCS was first heat inactivated (56^{o}C in water bath for 1 hour) and stored at -20^{o}C in 10 ml aliquots.

The tissue culture medium used for foetal and neonatal organ culture was Dulbecco's Modified Eagle's Medium (DME) (GIBCO, New York, NY. USA.) obtained in dry powder sachets and prepared by the Institute's Media Department (see Appendix 1 for method) and supplemented with 5% FCS.

2.2.3 Dissection of foetal and neonatal mouse pancreas

Dissecting instruments, forceps and scissors were sterilised by autoclaving. Two separate sets of instruments were used; one set for dissecting the skin and one set for dissecting the abdomen. Pregnant mice were killed by asphyxiation in CO_2 and pinned out on a cork board. The skin was wiped with 70% ethanol and using one set of sterile instruments the skin was cut away from the abdomen. The abdomen was rinsed with 70% ethanol and cut open using the second set of sterile instruments exposing the abdominal cavity and the uterus. The uteri were removed and transferred to a petri dish containing KDS + 5% FCS. Care was taken not to cut the wall of the uterus or the intestines during dissection. All steps were carried out using an aseptic technique in the mouse rooms. All subsequent steps were performed in a laminar flow cabinet located in the tissue culture room, using new sets of sterile instruments for every step.

The foetuses were removed from the uteri and transferred to a second dish containing KDS + 5% FCS; this was carried out by tearing open the wall of the uterus and removing the foetuses with a pair of small toothed forceps. The abdominal cavity of the foetuses was exposed and the contents removed and transferred to a third dish of KDS + 5% FCS using small curved forceps. Using a pair of 1 ml syringes (Terumo syringe for U-100 Insulin) and 26G.1/2" (0.46 x 13 mm) sterile needles, the pancreas was carefully dissected from the stomach, duodenum and spleen taking care not to rupture the spleen during dissection to

minimise the transfer of "passenger leucocytes". The pancreas was rinsed in fresh KDS + 5% FCS to remove any free-floating cells and immediately placed in organ culture. For practical reasons, only the body and the tail of the foetal pancreas was removed representing approximately one half of the organ.

Neonates less than 24 hours old were decapitated and their bodies placed in 70% alcohol and then transferred into fresh KDS + 5% FCS. The pancreas was then dissected using the same sterile technique as previously described with the foetal pancreas.

2.2.4 Dissection of foetal pig pancreas

Foetal pigs were obtained at a gestational age of 70 - 100 days from sows that had been killed by stunning and exsanguination, specifically to obtain the foetal tissue. The foetuses were removed within 5 minutes of death and immediately placed in plastic bags into an ice/water slurry to produce rapid body cooling and were transported to the laboratory where all further procedures were performed. In two experiments the foetal pig tissue was obtained from a local abatoir and gestational age was estimated from weight and crown-rump length. There was a significant warm ischaemia time of about 15-30 min between the death of the sow and the placement of the foetal pig on ice in these two instances. The foetal tissue remains viable for many hours after rapid cooling. Before the foetal pigs were dissected they were immersed in 70% ethanol to sterilise the skin and then placed on a sterile towel in a laminar flow hood. The skin was removed by making an incision from the paravertebral mid-thoracic region anteriorly to about the mid-costal point, then distally to the hind leg and then dorsally towards the spine. Using new instruments the abdominal muscle layer was dissected so that the left lateral abdominal viscera was exposed. In the foetal pig the pancreas has a very distinctive appearance and is readly identified. To dissect the pancreas from the adjoining viscera it is necessary to detach it from the adjacent organs by grasping the tail of the pancreas with a pair of small curved forceps and carefully cutting the pancreas away from its attachments with iris scissors. Using a dissecting microscope the splenic vessels and connective tissue are cut away. The pancreas is then diced into approximately 1 mm^3 pieces using 26G needles mounted on 1 ml syringe barrels and then placed in organ culture.

2.2.5 Organ culture

The organ culture technique was based on the original method described by Mandel and Kennedy (1978), with some small variations. The dissected foetal or neonatal pancreas was placed on a strip of millipore filter resting on a block of surgical gelatin foam (Gelfoam, Upjohn Co., Kalamazoo, MI. USA) in 15 ml of DME + 5% FCS using a 9 cm diameter tissue culture petri dish. The gelatin foam absorbed the culture medium transporting nutrients to the tissue and keeping the tissues moist while they remained in the gas phase. The culture media were changed twice weekly and the foetal or neonatal mouse pancreas cultured for 7 to 21 days.

2.2.6 Culture conditions

2.2.6.1 "Conventional", high oxygen and low temperature organ culture

Cultures were maintained in a humidified incubator at 37°C, in a gas phase of 10% CO_2/90% air. These conditions will be referred to as "conventional conditions" (CC) of culture. The "high oxygen" (HiO_2) cultures, used 10% CO_2/90% O_2 as the gas phase for 2 - 3 days for pig and upto 21 days for mouse pancreas, after which time the tissues were transfered into 10%CO_2/ 90% air. This initial culture phase allows the O_2 toxicity to be directed against the most susceptible cells (the irrelevant acinar and possibly most immunogenic cells), while allowing the islet cells, and most importantly their precursors in the ducts, to survive.

The pancreas dishes were placed into a modular incubator chamber (Billups-Rothenburg, Del Mar, CA. USA.) containing 2 open petri dishes of DDW to keep the chamber humidified. The chamber was then sealed and placed in the incubator at 37°C.

Tissue that was to be cultured at "low temperature" (LT) conditions was placed in a chamber, as for HiO_2, using 10% CO_2/90% air and maintained in a dark room at 22°C (ie at room temperature).

2.3 TRANSPLANTATION TECHNIQUES

2.3.1 Transplantation of foetal and neonatal pancreas

The animals were anaesthetised, one at a time, by inhalation of methoxyflurane (Abbot Laboratories, North Chicago, Ill. USA) using a beaker containing cottonwool and 20 ml of anaesthetic. Once the mice were unconscious, they were transferred to a dissecting microscope and anaesthesia maintained with the aid of a nose bottle containing methoxyflurane.

Illumination was provided by a 3-arm Schott Mainz KL150B optical fibre light which has the advantage of shadowless illumination and cold light resulting in minimal drying of the tissue.

The skin was wiped with 70% ethanol and a small left lateral incision was made exposing the abdominal wall. A short incision was then made in the abdominal wall muscle overlaying the kidney allowing the left kidney to be exteriorised by applying pressure to the abdomen. One explant was transferred to the surface of the kidney by pasteur pipette and at the same time the kidney was moistened with 1-2 drops of KDS/5% FCS. Using two pairs of jeweller's forceps a small hole was made through the capsule at the lower pole of the kidney. The capsule was slightly raised and the explant was tucked beneath the capsule. Using a pair of curved forceps to keep the kidney steady, the graft was then pushed to the upper pole by a gentle sliding action with a pair of jeweller's forceps. The kidney was returned to the abdominal cavity and the abdominal wall pulled together. The skin was closed with 1 or 2 surgical autoclips (Clay Adams, Parsippany, NJ. USA). The animal was allowed to recover under an infra-red lamp for 5-10 minutes to minimise body heat loss.

2.3.2 Graft nephrectomy

Each animal was anaesthetised and a left lateral incision was made through the skin and abdominal wall as described above. Using fine forceps, the left kidney was carefully freed from any adhesions to adjacent organs or the abdominal wall resulting from the previous operation. The kidney was slightly raised and renal pedicle, including the renal vein, renal artery and ureter, was clamped with a straight artery forceps and tied proximally to the clamp. The kidney was removed with a scalpel blade and the skin closed as above.

Grafts were scored for macroscopic size under the dissecting microscope on a 0-4+ scale.

2.4 HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ASSESSMENT

2.4.1 Tissue preparation for light microscopy and staining

All histology except immunoperoxidase staining was carried out by the Institute's Histology Department. The tissue was fixed in Bouin's Fixative which consists of 70% saturated picric acid, 25% formalin and 5% glacial acetic acid. The time for fixation depended on the size of the tissue and ranged from 15 min to 18 hr. Adult pancreas was fixed for 18 hr, pancreas grafts were fixed for 3-5 hr and cultured foetal pancreases were fixed from 15 min to

1 hour. I used Bouin's fixative because it is regarded as an excellent fixative for cytoplasmic staining required in Gomori's Aldehyde Fuchsin stain.

After fixation the tissue was washed in 70% ethanol to remove the picric acid and then placed in a Shandon processor 2LE (Shandon, Cheshire, England, UK.) and dehydrated through increasing concentrations of ethanol, cleared in chloroform and embedded using a Reichert Histostat (tissue embedding machine) in paraffin wax with a melting point of 56°C. The embedded tissue was sectioned at 4 μm with a Reichert-Jung 2040 (R. Jung Nubloch, Germany) autocut microtome, the sections floated on a 56°C water bath and picked up on glass slides. For staining, the sections were deparaffinized in three changes of xylene, hydrated through decreasing ethanol concentrations and alternate slides were stained with;

(1) Haematoxylin and Eosin (H&E):

1% Mayer's Acid Haematoxylin 10 min, rinse 3 min, Scotts tap water 2 min, rinse 3 min, and 1% aqueous Eosin 3 min, rinse well for about 2-3 min. Dehydrate through graded ethanol 70%, 80%, 90% and 3 changes of absolute ethanol, clear in 3 changes of xylene and mount in DPX (Fronine, Riverstone, NSW, Aus).

Haematoxylin and Eosin stain is a commonly used cytologic stain, staining the nucleus blue and cytoplasm red.

(2) Gomori's Aldehyde Fuchsin (GAF); Gomori *et.al.* (1950);

0.5% Aldehyde Fuchsin solution 30 min, rinse in 95% ethanol, rinse in tap water (check under the microscope for β cell staining), stain with Masson's Trichrome (BDH Chemicals Ltd, Poole, England, UK.) 20 min, rinse and differentiate in 0.5% acetic acid, rinse well for about 2-3 min, dehydrate through graded ethanol 70%, 80%, 90% and 3 changes of absolute ethanol, clear in 3 changes of Xylene and mount in DPX.

Staining results;

Insulin (β cells)	purple
Glucagon (α-cells)	red
Somatostatin (δ-cells)	green
Elastic tissue	purple
Muscle fibres	red
Collagen	green

2.4.2 Immunoperoxidase staining

2.4.2.1 Paraffin embedded tissue

The method used for immunoperoxidase staining procedure is a modification of the Sternberger (1979) method utilising a three antibody peroxidase anti-peroxidase (PAP) technique. Sections are first treated with normal swine serum (1:20) in MTPBS to inactivate endogenous peroxidase followed by incubation with normal serum of the species from which the linking antiserum has been obtained. This helps to reduce or eliminate non-specific background staining by blocking Fc receptor sites in the tissue. For a positive control, and for the non-specific binding control, sections of normal adult pancreas which have not been treated with the primary antibody were used. Antibodies used in this method are:

Anti-Porcine Insulin (Guinea pig) (ICN Immunobiologicals Code 65-104-1)

Rabbit Anti-Glucagon (Dakopatts Code A565)

Rabbit Anti Human Somatostatin (Dakopatts Code A566)

Swine Immunoglobulins to Rabbit Immunoglobulins (Dakopatts Code Z196)

PAP (Rabbit) (Soluble complex of Horseradish peroxidase and Rabbit Anti-Horseradish Peroxidase) (Dakopatts Code Z113)

Peroxidase-Conjugated Rabbit Immunoglobulins to Guinea Pig Immuno-globulins (Dakopatts Code P141).

The method is as follow;

1. Place slides overnight in a 37^{o}C oven
2. Place in a 70^{o}C oven — 30 min.
3. Rehydrate slides

{ Xylene for 15 min (3 changes) then through graded ethanol (three changes of ethanol 90%, 80%, 70% and water)}

4. 3% H_2O_2 in Methanol (15ml H_2O_2 in 500 ml Methanol)

{Elimination of endogenous peroxidase activity} — 5 min.

5. Rinse in MTPBS — 2x5 min
6. Normal Swine Serum 1:20 — 20 min
7. Primary antibody — 1 hr.

For Insulin, Anti-Porcine Insulin (Guinea pig) — 1:1000 in MTPBS

For Glucagon, Rabbit Anti- Glucagon 1:400 in MTPBS

For Somatostatin, Rabbit Anti Human Somatostatin 1:400 in MTPBS

8. Rinse in MTPBS 3x 5 min

9. Secondary antibody 30 min

For Insulin, Peroxidase-Conjugated Rabbit Immunoglobulins to Guinea Pig Immunoglobulins.

1:20 in MTPBS

For Glucagon and Somatostatin, Swine Immunoglobulins to Rabbit Immunoglobulins

1:20 in MTPBS

10. Rinse in MTPBS 3x 5 min

11. Third antibody 20 min.

For Glucagon and Somatostatin I used PAP (Rabbit) (Soluble complex of Horseradish peroxidase and Rabbit Anti-Horseradish Peroxidase) 1:100 in MTPBS

12. Rinse in MTPBS 3x5 min.

13. Place slides on a rack in a fume hood and freshly made DAB solution (Sigma D-5637 3,3'-Diaminobenzidine tetrahydrochloride Grade II) freshly made. 3 min

(DAB solutions 10 µg of DAB, 10 µl of H_2O_2 in 10 ml MTPBS)

14. The DAB reaction was stoped by rinsing the sections in tap water.

15. Mayer's Acid Haematoxylin 2 min.

16. Wash in tap water 2-3 min.

17. Scott's Tap Water Substitute 10-20 sec.

18. Washed in tap water 2-3 min.

19. Dehydrate through graded ethanol 70%, 80% 90% and three changes of absolute ethanol.

20. Clear in 3 changes of Xylene and mount in D.P.X.

2.4.2.2. Avidin-biotin double staining method for immunoperoxidase

Antibodies used in this method are:

Anti-Porcine Insulin (Guinea pig) (ICN Immunobiologicals Code 65-104-1)

Rabbit Anti-Glucagon (Dakopatts Code A565)

Rabbit Anti Human Somatostatin (Dakopatts Code A566)

Swine Immunoglobulins to Rabbit Immunoglobulins (Dakopatts Code Z196)

PAP (Rabbit) (Soluble complex of Horseradish peroxidase and Rabbit Anti-Horseradish Peroxidase) (Dakopatts Code Z113)

Peroxidase-Conjugated Rabbit Immunoglobulins to Guinea Pig Immuno-Globulins (Dakopatts Code P141).

Anti-Gal(α1-3)Gal (Biotinylated, Griffonia simplicifolia Lectin I (IB4-biotin) (Vector Laboratories Inc. Burlingame, CA, USA)

The method is only used for studies described in chapter 5 and is as follow;

1. Paraffin embedded sections 3-5 μm (Bouin's Fixed)
2. Place slides overnight in a 37°C oven
3. Place slides in a 70°C oven for 30 min.
4. Rehydrate slides

{ Xylene for 15 min (3 changes) then through graded ethanol (three changes of ethanol 90%, 80%, 70% and water)}

5. Place slides in 3% H_2O_2 in Methanol 5 min.
6. Place the slides in a moist chamber and wash in MTPBS 2x5 min
7. Dry around the sections and apply sufficient blocking serum 20 min

{5% FCS in MTPBS}

8. Flick off blocking serum and dry around the section.
9. Cover the sections with first antibody 100 μl for 3-4 hr.

Primary Ab-mix; (for insulin I used Anti-Porcine Insulin (Guinea pig) 1:500 in MTPBS; ant-Gal (α1-3)Gal 1:50. Final dilution of anti-insulin 1:1000 and Gal(α1-3)Gal 1:100

10. Rinse slides in MTPBS 3x5 min.
11. Wipe around the slides and add 100 μl of the 2nd antibody 30 min.

Secondary Ab mix; (For insulin I used Peroxidase-Conjugated Rabbit Immunoglobulins to Guinea Pig Immuno-Globulins 1:20 for Gal(α1-3)Gal use avidin-alkaline phosphatase 1:100) Final dilution for insulin 1:40 and for avidin-alkaline phosphatase 1:200.

30 minutes

12. Rinse slides in MTPBS. 3x5 min.

Visualise the reaction for Gal(α1-3) as follows:

Gal (α1-3)Gal with Fast Blue (biotin)

-5 mg napthol AS-MX phosphate (Sigma) is dissolved in 250 µl N,N"-dimethylformamide and then added to 40 ml Tris-HCl buffer (0.1M, pH 8.5).

-10 mg fast blue BB BASE (Sigma) is suspended in 250 µl 2M HCl and 250 ml 4% $NaNO_2$ solution is then added to the fast blue BB BASE. After 1-2 minutes this mixture is carefully dissolved in 40 ml Tris-HCL buffer.

- To inhibit endogenous alkaline phosphatase 2mM levamisole (Sigma) is added to the substrate solution.

- This solution is then filtered using a 0.8 µm filter unit.

- The reaction is allowed to proceed for 15-60 min at 37°C by vertical incubation in a Coplin jar.

13. Wash the slides thoroughly with MTPBS. 3x5 min.

14. Visualise the reaction for insulin as follows:

Insulin with 3-amino-9-ethylcarbozole

- 4 mg 3-amino-9-ethylcarbozole is dissolved in 250 µl N,N"-dimethylformamide and then added to 9.75 ml sodium acetate buffer (0.05 M, pH 5.0).

- Before use add 50 µl 3% H_2O_2.

- Allow reaction to proceed for 7-10 min at room temperature by horizontal incubation in the fume hood.

15. Wash slides in DDW for 5 min

16. Coverslip in aqueous solution

2.4.2.3 Frozen Tissue Preparation

The freezing technique employed was similar to that described by Johnson and Walker (1986)

The fresh pancreas and/or graft was placed on a film of OCT compound (Lab-Tek Division, Miles Laboratories Inc., Napierville, Ill. USA.) in disposable specimen vinyl mould (Cryomold, Lab-Tek Division, Miles Laboratories Inc., Napierville, Ill. USA). The mould was subsequently completely filled with additional OCT compound.

Previously, approximately 60 ml of iso-Pentane (BDH Chemicals Australia Pty. Ltd., Kilsyth, Vic. Aus.) was placed in a stainless steel round dish, which was placed on a stainless

steel tray filled with liquid nitrogen resting in an ice bucket filled with dry ice. The specimen mould was slowly immersed into the iso-Pentane, the tissue and the OCT was allowed to completely freeze. The block was wrapped in plastic film (Gladwrap), and aluminium foil and stored in a -20°C freezer.

Specimen blocks were sectioned using a Reichert-Jung 2800 Frigocut E cryostat (Heidelberg, Germany) at -20°C. The blocks were cut at 6 μm, placed on a gelatin-coated slides and fixed for 5 min in acetone at 4°C.

2.4.2.4 Gelatin coating of microscope slides

The reason for coating the slides is to provide an adhesive film for the tissue to adhere to so that it does not lift during the staining.

The method used for gelatin coating the slides is as follows:

The slides are washed in 70% alcohol and allowed to air dry, and are then dipped into the gelatin solution (freshly made), the slides are then allowed to dry in a hot oven at 56°C for 3 hours, and are stored at 4°C in dust free slide boxes. The gelatin solution was prepared by dissolving 5.0 g bacteriological gelatin powder (BDH Chemicals Ltd., Poole, England, UK) in 500 ml distilled water at 70°C on a hotplate using a magnetic stirrer, 0.50 g chromic potassium sulphate (Ajax Chemicals, Sydney, NSW. Aus) was added once the gelatin was dissolved. The solution was allowed to cool to exactly 21°C before the slides were dipped.

2.4.2.5 Avidin-biotin method for immunoperoxidase staining

For lymphocyte phenotyping used in chapter 5.

The strong affinity of avidin for biotin and the mild biotinylation process makes this method more sensitive than other methods (eg direct and indirect methods). This method requires a biotinylated antibody as a link antibody, where the biotin is covalently attached to the antibody. Open sites on the avidin biotin complex bind to the biotin on the link antibody. The biotinylated antibody does not have to be added in excess since free Fab sites are not needed for binding. The sequence of reagent application is primary antibody, biotinylated secondary antibody, preformed avidin-biotin-enzyme complex (ABC) substrate solution. For positive controls sections of normal adult mouse spleen stained with both primary and secondary antibodies were used whereas in the negative control sections normal adult mouse spleen that

had not been treated with primary antibody were used. For this study the following primary antibodies were used:

Pan T cell markers; Thy 1.2 (30-H12) IgG_{2b} anti rat (Lou/WS1/M)

Anti–CD3 (KT3); IgG_{2a} anti-rat (Lou/WS1/M)

T cytotoxic suppressor marker ($T_{c/s}$); anti-CD8 (53-6.7) IgG_{2a} (Lou/WS1/M)

T helper inducer marker ($T_{h/1}$); anti-CD4 (H129) IgG_{2a} anti-rat (Lewis)

Marker for activated T & B cells; IL-2R (PC61) $IgG_{1/K}$ anti-rat

Macrophage markers; F4/80 IgG_{2b} anti-rat (HO.B2) and Mac-1 (Rat anti-mouse)

B cell marker; Sheep anti-mouse IgG.

The method is as follow:

1. Fix in acetone at 4°C	5 min
2. Wash in MTPBS	
3. Blocking serum	20 min

(For T cells 1 ml MTPBS, 10 µl Rabbit anti-mouse IgG (H+L) antisera and 7µl sheep serum, for B cells 1 ml MTPBS and 7µl sheep serum)

4. Primary antibody	2 hr
5. Wash in MTPBS	3x 5 min
6. Second antibody	1 hr

If first antibody was anti-rat I used sheep anti-rat (1:500 in MTPBS + 0.1% BSA) 1 hour as the second antibody, if first antibody was anti-mouse than I used Rabbit anti mouse IgG Biotinylated

7. Rinse in MTPBS	3x 5 min
8. Avidin-biotin complex	30 min
9. Wash in MTPBS	3x 5 min
10. DAB	3 min

(DAB solution;10 mg DAB, 13.6 mg Immadazale 15µl H_2O_2 in 20 ml MTPBS)

11. Wash in normal saline	5 min

(stops the reaction)

12. 5% copper sulphate	15 min
13. Wash in tap water	5 min

14. Stain with Mayer's haematology	1 min
15. Wash in tap water	3 min
16. Scott's tap water substitute	30 secs

17. Dehydrate in ethanol, cleared in xylene and cover slipped in D.P.X.

2.4.2.6 Microscopy

The histological sections were examined with a Nikon AFX IIA microscope with x4, x10, x25, x40, and x100 (oil) objectives. All sections were assessed and appropriate sites photographed with a Nikon FX-35A camera. For black and white photographs Kodak Panatomic-X film (ASA 32) was used with a N16 neutral density filter. For colour photographs Kodak Gold III (ASA 100) was used with N16 neutral density filter.

2.4.2.7 Histological assessment of the graft.

The pancreatic isografts were assessed on an arbitrary scale (0-4+), for the presence of islets and the number and distribution of α, β and δ cells. The iso-, allo- and xenografts were assessed for the presence of infiltration and graft size (table 1). Diabetic adult pancreas was assessed for the presence of islets and insulitis.

Table 1

Graft score

0 = No evidence of islet tissue, scar.

1+ = Scattered islet cells with heavy infiltrate.

2+ = Obvious foci of differentiated islet cells.

3+ = Well differentiated graft with scattered infiltrate.

4+ = Large differentiated graft, no infiltrate.

Infiltrate score

0 = No infiltrating cells.

1+ = Scattered foci of perigraft small lymphocytes.

2+ = More extensive foci with some mononuclear cells intra-graft.

3+ = Extensive intra-graft infiltrate, minor endocrine cell loss.

4+ = Pleomorphic mononuclear cell infiltrate with massive islet loss.

2.5 RADIOIMMUNOASSAY TECHNIQUES

Radioimmunoassay is a quantitative immunochemical technique utilising antigen labelled with radioactive nuclide. The technique depends upon the ability of the antigen to inhibit the formation of a complex of labelled antigen and specific antibody. The principles of the reaction are as follows:

A* + B → A*B

(Labelled antigen) (Specific antibody) (Bound labelled antigen)

+

A (Antigen in serum or standards)

A.B (Bound antigen)

The binding of labelled antigen is inversely proportional to the concentration of unlabelled antigen present.

2.5.1 For insulin

2.5.1.1 Extraction of insulin from tissue

Insulin extraction was achieved by homogenisation of the tissue in a glass homogeniser (Kontes, Vineland, NJ. USA.) with 0.2M acid ethanol (70% ethanol, 0.2M HCl). The tissues studied were cultured foetal pancreas, pancreatic grafts and whole adult pancreas. Cultured foetal pancreas and pancreatic grafts were homogenised in 3 ml 0.2M acid ethanol, stored at 4°C overnight and centrifuged at 3,000 rpm at 4°C for 10 min using a Beckman GPR tabletop centrifuge. The supernatant was retained and stored at -20°C until ready for assay. Whole adult pancreases were homogenised as above except that on the second day after removing the supernatant the pellet was resuspended in a further 3 ml 0.2M acid ethanol and stored at 4°C overnight. Following centrifugation the second supernatant, totalling 6 ml, was pooled and stored at -20°C for assay.

2.5.1.2 Reagents for insulin radioimmunoassay

The buffer used in this assay was 0.35% bovine serum albumin (BSA) (Fraction V, CSL, Parkville, Vic. Aus) in MTPBS. The standards used were rat insulin (Novo,

Copenhagen, Denmark) received as 100 μg freeze dried in bottles then was diluted with buffer to a concentration ranging from 0.1 to 48 ng/ml. These were then aliquotted in 300μl and stored at -20°C. The primary antibody was guinea-pig anti-porcine insulin (Wellcome Laboratories, Beckenham, UK) and reagents diluted 1:50,000 with BSA Radio-labelled ^{125}I-insulin tracer was produced by the Isotope Laboratory at the Institute, using pig monocomponent insulin, (Novo) purified on cellulose (Yalow and Berson, 1960) and iodinated to a specific radioactivity of 100-150 μCi/μg by stepwise addition of chloramine-T (Roth and Gorden 1968). The second antibody was a solid phase antibody-coated cellulose using donkey anti-guinea pig IgG (IDS, Usworth Hall DC, USA). Tubes were centrifuged in a Beckman GPR tabletop centrifuge, and the number of β cells in the pellet counted on a Wallace LKB-1260 multi-gamma counter for 60 sec.

2.5.1.3 Method for radioimmunoassay

The method used was a modified version of the original Morgan and Lazarow method (1963) using a double antibody system which is reliable and adaptable to routine use on a large scale. The samples used were serum, tissue extracts from cultured pancreas, pancreatic grafts, whole adult pancreas and culture medium collected from pancreatic cultures. Two controls were used (High and Low), ten insulin standards ranging from 0.1 to 48 ng/ml, and non-specific binding (NSB) where the primary antibody was substituted with buffer containing 2 μl/ml normal guinea pig serum. Standards and samples were assayed in duplicate (12 x 75 mm plastic tubes) according to the following method:

1. 100 μl of buffer in all tubes (except total count)
2. 50 μl insulin standard or unknown sample.
   Standards included 0.1, 1, 2, 4, 8, 12, 16, 24, 32 and 48 ng/ml were diluted in MTPBS.
   Unknown samples were diluted 1:50 for culture medium; 1:600 for foetal pancreas extracts and 1:1,200 for pancreatic graft and whole adult pancreas extracts.
3. 100 μl of primary antibody: guinea pig anti-porcine insulin.
   (1:50,000 final dilution, containing 2 μl/ml normal guinea-pig serum)
   (All tubes except total count and non specific binding)
4. Vortex gently and incubate for 5 hr at room temperature (22°C)
5. 100 μl ^{125}I-insulin, diluted to 25,000 cpm.

6. Vortex and incubate for 36-40 hr at 4°C

7. 100 µl solid phase secondary antibody coated cellulose: donkey anti-guinea-pig IgG (except total count)

8. Vortex and incubate for 30 minutes at room temperature

9. 1 ml deionised water and centrifuge at 3,000 rpm (2,200 x g) for 2 min at 4°C and supernatant removed by suction pump

Count pellet for 60 sec on a multi-gamma counter

The final measurement was the radioactivity in the antibody-bound tracer. Standard curves were constructed by plotting the computerised counter values on the Y axis and concentration of the standards on the X axis. The unknown samples were then automatically extrapolated and given in ng/ml.

2.6. PREPARATION OF MONOCLONAL ANTIBODIES

2.6.1 Production of ascites.

Ascites was produced in BALB/c or CBA nude mice. The mice had previously been primed with 0.3 ml pristine (Aldrich Chem Co. Inc., Milwaukee, Wis. USA) 4 days before the cells were ready to be injected ip. Most ascites came up within 3 weeks of initial injection. Once the peritoneal cavity had become swollen with ascitic fluid the mice were sacrificed and the ascites collected. The amount of antibody in the ascites was determined by an enzyme-linked immunosorbert assay (ELISA).

2.6.2 ELISA method for quantitating antibody content of ascites

Ninety-six well, flat bottomed ELISA plates (Nunc, Denmark) were coated with 100 µl of the capture antibody, goat-anti-rat IgG (Chemicon Int., Los Angeles, CA. USA). The coating buffer was composed of:

0.02M $NaHCO_3$ (BDH) in distilled water

0.02M Na_2CO_3 (BDH) in distilled water

pH adjusted to 9.6

The wells were incubated overnight at room temperature in a perspex humidity chamber.

The following morning the coating antibody was flicked off and the wells washed 6 times in MTPBS containing 0.05% Tween 20 (Sigma Chemical Co., St Louis, MO. USA), 6

times in MTPBS and twice in distilled water before being dried by inverting the plates on a towel. A blocking solution containing 1% BSA (Sigma) dissolved in MTPBS +0.05% Tween 20 was added to each well in a volume of 200 µl and left for 1 hr. The blocking solution was then flicked off and the diluted ascites and standard added. The starting dilution for the ascites was usually 1:1000 and these were diluted in duplicate, by a factor of 2. The starting concentration for the standard was 1 mg/ml for rat IgG (Jackson Immunoresearch, USA) and was diluted, in duplicate by a factor of 2. 100 µl of each was added to the wells and left on for 1 hr. Negative controls to which only MTPBS + 0.05% Tween 20 + BSA was added, were always included to monitor non-specific staining of secondary antibody. The primary antibody was flicked off and the wells washed and dried as described previously.

100 µl of the peroxidase-conjugated goat-anti-rat IgG (F'ab 2) (Promega, Madison, WI. USA) was added to the wells and incubated for 1 hour.

The wells were then washed and dried and 100 µl/well of substrate added. The substrate was composed of: 10 ml 5-Aminosalicyclic acid (Sigma Chemical Co., St Louis, MO USA) and 5 µl 30% H_2O_2 (BDH). The substrate was left on for 45 min with the plate constantly being shaken on a plate shaker (Titerkek Flow Labs., Germany).

The plates were analysed on an ELISA plate reader (Titerkek Multiskan MCG Flow and Lab Systems, Finland) and read at an optical density of 450 nm.

The concentration of antibody was calculated by reading off a standard curve.

2.6.3 Purification of antibodies

2.6.3.1 Protein A column

The ascites was clotted in a 37°C water bath for 30-60 min and left at 4°C overnight. The following morning, the ascites was spun at 1800g for 30 min at 4°C. The supernatant was passed over glass wool (Corning Glass Works, Corning. NY. USA) lightly packed into a 2.5 ml syringe and diluted 1:2 with binding buffer (pH 8.9) (Bio-Rad Laboratories, Richmond, CA. USA) or 3M NaCl+1.5M glycine (pH 8.9).

The Protein A column (Bio-Rad Lab.) was connected to a spectrophotometer, chart recorder (ISCO) and pump (Pharmacia LKB Biotechnology Uppsala, Sweden) and pre-eluted with 10 ml of elution buffer (pH 3.0) (Bio-Rad Lab) or 0.1M glycine-HCL (pH 2.8) to ensure that the previously purified antibody has been eluted.

The column was then purged with 5 ml of binding buffer and the diluted ascites passed over the column. The eluate was collected and later passed over the column again. The column was washed with binding buffer until the trace returned to baseline and the antibody eluted and collected into a 50 ml tube containing Trizma Base (Tris{hydroxymethyl}aminomethane, Sigma Chemical Co., St. Louis, MO. USA).

The immunoglobulin subclasses were purified from mouse serum (Sigma, St. Louis MO. USA) using an elution stepwise pH gradient devised by Ey *et al,* (1978). IgG_1 is eluted at pH 6.0, IgG_{2a} at pH 5.0 and IgG_{2b} and IgG_3 at pH 4.0.

The purified antibody was dialysed against 2 litres of MTPBS in a measuring cylinder on a magnetic stirrer at 4°C overnight, with several changes of dialysis fluid, and protein content determined on a spectrophotometer at 280 nm.

The protein concentration was calculated using the formula:

$$= \frac{\text{Reading at 280 nm x 1/dilution factor}}{1.4}$$

= mg/ml of protein.

2.6.3.2 Protein G Column

The ascites was clotted in a 37°C water bath for 30-60 min and left at 4°C overnight. The following morning, the ascites was spun at 1800g for 30 min at 4°C. The supernatant was removed and dialysed against 20 mM phosphate Protein G binding buffer overnight at 4°C. The following day, the ascites was transferred to a 50 ml tube, spun at 1800g for 30 minutes at 4°C and passed over glass wool (Corning Glass Works, Corning. NY USA) lightly packed into a 2.5 ml syringe.

The Protein G column (Pharmacia LKB Biotechnology, Uppsala, Sweden) was connected to a spectrophotometer, chart recorder (ISCO), and pump (Pharmacia LKB Biotechnology, Uppsala, Sweden) and pre-eluted with 10 ml of 0.1M glycine-HCL elution buffer (pH 2.8).

The column was purged with 50 ml of 20 mM phosphate Protein G binding buffer and the ascites passed over the column. The eluate was collected and the column washed with

binding buffer until all unbound protein has been removed. The antibody was eluted with elution buffer and the eluate collected into a 50 ml tube containing Trizma Base.

The purified antibody was dialysed against MTPBS, with several changes of dialysis fluid, and protein content determined as described above.

2.6.3.3 Fluoresceination of antibodies

The method used for the fluoresceination of antibodies was obtained from Goding (1983). The purified antibody was concentrated using Ficoll (Pharmacia, Uppsala, Sweden) to yield a concentration of 4 mg in 0.5 ml (8 mg/ml) and transferred to a 1.8 ml Treff bullet-shaped tube (Treff, Switzerland). I used a molar ratio of fluorescein isothiocyanate (Sigma, St. Louis, MO. USA) (FITC): antibody 4:1, where 10 mg of FITC per mg of protein gave a molar ratio of about 1. An equal volume of FITC solution was added to the antibody. Thus, since I was using a molar ratio of 4 and I had 4 mg of protein in 0.5 ml, I required 4 x 10 mg x4 = 160μg of FITC dissolved in 0.5 ml of 1M carbonate buffer.

The FITC solution was added to the antibody and the tube sealed with adhesive film, wrapped in aluminium foil because the FITC is photosensitive, placed on a wheel and rotated at room temperature for 2-3 hr. The FITC-conjugated antibody was dialysed against 1 litre of MTPBS at 4°C for at least 48 hr, with frequent changes of dialysis fluid. The conjugated antibody was stored at 4°C in a 1 ml cryotube (Nunc, Denmark) with 15M sodium azide and 1:100 dilution of FCS. It was later titrated in a FACS to obtain the optimum concentration for staining.

2.6.3.4 Biotinylation of antibodies

Purified antibodies were biotinylated using a method described by Goding (1983). Approximately 1 mg of purified antibody was dialysed overnight at 4°C against 1 litre of 0.1M $NaHCO_3$ and transferred to a 1.8 ml Treff bullet-shaped tube. Immediately prior to use, 1.0 mg/ml of biotin succinimide ester (Calbiochem, La Jolla, CA. USA) was dissolved in DMSO (BDH) and 110 μl/ml of the biotin solution was added to the purified antibody. The tube was sealed with an adhesive film and rotated on a wheel for 4 hr at room temperature. The biotinylated antibody was dialysed overnight against 1 litre of MTPBS at 4°C. The dialysis fluid was changed at least once over this period. The antibody was stored at 4°C in 1 ml

cryotube with 15 mM sodium azide and 1:100 dilution for FCS, and titrated on the FACS to determine the optimum concentration for staining.

2.7 FLOW CYTOMETRIC ANALYSIS

The mice were killed by CO_2 asphyxiation and pinned out on a cork board. The ventral surface was thoroughly rinsed with 70% alcohol and a midline incision was made. Using blunt dissection the skin was separated from the underlying muscle. The skin was cut away and pinned to expose the peripheral lymph nodes. The inguinal, axillary, submandibular and the mesenteric lymph nodes were removed from the surrounding fatty tissue and placed on ice in a 10 ml tube containing 5 ml of KDS +5% FCS. The spleen was located and carefully dissected.

2.7.1 Lymph node suspension

Using the end of a 2.5 ml syringe plunger, the lymph nodes were gently mashed through a sieve into a petri dish. The sieve and the petri dish were rinsed with an additional 5 ml of medium and the suspension collected in a 10 ml tube and spun at 300g for 10 min at 4°C. The supernatant was decanted and the pellet resuspended in 3 ml of red cell lysing buffer and left on ice for 5 min. After spinning at 300g for 10 min, the supernatant was discarded and the pellet resuspended in 1.5 ml of KDS +5% FCS and counted on a haemocytometer.

2.7.2 Spleen cell suspension

The spleen was placed in a petri dish and the medium collected in a 2.5 ml syringe. Whilst holding the spleen with forceps and using a 26G needle, the spleen was distended by injecting the medium several times until the spleen became clear. The capsule was teased apart with the needle and the suspension collected in a 10 ml tube. The petri dish was rinsed with a further 5 ml of medium. This was also collected in the tube and the suspension was left on ice for 10 min to allow the debris to settle. Apart from the debris, the contents were transferred to another tube and spun at 300g for 10 min at 4°C. The supernatant was decanted and the pellet resuspended in 5 ml of red cell lysing buffer and left on ice for 10 min. The suspension was topped up with 5 ml of KDS +5% FCS and the pelleted at 300g for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 5 ml KDS +5% FCS and counted on a haemocytometer.

2.7.3 Peripheral blood suspension

Mice were bled from the retro-orbital venous plexus with a heparinized Micro-Haematocrit tube (Clay Adam, Parsipanny, NJ. USA.) into a 10 ml tube containing 5 ml of Alsevers medium. Approximately 100µl of blood was taken and the contents of the tube mixed by inverting several times. After spinning at 300g for 10 min at 4°C, the supernatant was removed and the pellet resuspended with 5 ml of red cell lysing buffer and left on ice for 10 min. The suspension was then spun at 300g for a further 10 minutes at 4°C, the supernatant decanted and the pellet resuspended in 1 ml of KDS+5% FCS and counted on a haemocytometer.

2.7.4 Staining of cells

Cells were aliquoted at 2 x 10^6 cells per well into a 96 well round bottomed tissue culture plate (Linbro, Flow Labs. McClean, VA. USA.) and spun for 3 min. The supernantants were flicked off and the pellets resuspended in 50 µl of appropriate primary antibodies {anti-CD3 rat IgG_{2a} (KT3), anti-CD8 rat IgG_{2a} (53-6.7), anti-CD4 rat IgG_{2b} (GK1.5)} and anti-B cells (sheep anti-mouse IgG) and left on ice for 30 min. A negative control was always included if a secondary antibody was used. These cells were resuspended in KDS + 5% FCS for the duration of the primary antibody incubation. The cells were subsequently washed with 200µl of medium and spun at 300g for 3 min. The supernatant was flicked off and the cells resuspended in 50 µl of a conjugated secondary antibody, if necessary. Secondary antibodies were FITC-conjugated goat-anti-rat IgG (Kirkegard and Perry Lab Inc., Maryland, USA) for the rat antibodies, FITC-conjugated sheep-anti-mouse IgG (Silenus, Vic. Aus.) for mouse antibodies and PE-conjugated streptavidin for biotinylated antibodies. The cells were incubated on ice for 30 min.

Then, the cells were washed with 200µl of medium and spin for 3 min at 200g. The supernatant was flicked off and the pellets resuspended in 200µl of a 8µl/ml propidium iodide solution (stock solution of 400µg/ml) (Calbiochem Corp., San Diego, CA. USA) and analysed on a flow cytometer (FACS II, Becton Dickinson, Mountain View, CA. USA).

CHAPTER 3 THE EFFECT OF GESTATIONAL AGE AND TIME IN ORGAN CULTURE ON THE FUNCTION OF ISLET ISOGRAFTS

3.1 INTRODUCTION

This chapter will deal with the effects of transplantation of syngeneic cultured foetal and neonatal pancreas in a drug-induced murine model of diabetes. The aim of the experiments was to compare the morphological and functional aspects of foetal and neonatal pancreas, using 13d, 14d, 15d and 17d gestation and new born tissue. Furthermore, the time in organ culture was studied in all groups, comparing uncultured tissue to 7d, 14d and 21d in "conventional culture" (CC) conditions. In the morphological studies the assessment was based on the microscopic appearance and RIA was used for quantitative insulin extraction of the pancreas. The assessment for functional studies was based on the reversal time of the diabetic animals to normoglycaemia assessed by random blood glucose (RBG) and body weights (BW) measured every 2 weeks. The differentiation of the graft was studied by light microscopy and immunochemistry.

3.2 MATERIALS AND METHODS

3.2.1 Donor Tissue

Each foetus was aseptically removed from the uterus, and the pancreas for culture and transplantation was isolated under sterile conditions using a dissecting microscope. The organ culture technique was previously described in chapter 2. Briefly, foetal distal fore-gut and adjacent midgut containing the pancreatic anlagen was dissected and the body and tail of the pancreas removed and placed in culture on top of a Millipore filter strip which rested on Gelfoam blocks, under CC conditions (humidified incubator at pH 7.2 in 10% CO_2 and 90% air at 37^{o}C) for 7d, 14d, or 21d. The tissue culture medium was mouse tonicity Dulbecco's Modified Eagle's medium (DME) containing 1g per litre glucose, penicillin (60 mg per 100 ml), streptomycin (100 mg per 100 ml) and supplemented with 5% foetal calf serum (FCS). The DME was replaced twice weekly and 1 ml samples were collected and stored at -20^{o}C for RIA to measure the chronic release of insulin into the media. At each organ culture time point a

sample of the pancreas was placed in Bouin's fixative and processed for histological assessment.

3.2.2 Recipient mice

Eight week old female BALB/c mice received an intravenous injection of freshly prepared STZ, 250 mg/kg body weight. The animals included in the transplantation study were those that developed stable diabetes, as defined by two RBG (2 weeks apart) estimations greater than 15 mmol/L. Four weeks after the induction of diabetes, each animal was transplanted with a single foetal or neonatal pancreas, either fresh or after culture for 7d, 14d, 21d in CC conditions. The operation procedure was previously described in chapter 2. The grafted animals had free access to mouse pellets (Barastoc, Vic. Aus.) and acid-water, and were checked daily. Diabetic mice were given 1 to 2 U of insulin (Ultralente MC CSL/Novo Vic. Aus.) per day and additional normal saline per day ip to replace body fluids. RBG estimation and BW measurements were performed at 2 weekly intervals for 20 weeks following grafting. On each occasion 100 µl of blood was removed from the ophthalmic venous plexus (Riley *et al.*, 1960) using 100 µl Microcap pipettes. The blood was centrifuged for 5 min at 1000 g and a 10 µl serum sample was measured on a Beckman II Glucose Analyser as described in chapter 2.

To confirm that diabetes had not reversed spontaneously independently of transplantation, the kidney with the graft *in situ* was removed by a nephrectomy, as described in chapter 2. The graft was carefully dissected from the kidney with a scalpel blade. Two to three grafts per experimental group were taken for histological assessment. They were fixed in Bouin's fluid, embedded in paraffin and each graft site was serially sectioned at 3-4 µm and alternate slides were stained with haematoxylin and eosin (H&E) for a general survey of islet morphology, and Gomori's aldehyde fuchsin (GAF) or immunoperoxidase to identify α, β, and δ cells. The remaining grafts (6 to 8) were placed in acid-ethanol and homogenised to determine the insulin content of the graft using the RIA method described in chapter 2.

3.3 RESULTS

3.3.1 Development of endocrine cells in cultured foetal pancreas

Foetal pancreases from each of the gestational ages was extracted in acid ethanol and the insulin content determined in both uncultured and cultured explants. Histological

examination of freshly isolated uncultured 15d and 17d foetal pancreas showed that it consisted predominantly of acinar tissue. Scattered throughout the pancreas were ducts and small immature endocrine cells, some of which were positive by immunoperoxidase staining for insulin, glucagon or somatostatin. Fig. 3.1 a, b, c, d, e, shows the distribution of endocrine cells in a normal adult pancreas stained with H&E, GAF, insulin, glucagon, and somatostatin. By day 7 in CC conditions the acinar tissue rapidly degenerated and disappeared, whereas the number of β cells dramatically increased forming small oval shaped islets scattered throughout the tissue (Fig. 3.2 a). A small number of α and δ cells was also found scattered mostly at the periphery of the islets (Fig. 3.2 b and c). Mitotic activity of islet cells was still high as was ductal cell proliferation. By day 14 of organ culture the islets of the 15d and 17d foetal pancreas had increased in size, due mostly to the increasing numbers of β, α, and δ cells, and closely resembled adult islets (Fig. 3.3 a,b,c). In the 13d and 14d foetal pancreas the islets were smaller, with high mitotic activity and numerous undifferentiated endocrine cell aggregates. After 21d of CC conditions the 15d and 17d foetal pancreas underwent a dramatic change in that many of the islets began to lose their islet morphology and had grossly diminished insulin staining, while the α and δ cells appeared to suffer no damage as they increased in number (Fig. 3.4 a, b, c). The 13d and 14d foetal pancreas at day 21 in culture had increased in size with large numbers of well differentiated β cells and resembled those of the 15d and 17d foetal pancreas in 14d cultures.

3.3.2 Insulin production *in vitro* by foetal and neonatal pancreas

The *in vitro* insulin production by the cultured foetal and neonatal pancreas was assessed both in terms of hormone secretion into the culture medium (chronic release) and by the content of insulin within the tissue, using the RIA method. However, the results do not take into account any loss of insulin due to degradation during organ culture or during the storage of samples at -20°C prior to assaying.

The culture medium was changed and aliquots collected twice weekly. At almost all gestational ages insulin secretion was minimal, almost the same as the background, during the first collection on day 3 of organ culture. Insulin secretion rapidly increased by day 7, reaching peak levels by the second week in the 15d and 17d foetal pancreas whereas the 13d and 14d

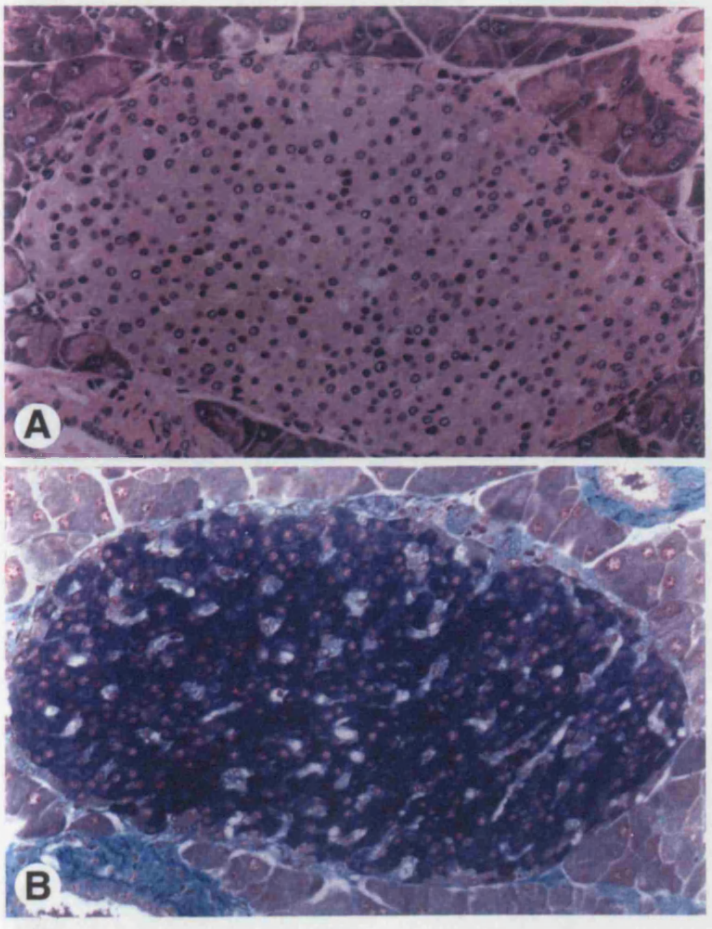


Fig. 3.1: **(a)** Normal adult mouse (aged 100 days) islet stained with H&E (x250).
(b) Normal adult pancreatic islet showing β cells stained with GAF (x250).

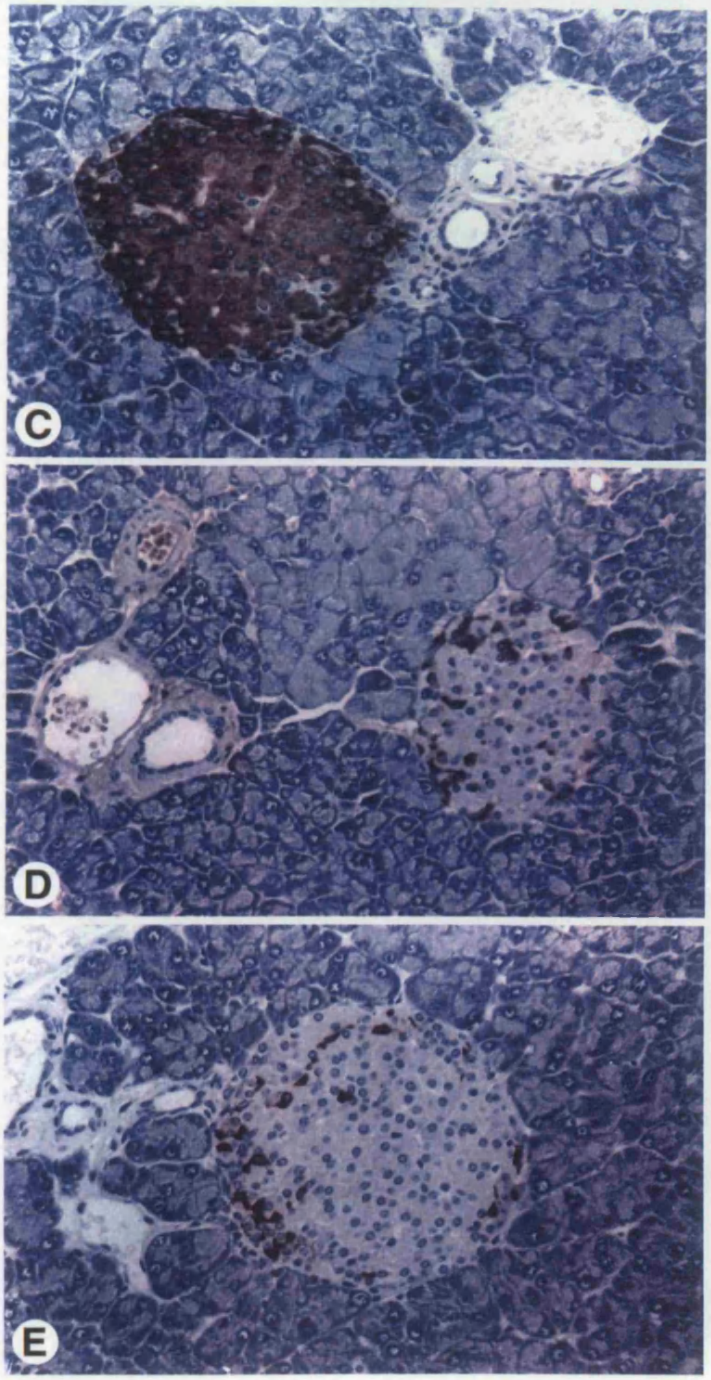


Fig. 3.1: **(c)** Normal adult mouse pancreas stained with IMP for insulin (x250).
(d) Normal adult pancreas stained with IMP for glucagon (x250).
(e) Normal adult pancreas stained with IMP for somatostatin (x250).

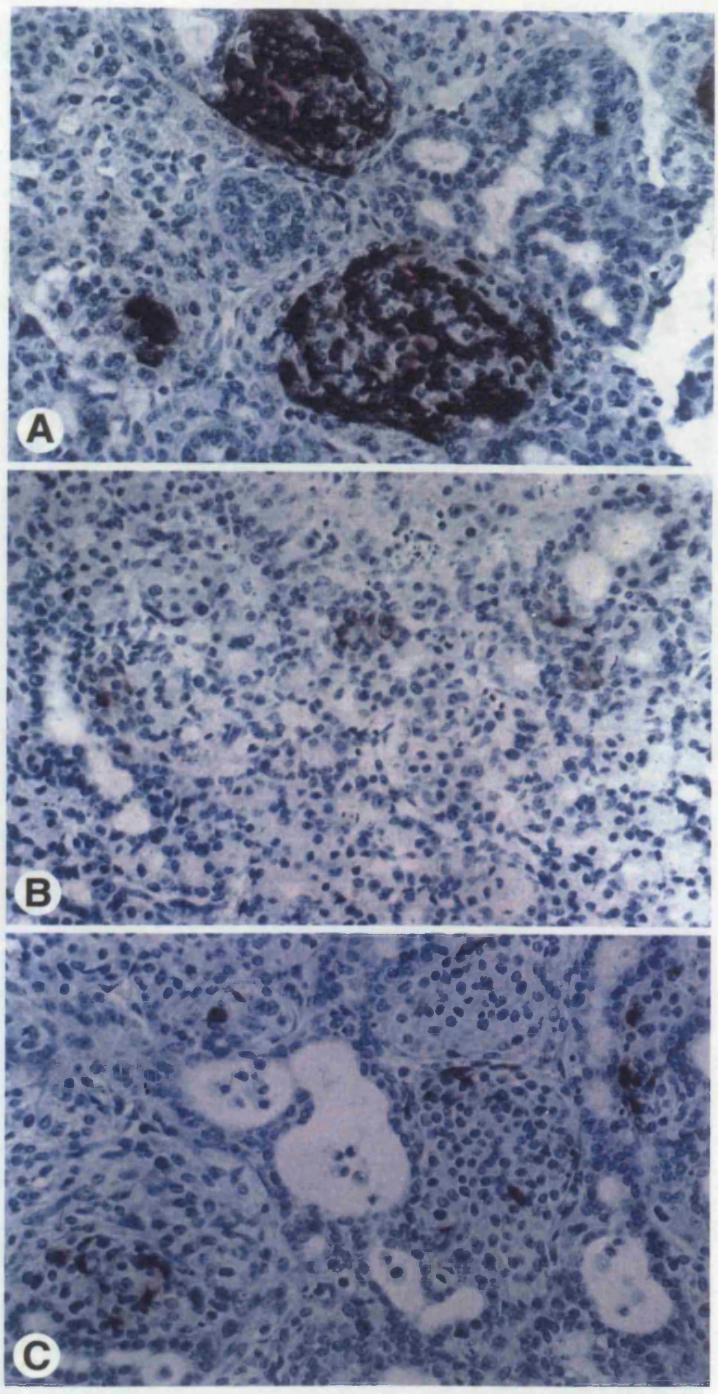


Fig. 3.2: (a) Seventeen-day foetal pancreas cultured under CC for 7 days and stained with IMP for insulin (x250).
(b) Seventeen-day foetal pancreas cultured under CC for 7 days and stained with IMP for glucagon (x250).
(c) Seventeen-day foetal pancreas cultured under CC for 7 days and stained with IMP for somatostatin (x250).

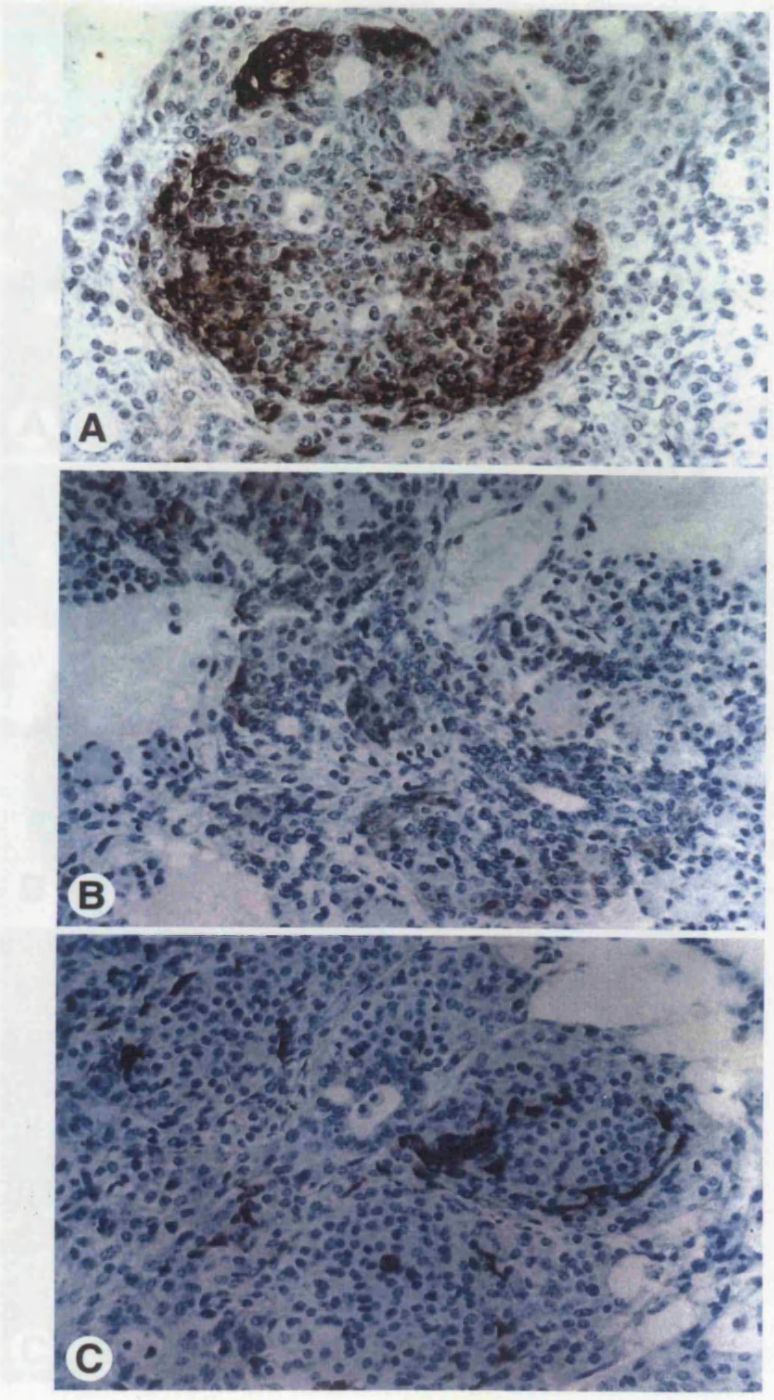


Fig. 3.3: (a) Fifteen-day foetal pancreas cultured under CC for 14 days and stained with IMP for insulin (x250).
(b) Fifteen-day foetal pancreas cultured under CC for 14 days and stained with IMP for glucagon (x250).
(c) Fifteen-day foetal pancreas cultured under CC for 14 days and stained with IMP for somatostatin (x250).

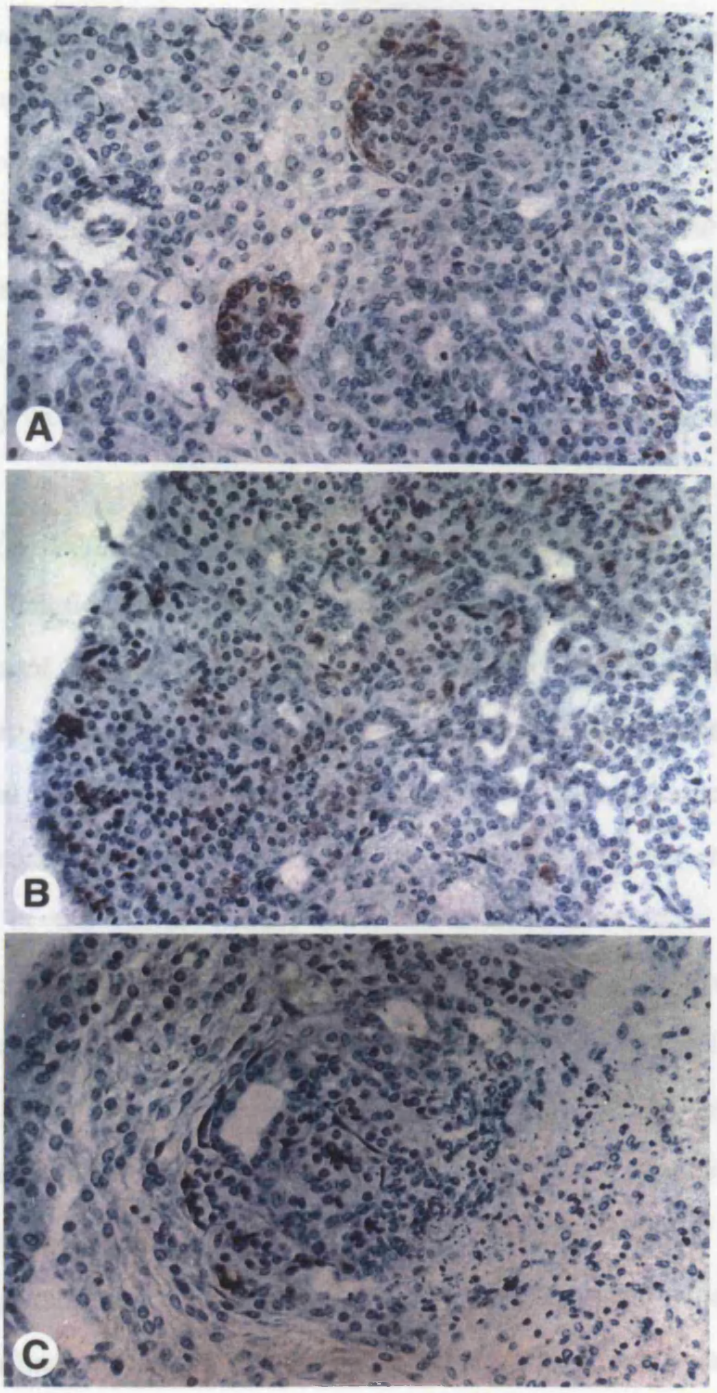


Fig. 3.4: (a) Seventeen-day foetal pancreas cultured under CC for 21 days and stained with IMP for insulin (x250).
(b) Seventeen-day foetal pancreas cultured under CC for 21 days and stained with IMP for glucagon (x250).
(c) Seventeen-day foetal pancreas cultured under CC for 21 days and stained with IMP for somatostatin (x250).

foetal pancreas reached their peak by day 21. The level of insulin then rapidly declined during the third week of culture for the 15d and 17d foetal pancreas (Fig. 3.5).

The somewhat surprising observation was the enormous variation in the insulin secretory capacity of the tissue from the different gestational ages. In the 13d and 14d gestation ages insulin secretion steadily increased during the 21d in culture and was maximal at the point when organ culture was terminated, whereas in the 15d and 17d foetal pancreas insulin secretion rapidly increased to peak at 14d, followed by a rapid decrease during the third week.

3.3.3 Function of the cultures after iso-transplantation.

Graft function was assessed both in terms of; a) the time of reversal to normoglycaemia of the recipient; and b) the insulin content of the graft on its removal. In the uncultured group I found that it took 12 weeks *in situ* to reverse diabetes in the animals that received 14d and 15d foetal pancreas cultures whereas the animals in the groups which received 13d, 17d and neonatal pancreas it took 20 weeks to reverse and they still had RBG's levels at the upper end of the normal range. Once the graft was removed all recipients became hyperglycaemic with RBG readings of 15 to 25mmol/L, with the exception of the neonatal group which stayed around 10 mmol/L. The uncultured neonatal group I started with 8 animals and, since the grafts were not functioning, the very sick diabetic animals died during the duration of the experiment and only 3 animals were left; these had been mildly diabetic to start with (Fig. 3.6 a).

In the groups that received pancreas cultures for 7d most animals reversed within 6 to 8 weeks of grafting and stayed normoglycaemic until the grafts were removed. All groups reversed by week 14 and all animals became hyperglycaemic once the grafts were removed with RBG's levels averaging around 25mmol/l (Fig. 3.6 b). The transplants that were cultured for 14d were able to reverse diabetes within 8 weeks after transplantation and maintain normal RBG levels (Fig. 3.6 c). The 17d foetal pancreas kept in culture for 21d did not function as well as the grafts from the other gestational ages. It took 18 weeks to reverse recipients to normoglycaemia whereas the rest of the 21d organ cultures reversed diabetes within 8 weeks (Fig. 3.6 d).

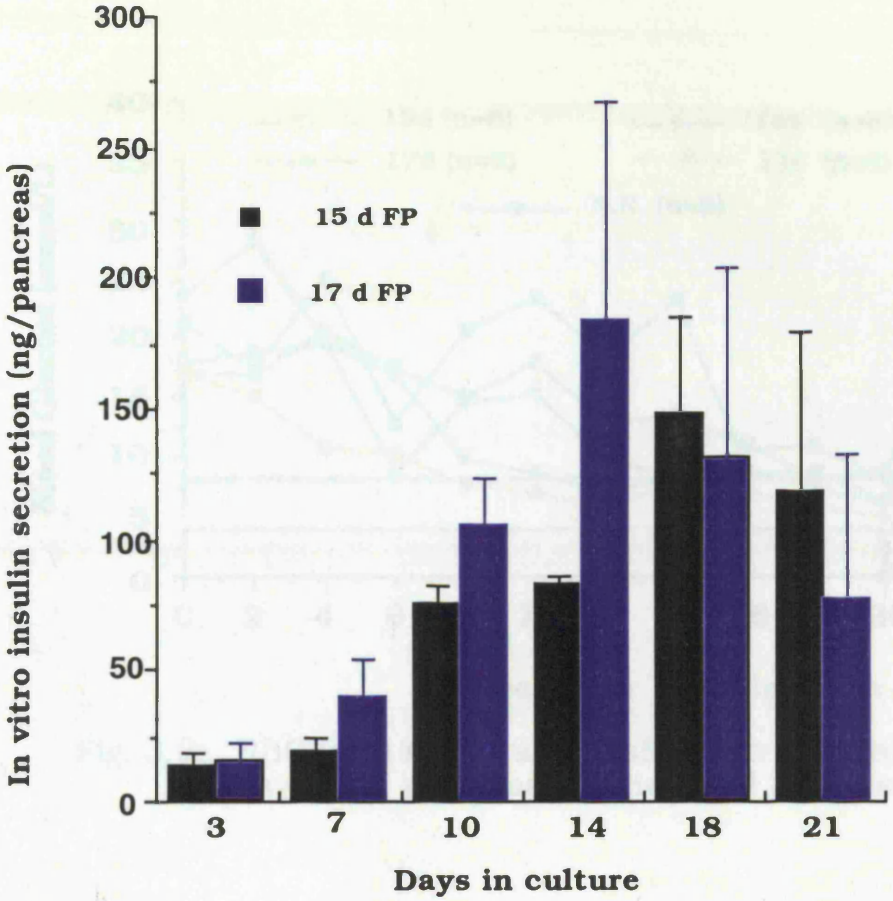


Fig. 3.5 Insulin secretion in organ culture from 15 and 17 day foetal mouse pancreas (mean ± SD).

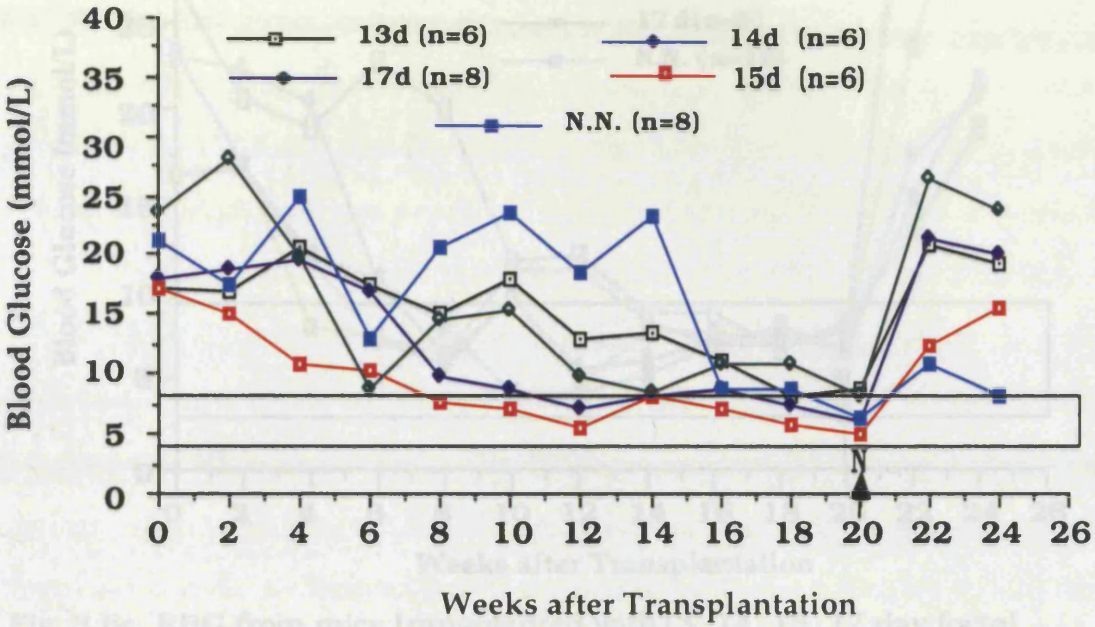


Fig. 3.6a. RBG from mice transplanted with uncultured13, 14, 15 and17 day foetal pancreas and neonatal pancreas (N= nephrectomy).

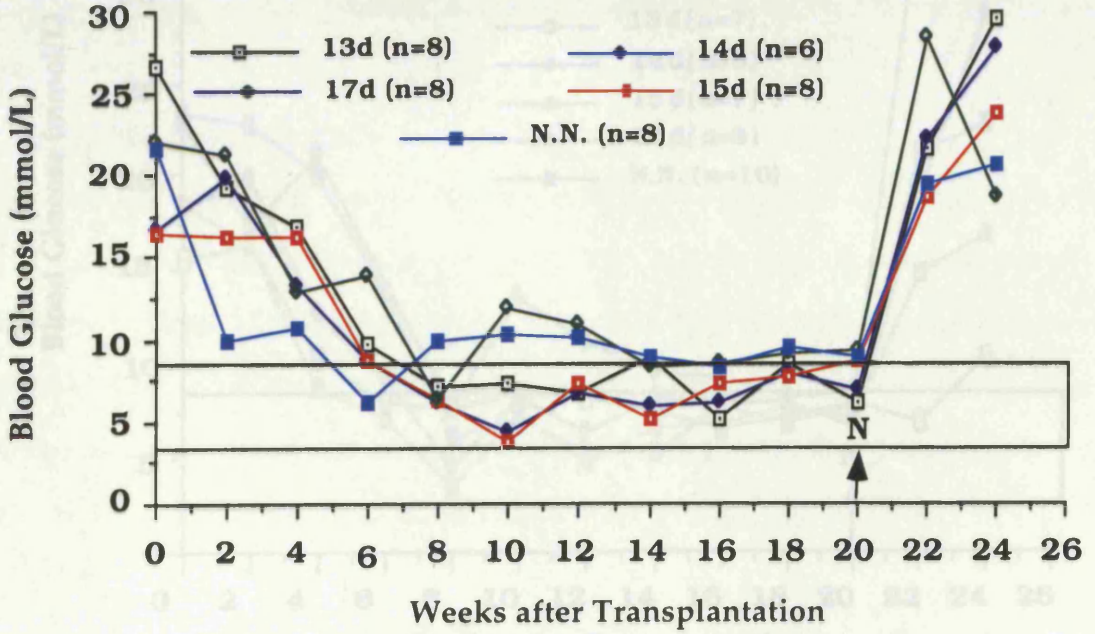


Fig. 3.6b. RBG from mice transplanted with13, 14, 15, 17 day foetal pancreas grown under CC for 7 days.

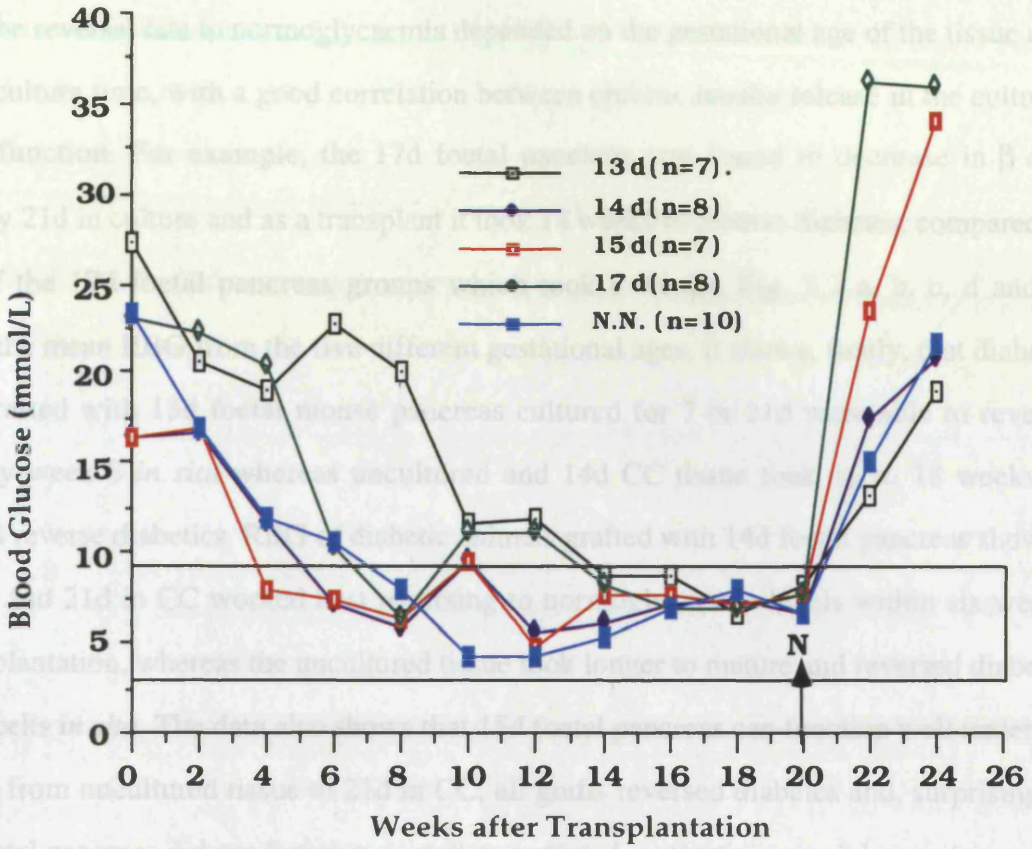


Fig. 3.6c. RBG from mice transplanted with 13, 14, 15, 17 day foetal and neonatal pancreas grown under CC for 14 days.

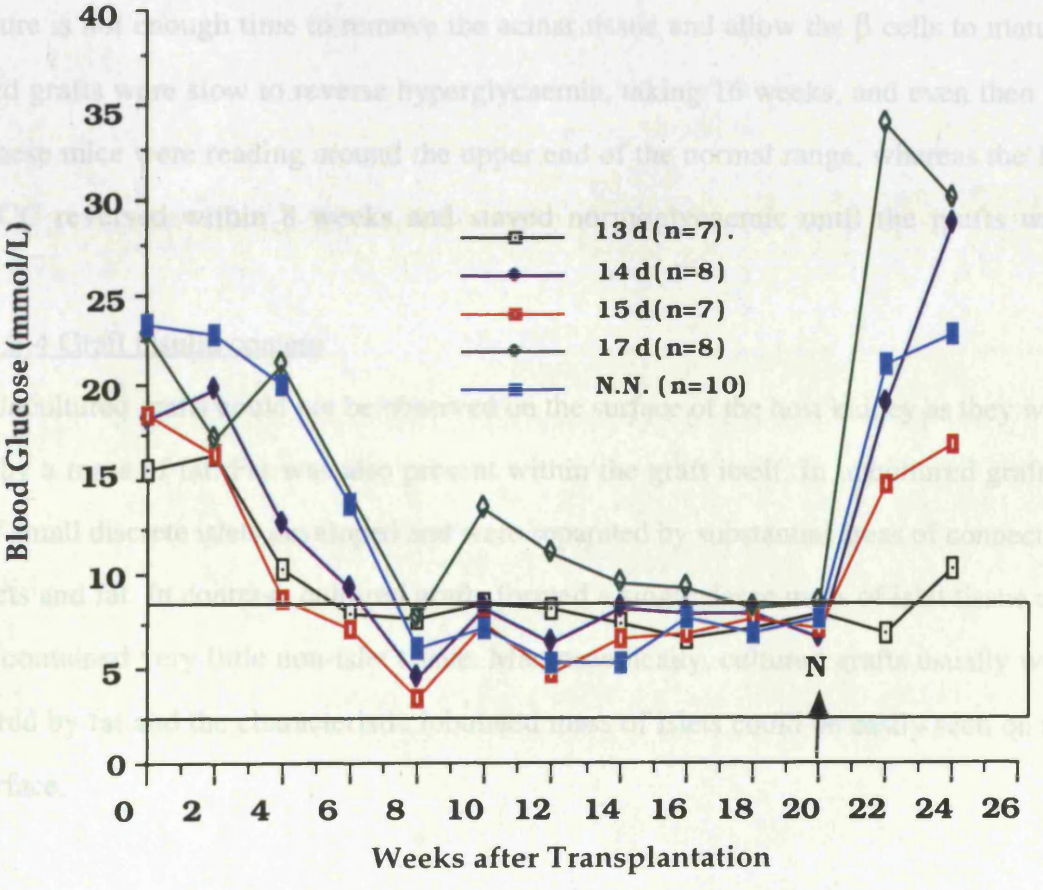


Fig. 3.6d. RBG from mice transplanted with 13, 14, 15, 17 day foetal and neonatal pancreas grown under CC for 21 days.

The reversal rate to normoglycaemia depended on the gestational age of the tissue and the organ culture time, with a good correlation between chronic insulin release in the cultures and graft function. For example, the 17d foetal pancreas was found to decrease in β cell numbers by 21d in culture and as a transplant it took 18 weeks to reverse diabetes, compared to the rest of the 17d foetal pancreas groups which took 8 weeks. Fig. 3.7 a, b, c, d and e, illustrates the mean RBG from the five different gestational ages. It shows, firstly, that diabetic animals grafted with 13d foetal mouse pancreas cultured for 7 or 21d were able to reverse diabetes by week 8 *in situ* whereas uncultured and 14d CC tissue took 16 to 18 weeks to mature and reverse diabetics. RBG of diabetic animals grafted with 14d foetal pancreas showed that 7, 14, and 21d in CC worked best reversing to normoglycaemic levels within six weeks after transplantation, whereas the uncultured tissue took longer to mature and reversed diabetes after 10 weeks *in situ*. The data also shows that 15d foetal pancreas can function well under all conditions from uncultured tissue to 21d in CC, all grafts reversed diabetes and, surprisingly, the 17d foetal pancreas did not function as well as expected, as the tissue took longer to reverse diabetes and most of the groups had a mean RBG around the upper end of the normal range. Finally, the results show that uncultured neonatal pancreas does not work at all as a graft and 7d in culture is not enough time to remove the acinar tissue and allow the β cells to mature. Uncultured grafts were slow to reverse hyperglycaemia, taking 16 weeks, and even then the RBG of these mice were reading around the upper end of the normal range, whereas the 14d and 21d CC reversed within 8 weeks and stayed normoglycaemic until the grafts were removed.

3.3.4 Graft insulin content

Uncultured grafts could not be observed on the surface of the host kidney as they were obscured by a mass of fat. Fat was also present within the graft itself. In uncultured grafts a number of small discrete islets developed and were separated by substantial areas of connective tissue, ducts and fat. In contrast, cultured grafts formed a single, large mass of islet tissue and the grafts contained very little non-islet tissue. Macroscopically, cultured grafts usually were not obscured by fat and the characteristic lobulated mass of islets could be easily seen on the kidney surface.

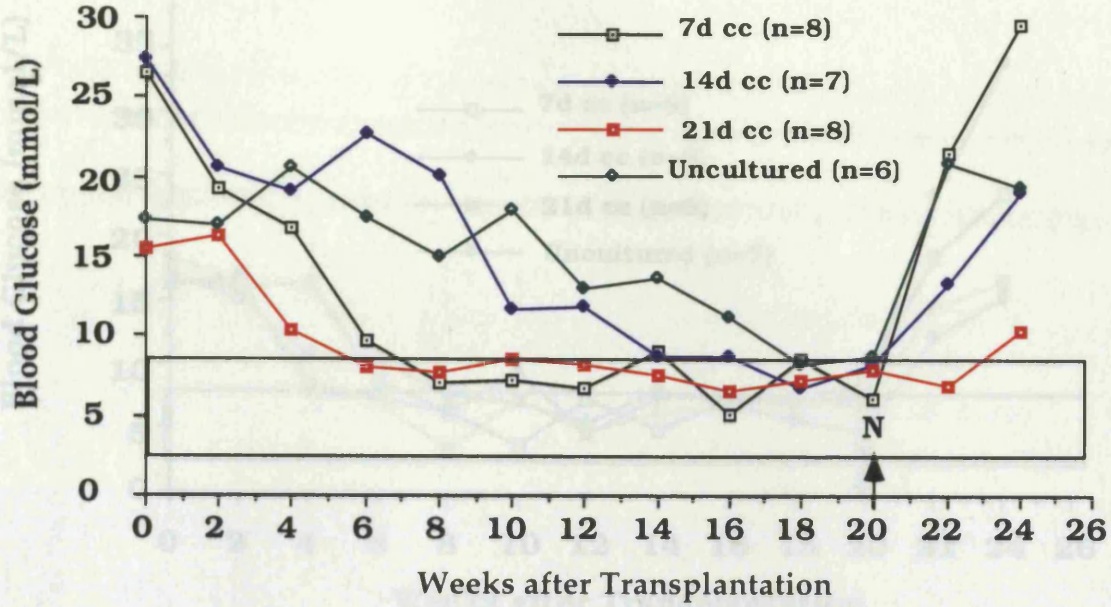


Fig 3.7a. RBG from animals grafted with 13 day BALB/c foetal pancreases, uncultured, and grown under CC for 7, 14 and 21 days

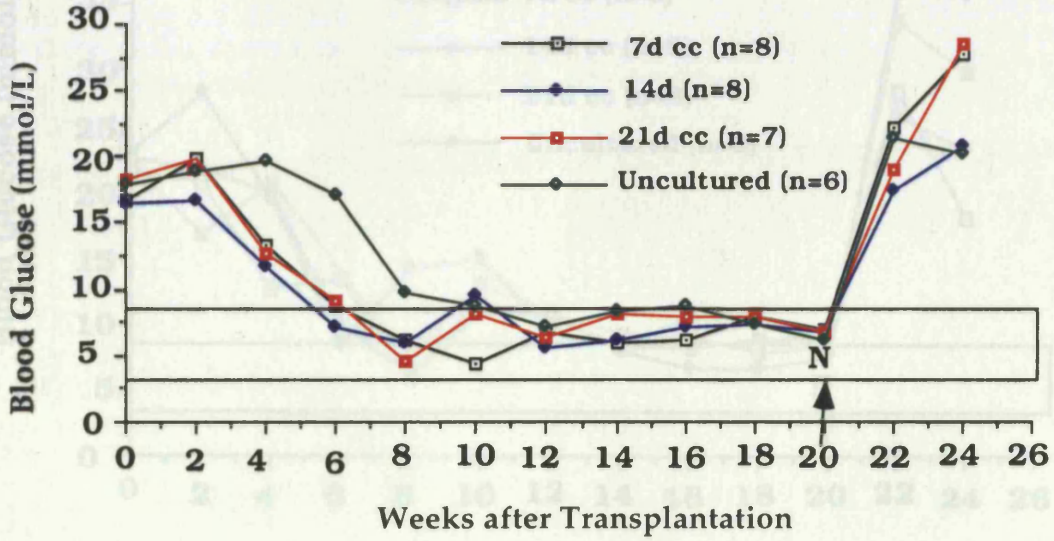


Fig 3.7b. RBG from animals grafted with 14 day BALB/c foetal pancreases, uncultured, and grown under CC for 7, 14 and 21 days

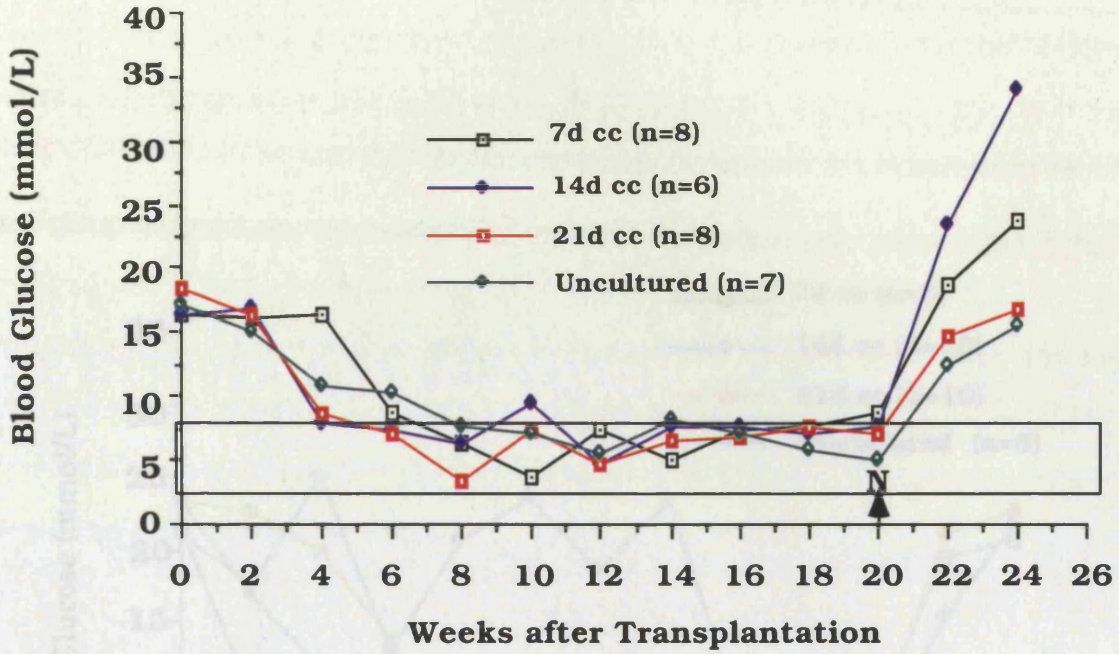


Fig. 3.7c. RBG from animals grafted with 15 day BALB/c foetal pancreases uncultured, and grown under CC for 7, 14, and 21 days.

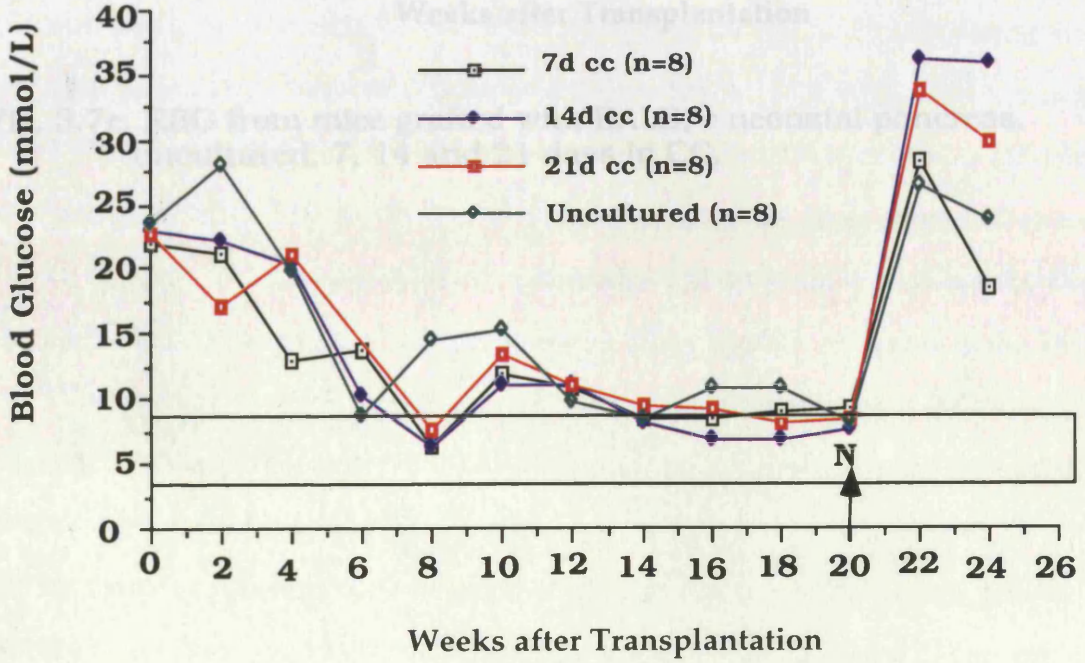


Fig. 3.7d. RBG from animals grafted with 17 day BALB/c foetal pancreases uncultured, and grown under CC for 7, 14, and 21 days.

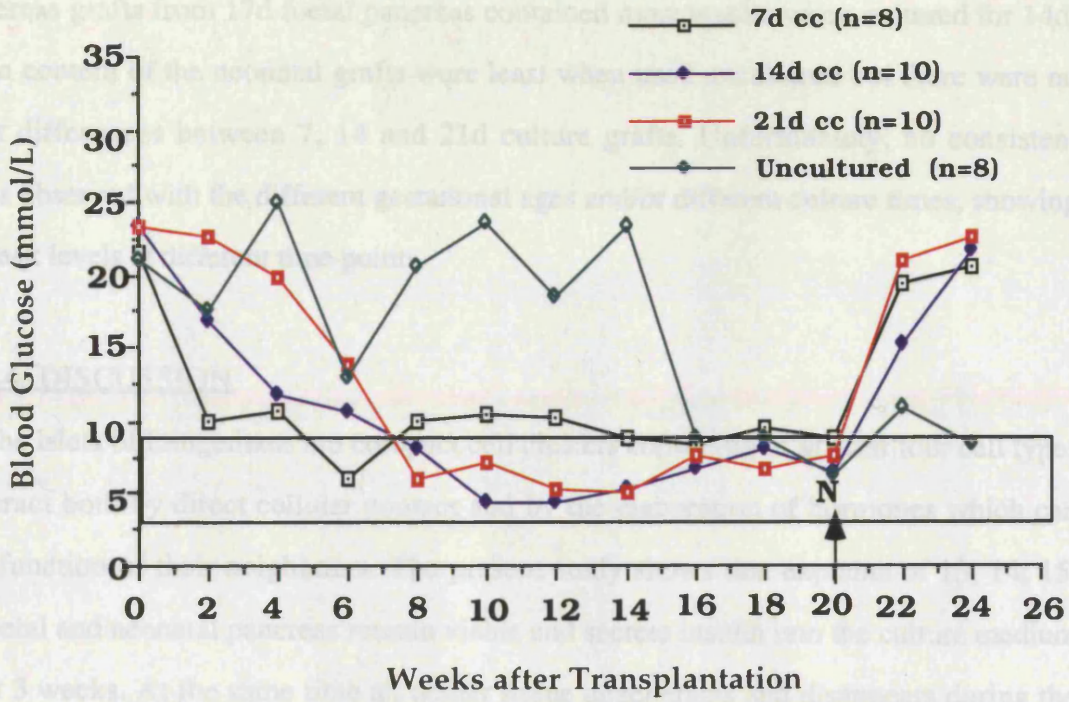


Fig. 3.7e. RBG from mice grafted with BALB/c neonatal pancreas, uncultured, 7, 14 and 21 days in CC.

The pancreatic grafts from each of 20 groups were extracted in acid ethanol and their insulin content determined. Fig. 3.8 shows that grafts from 13d gestation foetal pancreases show hardly any variation in the insulin content of uncultured, 14d and 21d CC grafts, whereas grafts cultured for 7d had a significant increase in their insulin content. Also grafts from the 14 and 15d foetal pancreases showed no significant pattern between the uncultured and cultured tissue, whereas grafts from 17d foetal pancreas contained most insulin when cultured for 14d. The insulin content of the neonatal grafts were least when used uncultured but there were no significant differences between 7, 14 and 21d culture grafts. Unfortunately, no consistent pattern was observed with the different gestational ages and/or different culture times, showing different peak levels at different time points.

3.4. DISCUSSION

The islets of Langerhans are compact cell clusters consisting of at least four cell types which interact both by direct cellular contact and by the elaboration of hormones which can affect the function of their neighbours. The present study shows that explants of 13, 14, 15, and 17d foetal and neonatal pancreas remain viable and secrete insulin into the culture medium for at least 3 weeks. At the same time all acinar tissue degenerates and disappears during the first week of organ culture. The endocrine tissue invariably undergoes a rapid proliferation and differentiation which corresponds with the large increase in β cells, and islet cells in mitosis seen in the histological study. During the first 2 weeks of culture, the islets consist of up to 75% β cells and by about 14d of organ culture the islets appear mature as the α, β, and δ cells gradually distribute into their characteristic arrangement. After 3 weeks of culture in the 17d foetal and neonatal cultured pancreas the number of insulin-positive β cells rapidly declines and remains low, whereas the 13, 14, and 15d foetal pancreases are still maturing and increasing their insulin-producing β cells.

The necessity and requirements for organ culture of foetal or neonatal pancreas are largely dependent on how the tissue will subsequently function as a graft. The basic requirements for successful graft function are the presence of β cells in sufficient number to effectively ameliorate diabetes and to respond to appropriate stimuli. Cultured foetal pancreas

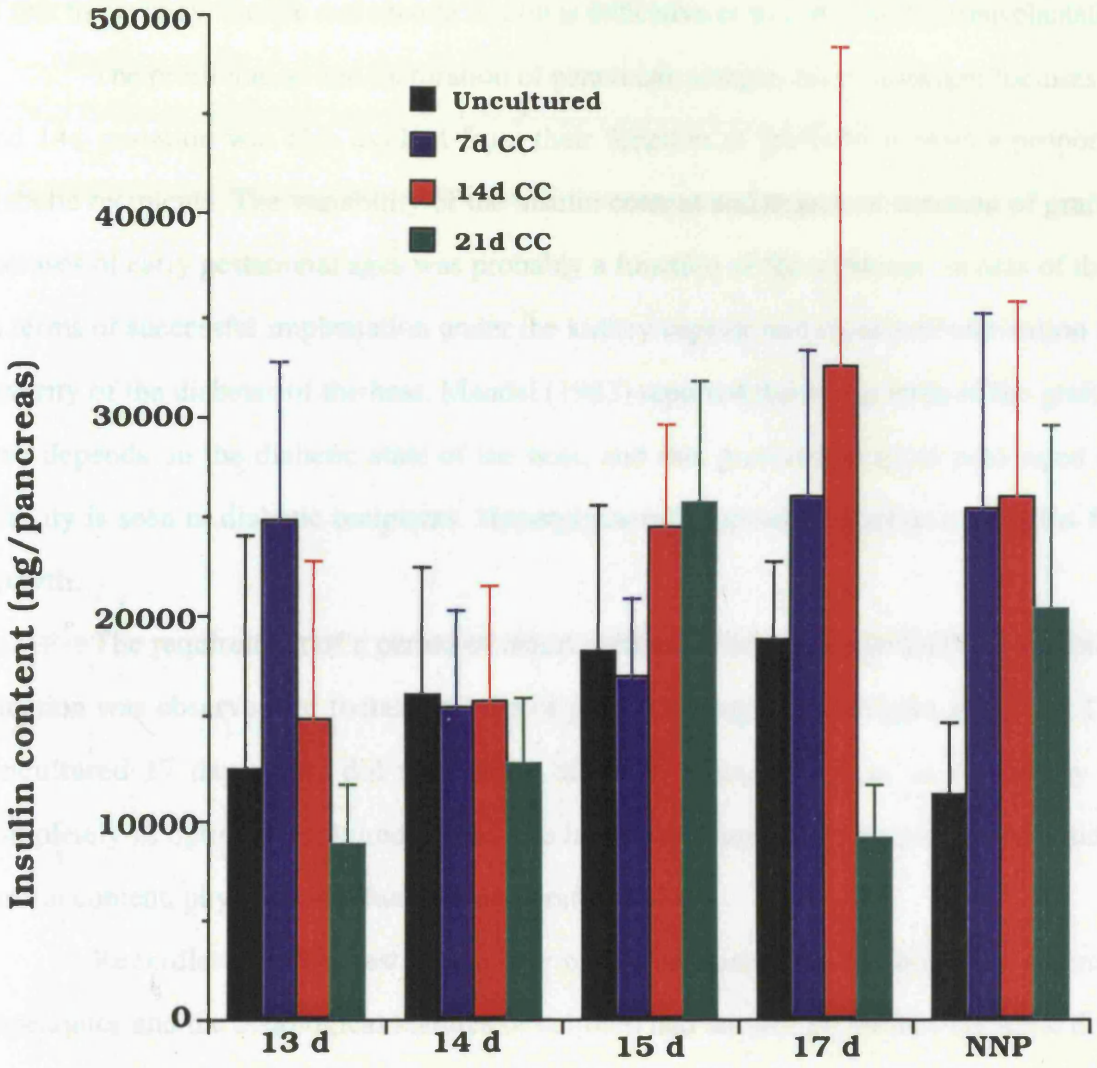


Fig. 3.8 Insulin content of grafts from 13, 14, 15 and 17 day foetal pancreas and neonatal pancreas; 20 weeks in-situ.

consists of numerous islets which closely resemble the appearance of adult islets and the ability of this tissue to synthesise and secrete insulin is indicative of its potential for transplantation.

The proliferation and maturation of pancreatic anlagen from immature foetuses of 13d and 14d gestation was also evident from their function as grafts in at least a proportion of diabetic recipients. The variability of the insulin content and degree of function of grafts from foetuses of early gestational ages was probably a function of the technical success of the graft, in terms of successful implantation under the kidney capsule and rapid vascularization and the severity of the diabetes of the host. Mandel (1983) reported that the growth of the graft *in situ* also depends on the diabetic state of the host, and that greater and more prolonged mitotic activity is seen in diabetic recipients. Hyperglycaemia appeared to act as a stimulus for islet growth.

The requirement of a period of organ culture before transplantation for optimal graft function was observed for foetal pancreas of gestational ages as divergent as 13 and 17 days. Uncultured 17 day grafts did ameliorate diabetes although neither as efficiently nor as completely as optimally cultured grafts. The latter were superior in all parameters studied, ie. insulin content, physiological function and graft histology.

Regardless of the gestational age of the explanted tissue, both the macroscopic appearance and the histological features of cultured and uncultured grafts were quite different. The histological appearance of cultured grafts from most foetuses was markedly superior to that of uncultured grafts. Cultured grafts consisted of a single mass of predominantly β cells. Uncultured grafts contained numerous dispersed small islets, ducts, connective tissue and copious amounts of adipose tissue. The proliferation of adipose tissue in association with uncultured (neonatal rat) isografts has been noted also by Banks *et al.*(1982). Whatever the mechanism, uncultured grafts of all gestational ages used in this study, did not function as well as optimally cultured grafts.

There are a number of questions which remain to be answered. These address the issue of optimum culture and gestational time for successful graft function eg. is insulin secretion or insulin content a more useful indicator of subsequent graft function? Does the culture time for optimum graft function correspond with the culture time required to effectively reduce the immunogenicity of the tissue?

Other variables in a successful graft function could be the diabetes state and the age of the recipient post-transplantation. Does chronic diabetes and chronic complications affect the vascularisation of the islet graft? In the next chapter I will investigate the role of chronic diabetes in the recipient and also the effect of recipients age on graft function.

CHAPTER 4 THE EFFECT OF CHRONIC DIABETES AND THE FUNCTION OF ORGAN CULTURED FOETAL ISLET ISOGRAFTS

4.1 INTRODUCTION

Transplantation of islets of Langerhans has been highly successful in producing euglycaemia in experimental induced diabetes but islet transplantation in humans has had little success. Amongst the reasons for this difference is the different nature of the disease in the animal models and in humans. In the animal models drug-induced diabetes or surgical pancreatectomy have been generally used although spontaneous diabetes in NOD mice is now favoured, whereas, in humans, diabetes is an autoimmune disease. Another major difference is the duration of the disease. In most animal models diabetes has been of short duration (weeks), in contrast to perhaps decades of disease before islet or pancreas transplantation is attempted in most patients. This means that islet grafts in humans are done when advanced microvascular disease is usually present in the recipient.

In chapter three it has been shown that foetal pancreas has the capacity to proliferate and differentiate *in vitro* for up to 3 weeks before a reduction of insulin-producing cells is seen. However, with transplantation into a syngeneic recipient the islet tissue continues to proliferate and differentiate until the animal has become normoglycaemic. The factors that may be important for *in vivo* islet function are vascularisation of the graft, and exposure to physiological stimuli such as glucose and other dietary factors.

The aim of this study was to see (1), whether chronic, (more than 6 months) drug-induced diabetes in mice had an effect on foetal isograft function and (2), whether there was an effect of pre-transplantation recipient age on graft function.

4.2 MATERIALS AND METHODS

BALB/c female mice were made diabetic with a single intravenous injection of streptozotocin (STZ, Sigma Chemical Co., St. Louis, USA.) at a dose of 250 mg/kg body weight. Based on previous experiments (chapter 3), tissue for transplantation was obtained from 17 day gestation BALB/c foetuses. The body and tail of the pancreas was dissected and placed in organ culture in Dulbecco's Modified Eagle's medium containing 5% foetal calf serum

and maintained at 37°C for 14 days in an atmosphere of 10% CO_2/90% air. Media were changed twice weekly.

The recipients were transplanted with 1/2 of a foetal pancreas under the left renal capsule as described in chapter 2. Animals with pre-graft RBG levels greater than 15 mmol/L were used as recipients and transplanted after having untreated diabetes for 2, 4, 8, 12, 16, 20, and 24 weeks. There were 7-10 animals per group, aged 8-10 weeks when injected with STZ. In a separate experiment two groups of 10 female BALB/c mice were used. In the first group the animals were 8-10 weeks and in the second group they were 32 weeks old. Both groups were made diabetic and received a graft within 4 weeks of STZ. All mice were bled to determine RBG levels at 2 week intervals after transplantation. After 18 weeks, the graft was removed by a left nephrectomy and the mice kept for 4 weeks and bled twice to establish that hyperglycaemia recurred. The host pancreas was removed and placed in acid ethanol for insulin extraction. Most of the grafts were extracted in acid ethanol for insulin content measurement, using the double antibody RIA previously described in chapter 2. The remaining grafts were taken for light microscopy, fixed in Bouin's fluid, dehydrated through increasing concentrations of ethanol, cleared in chloroform, embedded in paraffin wax and sectioned at 4 μm.

4.3 RESULTS

4.3.1 Effect of recipient age

Fig. 4.1 shows that RBG of both young (8-10 weeks) and old (32 weeks) mice made diabetic 4 weeks pre-transplantation reversed to euglycaemia within 8 weeks after transplantation and remained so until the grafts were removed by a left nephrectomy. The mean graft insulin content (± SD) between the groups showed the younger mice (n=9) had 13,400 ± 2,600 ng/graft and the older recipients (n=8) 17,400 ± 3,400 ng/graft. This difference was significant $P<0.01$ (student t test). The histology of both groups showed well developed and well vascularized islets with the presence of mitotic activity; showing that the age of the recipient did not interfere with the isograft growth, function or vascularization.

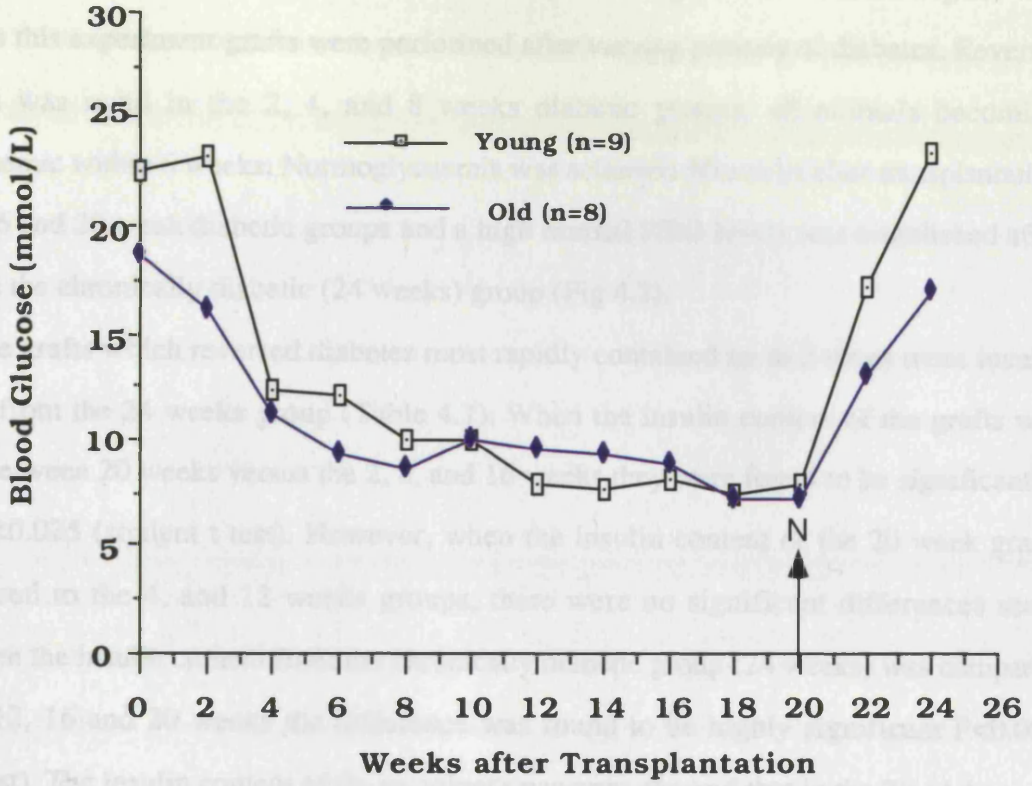


Fig. 4.1 RBG from animals grafted with BALB/C 17 day fetal pancreas cultured for 14 days cc.

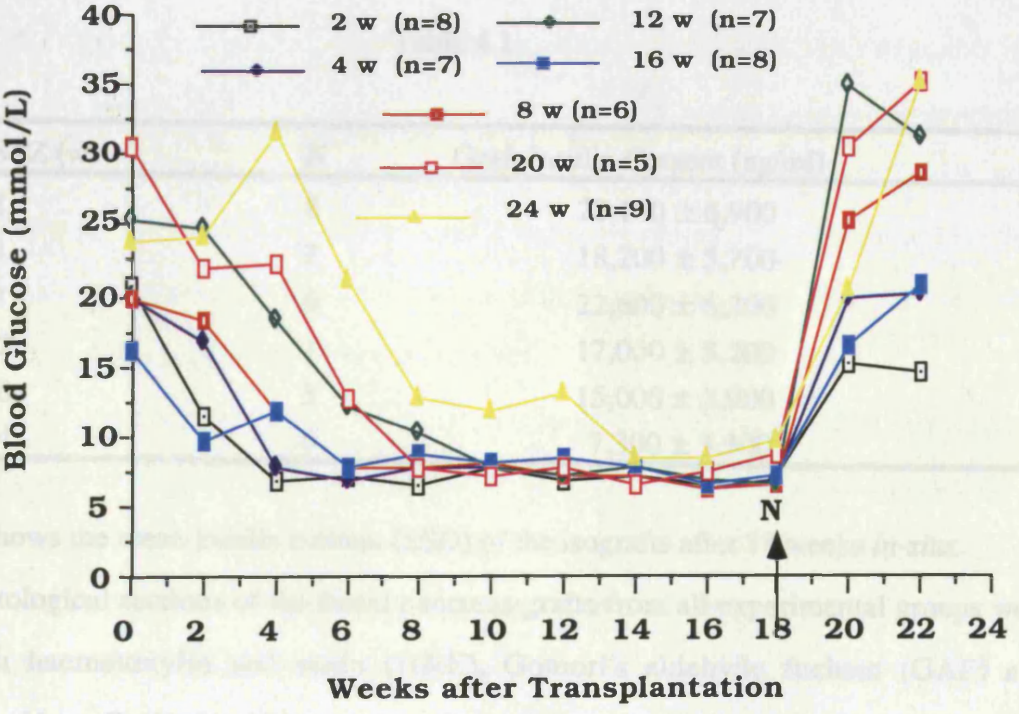


Fig. 4.2 RBG from animals with varying duration of diabetes grafted with BALB/C 17 day fetal pancreas cultured for 14 days cc.

4.3.2 The effect of duration of diabetes before transplantation

Acutely diabetic mice, each grafted with 1/2 of a cultured foetal pancreas, usually became euglycaemic within 4-6 weeks and remained normoglycaemic until the graft was removed. In this experiment grafts were performed after varying periods of diabetes. Reversal of diabetes was rapid in the 2, 4, and 8 weeks diabetic groups, all animals becoming normoglycaemic within 6 weeks. Normoglycaemia was achieved 10 weeks after transplantation in the 12, 16 and 20 week diabetic groups and a high normal RBG levels was established after 14 weeks in the chronically diabetic (24 weeks) group (Fig 4.2).

The grafts which reversed diabetes most rapidly contained up to 3 times more insulin than grafts from the 24 weeks group (Table 4.1). When the insulin content of the grafts was compared between 20 weeks versus the 2, 8, and 16 weeks they were found to be significantly, different $P<0.025$ (student t test). However, when the insulin content of the 20 week grafts was compared to the 4, and 12 weeks groups, there were no significant differences seen. Finally, when the insulin content from the chronically diabetic group (24 weeks) was compared to 2, 4, 8, 12, 16 and 20 weeks the difference was found to be highly significant $P<0.005$ (student t test). The insulin content of the recipient's pancreas showed that in the 2 and 4 weeks groups there was some regeneration of β cells, whereas in the other groups the β cells were completely destroyed.

Table 4.1

Time After STZ (w)	N	Graft Insulin Content (ng/ml)
2	8	22,100 ± 6,900
4	7	18,200 ± 5,700
8	6	22,600 ± 6,200
12	7	17,000 ± 5,200
20	5	15,000 ± 2,900
24	9	7,300 ± 3,300

Table: 4.1 Shows the mean insulin content (±SD) of the isografts after 18 weeks *in-situ.*

Histological sections of the foetal pancreas grafts from all experimental groups were stained with haematoxylin and eosin (H&E), Gomori's aldehyde fuchsin (GAF) and immunoperoxidase (IMP) for the presence of insulin, glucagon or somatostatin-producing

cells. One of the advantages of IMP staining over the GAF stain, is the semi-quantitive analysis of insulin, glucagon and somatostatin content that can be gained by the staining intensity of the cells.

H&E stain from all groups showed well developed and well vascularized islets and the presence of mitotic activity. When the grafts were stained with GAF and IMP (α, β, and δ-cells) the groups with 2, 4, 8, 12, 16 and 20 weeks of pre-transplant diabetes showed well developed islets and the β cells accounted for 60-80% of islet cells (Fig. 4.3 a, b, c, and d). However, the 24 week group showed fewer and poorly stained β cells throughout the islets, correlating well with the macroscopic assessment of the grafts, which appeared to be smaller than in the other groups.

4.4. DISCUSSION

Islet transplantation is highly successful in treating acute diabetes in mice, as shown previously in chapter 3, however few reports deal with chronic experimental diabetes. The present study shows that foetal tissue retains the capacity for proliferation, and correlates functional activity with insulin content, histological appearance and mitotic activity. It also suggests that chronic diabetes in the recipient in the pre-transplant period can impair the function and growth of foetal pancreas isografts. Studies by Cuthbertson *et al.*, (1988) showed that part of that impairment can be overcome by good pre-transplant control of diabetes by implanting an intraperitoneal continuous insulin infusion (CII) pump. However, even with good insulin induced control, graft function was not as good as in the mice with acute uncontrolled diabetes. The authors concluded that at least two factors may affect the function of transplanted organ cultured foetal islets, the duration of the diabetes and the control of hyperglycaemia in the immediate pre-transplant period.

Possibly an important reason for poor graft function in chronic diabetes is impaired vascularization of the tissue. In most organ transplants vascular anastomosis produces immediate perfusion of the transplant organ, and the microvascular bed of the graft is that of the donor. However, in foetal pancreas islet transplantation the capillary bed of the graft slowly develops by ingrowth of vessels from the recipient. In humans with chronic diabetes it is well documented that widespread microangiopathy occurs. In mice with drug induced diabetes of

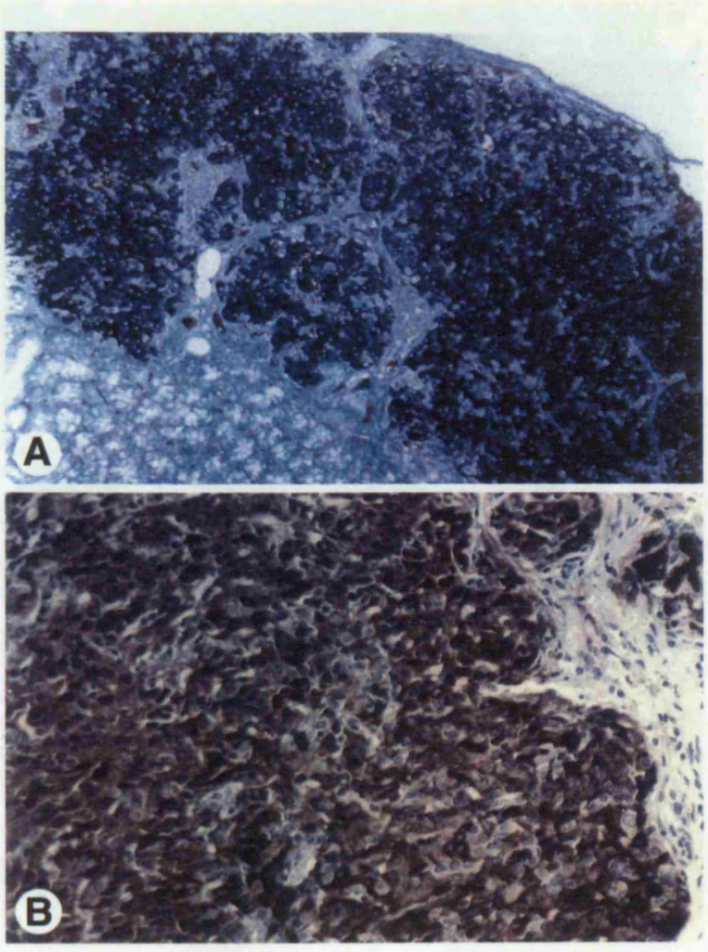


Fig. 4.3: (a) Isografts from BALB/c mice with 8 weeks pre-transplant diabetes after 18 weeks in situ GAF (x100).
(b) Isografts from BALB/c mice with 16 weeks pre-transplant diabetes after 18 weeks in situ IMP for insulin (x250).

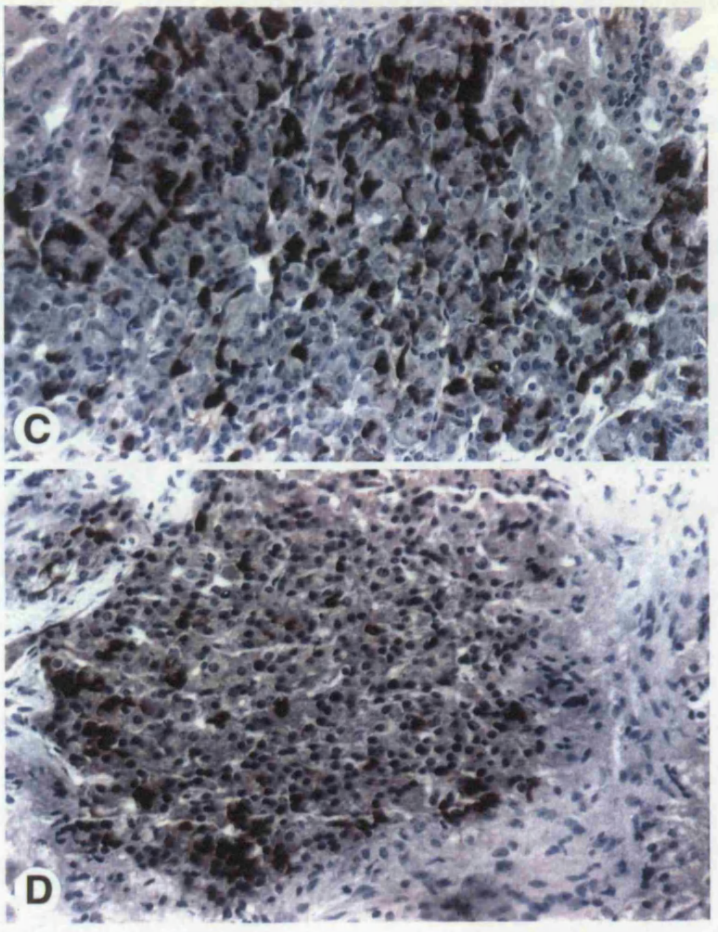


Fig. 4.3: **(c)** Isografts from BALB/c mice with 16 weeks pre-transplant diabetes after 18 weeks in situ IMP for somatostatin (x250). **(d)** Isografts from BALB/c mice with 16 weeks pre-transplant diabetes after 18 weeks in situ IMP for glucagon (x250).

around 6 months duration, microangiopathy is minimal; however basement membrane thickening does occur in the kidney (Cuthbertson and Mandel, 1987). After 12 months of diabetes a significant difference is seen in the retinal and glomerular basement membrane thickness between diabetic and control mice (Gillies and Mandel, 1990). Although this study has not directly shown that impaired angiogenesis occurs, impairment of vascularization could interfere with engraftment and may result in diminished graft size and function.

Finally, this study could have important implications in human islet transplantation, where microvascular angiopathy is a common diabetic complication, and islet grafts, particularly foetal, may require rapid vascularization in order to mature and function optimally. Thus, the duration of diabetes is an important non-immunologic determinant of graft function that needs to be considered.

CHAPTER 5 ORGAN CULTURED FOETAL ISO-, ALLO-, AND XENOGAFTS (PIG) IN NON-IMMUNOSUPPRESSED NON-OBESE DIABETIC (NOD) MICE

5.1 INTRODUCTION

The response of non-immunosuppressed NOD mice to an isograft (H-2^{g7}), MHC-mismatched allograft (CBA, H-2^{k}; BALB/c, H-2^{d}), and xenograft (pig) of foetal pancreas was assessed by light microscopy. In this chapter I describe the natural history of islet transplants in NOD/Lt mice, a strain that develops spontaneous autoimmune diabetes. There are striking differences in the response that a host mounts against 3 types of free transplants of organ cultured foetal pancreas; an autoimmune recurrence of disease in isografts that is T cell mediated and β cell specific, a strong alloresponse that is predominantly mononuclear, and a qualitatively quite different response against the xenografts that is dominated by eosinophils and macrophages and may require different forms of immunosuppression for its control. However, HAR is not a problem in this model of xenotransplantation and when immunosuppression is used that does not prevent infiltration of the isograft site or prevent rejection of an allograft, the xenograft is still temporarily protected (Bernard *et al,* 1992). Also, the ability of the foetal pig xenografts to grow and differentiate into adult islets was studied *in vivo* in CBAnu/nu mice.

5.3 MATERIAL AND METHODS

Recipients were adult male and female NOD mice, either diabetic or pre-diabetic, of two inbred sublines; a low diabetes incidence NOD/Wehi line and a high diabetes incidence NOD/Lt line (Baxter and Mandel, 1991). Mice were observed for cage wetting and weight loss and when this was noted random blood glucose (RBG) measurements were made using a Beckman Astra 4 multichannel analyser. Mice with a RBG >15mmol/L were treated with daily insulin injections (Ultralente, 2-4U/day) before and after transplantation. Donor tissues for iso- and allografts were from 16-18 day foetal mouse pancreas from age dated pregnancies and for xenografts from foetal pigs of various gestational ages, generally between 60-100 days.

Tissue for transplantation was removed aseptically from the foetal donors and placed in organ culture, as previously described in chapter 2. The foetal mouse pancreas was kept in

10%CO_2/90% air for 7-14 days and the foetal pig pancreas cultured for 2-3 days in 90%O_2/10%CO_2 and further 2-5 days in 10%CO_2/90% air. Transplantation was of single pieces of tissue placed under the renal capsule; in some instances grafts were placed under the capsule of both kidneys and in some experiments each mouse was grafted with isogeneic, allogeneic and xenogeneic tissue. The methods used have been described in chapter 2. Samples of tissue that was used for transplantion was also taken for histologic examinantion to confirm that it was viable.

In a separate experiment athymic nude CBA female mice were transplanted with foetal pig pancreas so that the development of the tissue in a recipient unable to reject the graft could also be assessed. Five animals were killed at each time point for histological assesment on days 7, 14, 21, 28, 35 and 90 post-transplantation.

Grafts were removed from non-immunosuppressed mice at intervals from day 1 post-transplantation by removing the entire kidney which was either fixed in Bouin's fluid or frozen in isopentane in liquid N_2 for phenotyping of the infiltrating cells. The Bouin's fixed kidneys were placed in 70% ethanol for 24 hours and the graft sites identified and cut out for routine processing for light microscopic examination. The specimens were coded and paraffin-blocked specimens were serially sectioned at 4 μm. Alternate slides were stained with H&E for a general examination of the graft site, with GAF to detect β cells, mast cells and connective tissue elements (elastin and collagen), and unstained slides were processed for anti-hormone immunocytochemistry to detect insulin, glucagon and somatostatin.
The graft sites were scored for graft survival and for the type of infiltrate present, as described in chapter 2.

5.3 RESULTS

5.3.1 Pre-transplant morphology and development in nude mice.

Examination of the foetal mouse pancreas after 7-14 days *in vitro* showed that there was excellent survival of islets and ducts but loss of exocrine tissue (Fig 5.1a). The foetal pig pancreas after a few days *in vitro* was similar except that there was less obvious development of islets and the tissue consisted of ducts, small clusters of islet cells and no viable acinar tissue (Fig. 5.1b). To confirm that this tissue could develop and differentiate into islets after

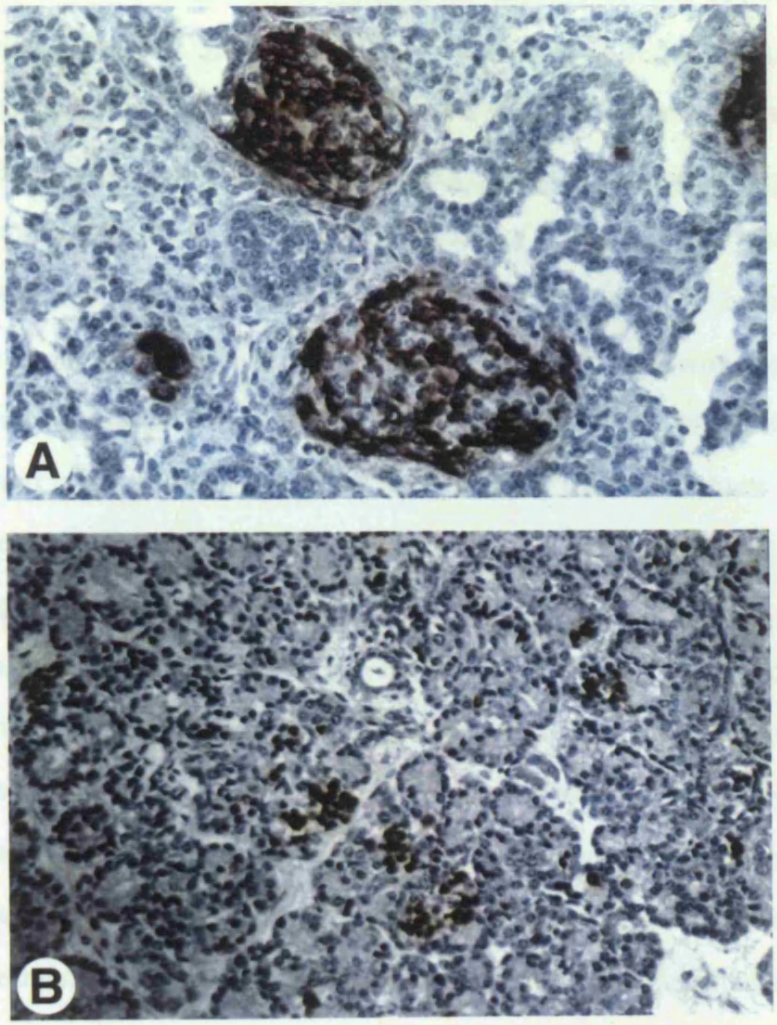


Fig. 5.1: (a) Foetal mouse pancreas (17 days gestation) 14 days in 10%CO_2/90% air, showing well developed islets and ducts (H&E, 250x).
(b) Foetal pig pancreas (70 days gestation) 2 days in 90%O_2/10%CO_2 followed by 3 days in 10%CO_2/90% air, the tissue consists of ducts and small clusters of islet cells (IMP for insulin, x250).

transplantation, nude mice that cannot reject allo- or xenogeneic grafts were examined. These confirmed that the grafts were viable and that post-transplant differentiation could occur. The xenografts *in vivo* were histologically examined on days 7, 14, 21, 28, 35 and 90 post-transplantation. On day 7 small areas of islet like clusters appeared with large numbers of immature endocrine cells and many ducts by day 14 the β cell mass increased with many more islets forming clusters (Fig 5.2 a). On day 35 the foetal pig pancreas had developed into a well defined adult-like appearance with islets resembling those of the mature pancreas and the β cells were well differentiated staining positive with GAF (Fig 5.2 b). Three months post-transplantation the xenografts were well developed and differentiated with more than 90% of the graft being mature β cells (Fig 5.2 c). Hence, *in vivo* the foetal pig pancreas has the ability to grow and differentiate and form islets from the immature tissue and ducts to resemble the adult endocrine pancreas.

5.3.2 Isografts in pre-diabetic recipients

One day after transplantation the isograft sites were oedematous but there was only a minimal cellular response comprising mostly a few neutrophils and macrophage-like cells. The transplanted tissue was obvious and resembled closely the pre-transplant appearance of the organ cultured foetal pancreas that was used for that transplant. The graft sites began to be invaded by mononuclear cells (MNC) by about the 3rd day. The infiltrating cells were generally small with the appearance of typical lymphocytes. The infiltrate was often sparse or even absent and in pre-diabetic recipients was always peri-islet and spared the islets. When the recipients were sacrificed and the severity of the insulitis in the pancreas scored there was a good correlation between the intensity of the insulitis around the islets and the severity of the infiltrate in the graft site after 14 days post-transplantation (Fig 5.3 a and b). Well stained β cells were present in the graft within days of transplantation. Immunocytochemistry showed that all major lymphocyte subsets were present but B cells (sIg or B220+ve) were relatively sparse while typical T cells (CD3/Thy1+ve) and the two major T cell subsets (CD4+ve and CD8+ve) were common with usually more CD4+ve than CD8+ve cells (Fig. 5.4 a, b and c). Macrophages (F4/80) were common and were scattered throughout the graft site. Granulocytes were usually absent or at most infrequent. Hormone immunocytochemistry showed that the three major endocrine cell types (α, β and δ) were well preserved.

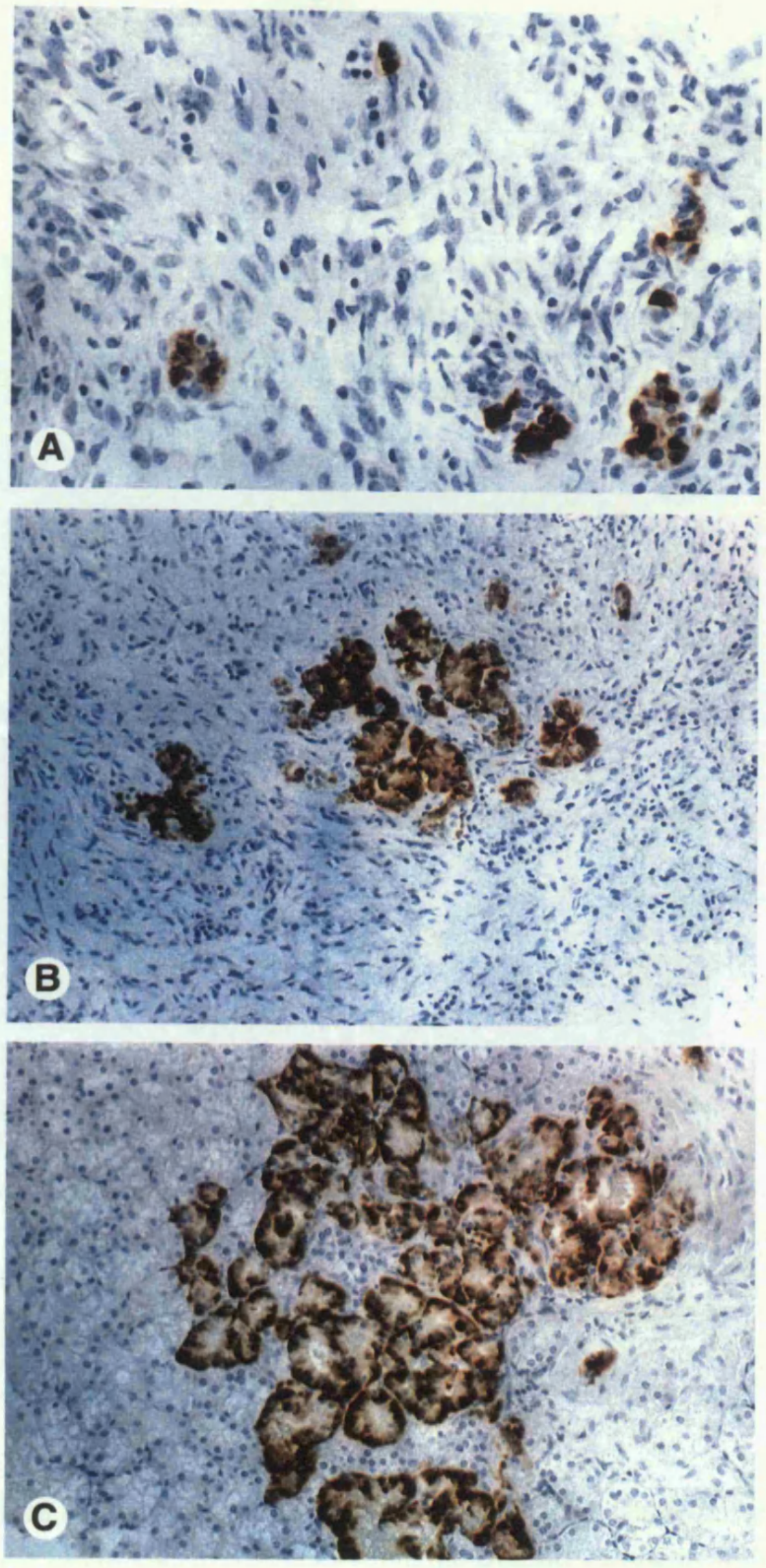


Fig. 5.2: Xenografts in nude mice at days 14, 35 and 90 post-transplantation.
(a) Xenograft at day 14 post-transplantation showing small clusters of islets stained with IMP for insulin (x250).
(b) Xenograft at day 35 post-transplantation showing well developed islets stained with IMP for insulin (x250).
(c) Xenograft at day 90 post-transplantation showing well developed islets with 90% of the graft been β cells stained with IMP for insulin (x250).

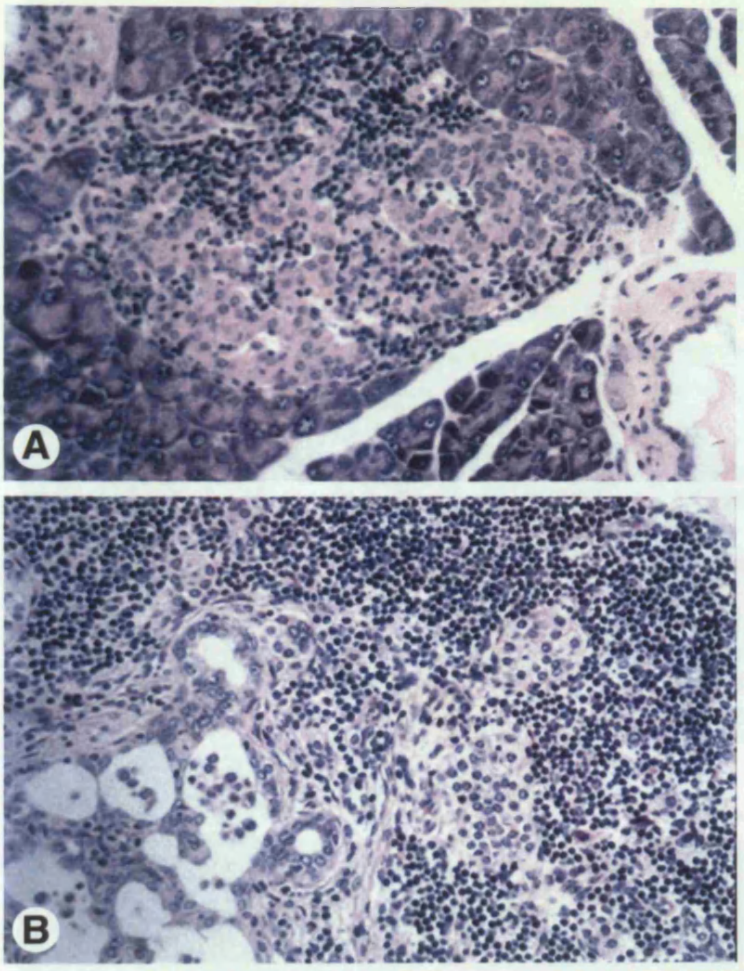


Fig. 5.3: Pancreas and isograft from NOD/Lt female mouse. The severity of insulitis (recurrence of the disease) in the isograft correlates with the severity of insulitis in the host pancreas.
(a) Host pancreas with marked insulitis (H&E, 250x).
(b) Isograft showing similar severity of insulitis to the host pancreas 14 days post-transplantation (H&E, x250).

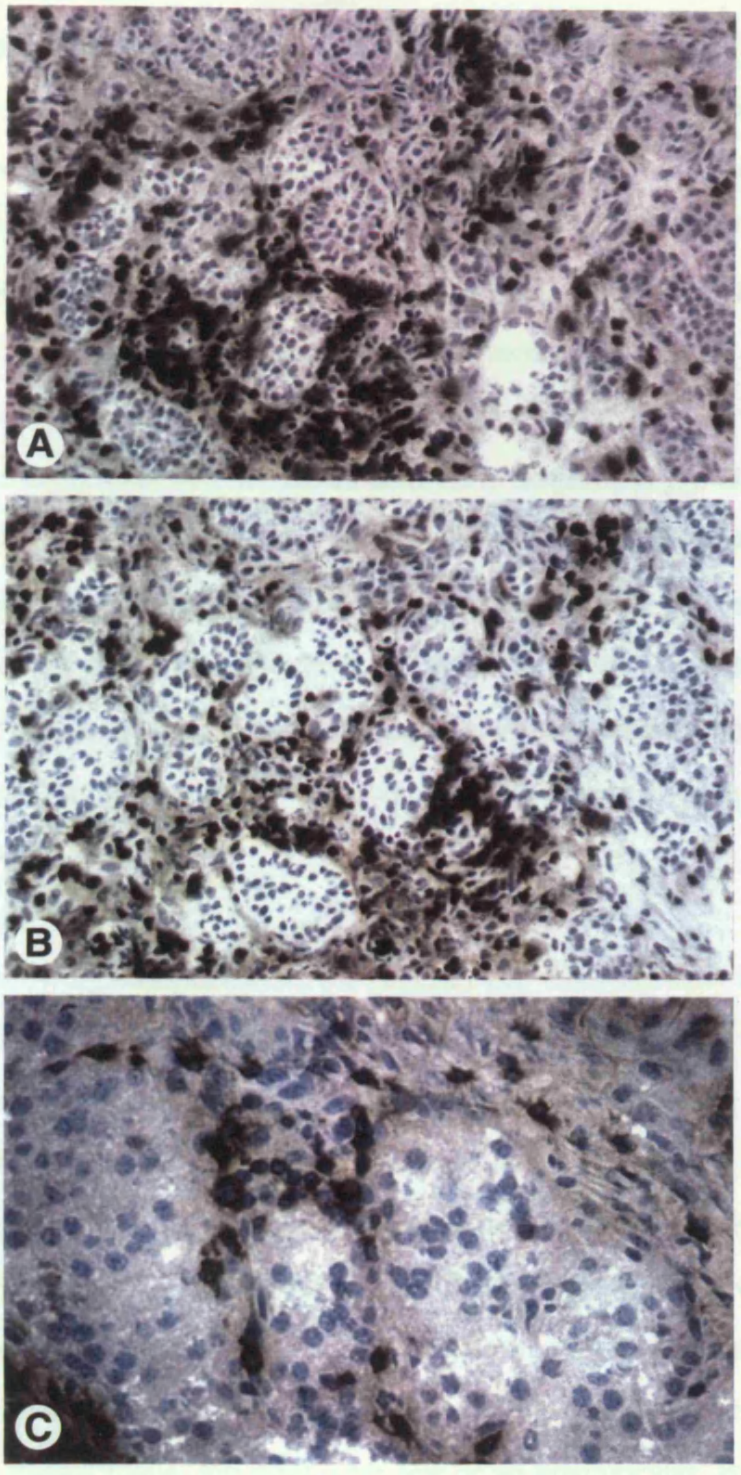


Fig. 5.4: Isograft from pre-diabetic NOD/Lt female mice 14 days post-transplantation. Frozen section stained for T cells by Thy 1.2 and T cell subsets for CD4 and CD8 positive cells.
(a) Isograft showing positive cells for Thy1.2, (x250).
(b) Isograft showing the presence of CD4+ T cells, (x250).
(c) Isograft showing the presence of CD8+ T cells, (x250).

5.3.3 Isografts in diabetic recipients

In contrast to the appearance of isografts in pre-diabetic recipients, the graft sites in non-immunosuppressed diabetic mice were rapidly invaded by MNC but in this instance the invading cells entered the islets and quickly destroyed most of the β cells. By 14 days post-transplantation there was generally complete destruction of β cells and immunocytochemistry showed that whereas β cells were absent α and δ cells remained. This was also quite apparent in GAF stained specimens where obvious islets and well developed ducts were present but no GAF positive cells remained. This was in marked contrast to isografts in pre-diabetic animals that had well stained β cells despite often marked peri-islet infiltration. The appearance of the grafts (Fig. 5.5 a,b and c) resembled that of the islets in the recipient's pancreas which also showed a selective loss of β cells (Fig 5. 5 d,e and f). That β cell damage or degranulation was not due to diabetes *per se* was established from experiments where spontaneously diabetic mice were immunosuppressed and even in the presence of quite severe hyperglycemia some granulated β cells remained in the grafts.

5.3.4 Allografts

On the day following transplantation, the allograft site was essentially identical to the appearance of an isograft. The response against MHC-mismatched allogeneic islets occurred rapidly and a slight but obvious cellular response was present in the graft site by 2 days. At that time there was a sparse infiltrate of mainly small MNCs that were frequently still within the lumen of small vessels. The severity of infiltration was greater by the 3rd day and by 4-5 days there was a large infiltrate consisting almost solely of MNC some of which were large and blast-like and others had the appearance of macrophages. Immunocytochemistry showed that CD4+ve, CD8+ve and F4/80+ve cells were all present in large numbers and these cells were starting to invade the grafted tissue, but there were few or no granulocytes. By 5 and 7 days there was some damage to the graft but some islets and ducts were generally still well preserved despite the presence of a marked mononuclear cell infiltrate (Fig. 5.6 a). By 9-12 days there was little or no evidence of viable grafted islets but there was a heavy infiltrate of predominantly mononuclear cells many of which were large and blast-like (Fig. 5.6 b). Hormone-positive cells were only sometimes still detectable by immunocytochemistry, but ducts were still

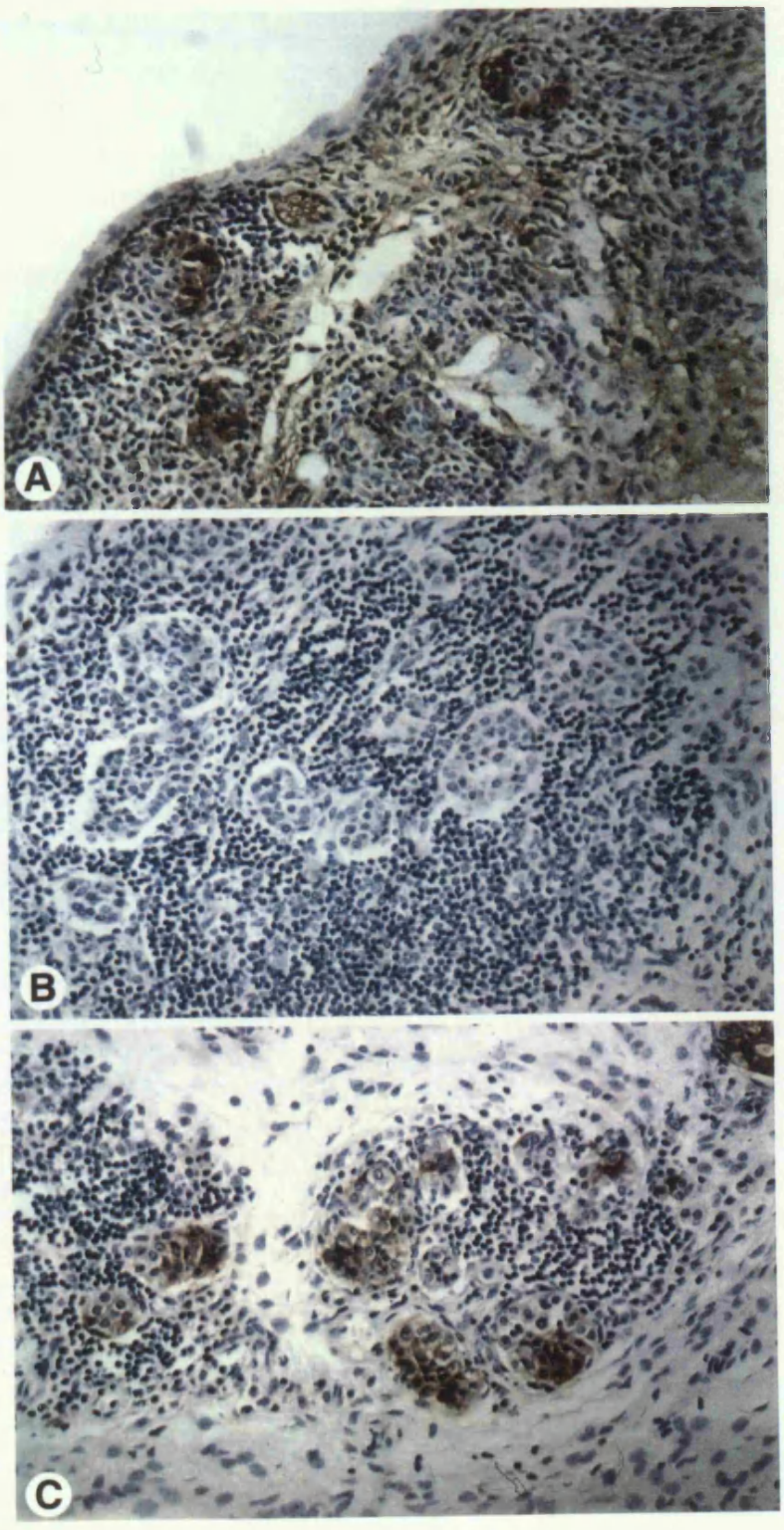


Fig. 5.5: Isografts of foetal mouse pancreas transplanted into diabetic NOD/Lt mice 14 days post-transplantation showing peri-and intra-islet infiltration.

(a) IMP staining showing positive α cells (for glucagon x250).

(b) IMP staining showing no β cells (for insulin, x250).

(c) IMP staining showing positive δ cells (for somatostatin x250).

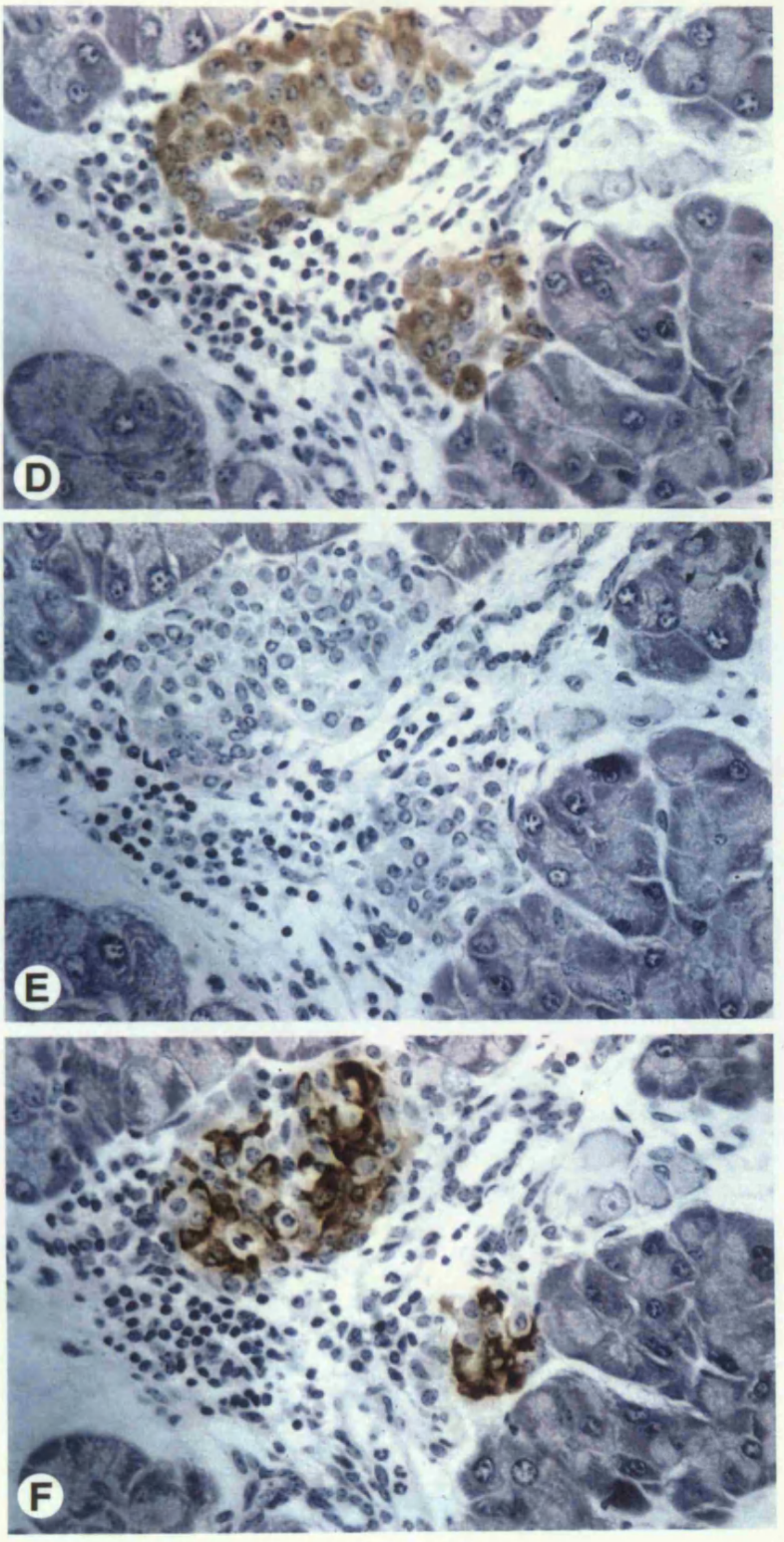


Fig. 5.5: Adult pancreas from a diabetic NOD/Lt mouse showing an atrophic islet with a selective loss of β cells.

(d) IMP staining showing positive α cells (for glucagon x250).

(e) IMP staining showing no β cells (for insulin, x250).

(f) IMP staining showing positive δ cells (for somatostatin x250).

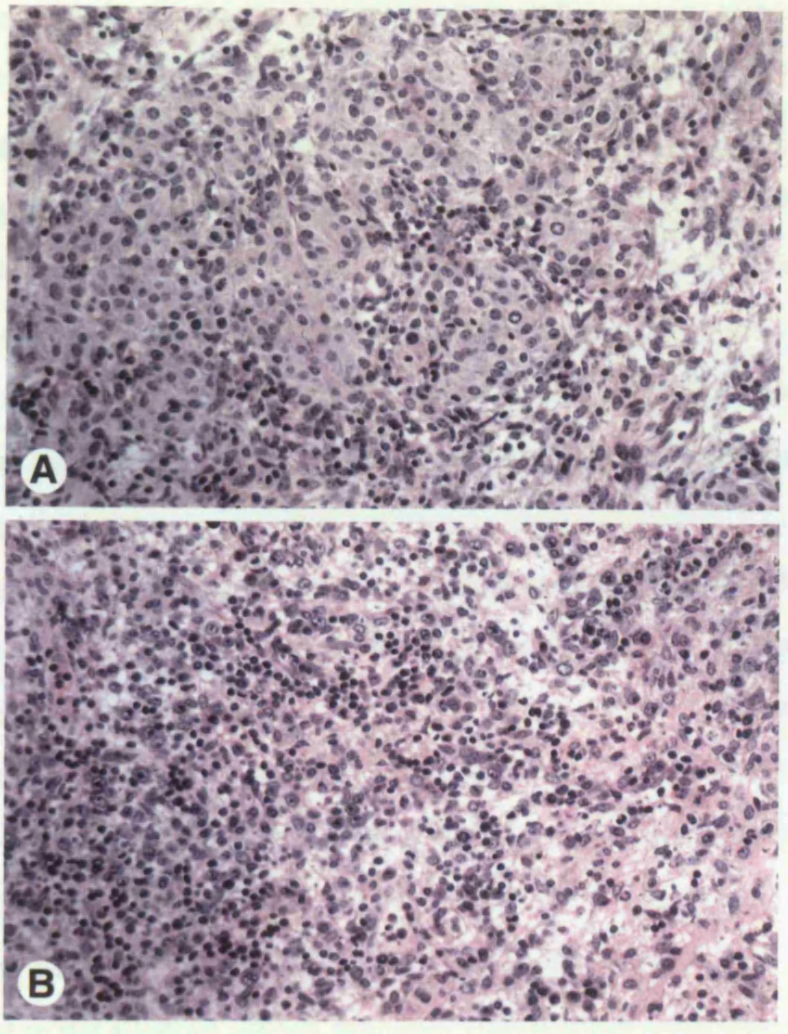


Fig. 5.6: Allografts of foetal mouse pancreas transplanted into NOD/Lt mice.
(a) 5 day post-transplantation the islets and ducts preserved with some MNC infiltrate (H&E, x250).
(b) 9 day post-transplantation some viable grafted islets still present. With heavy infiltrate predominantly mononuclear cells (H&E, x250)

sometimes seen embedded in a large infiltrate. By 14-15 days there was no more evidence of a graft but a moderately heavy MNC infiltrate was still evident (Fig. 5.6 c). After this time the infiltrate started to diminish and collagen in the graft site increased so that the site was converted to a mildly cellular scar. By 21 day the resolution of the inflammatory response was well advanced and by 28 days there was only a thin relatively acellular scar left (Fig. 5.6 d).

5.3.4 Xenografts

The xenograft site resembled the iso- and allograft sites on day 1 with oedema and only a minimal cellular reaction but with quite large amounts of graft tissue. The site began to become infiltrated only after day 3 when a few MNCs were seen usually within the lumina of small vessels. By the 4-5th day there was a variable but often slight peri-graft infiltrate of mainly mononuclear cells but by day 7 the infiltrate was often large and was clearly destroying the graft but was quite variable in severity in different specimens (Fig. 5.7 a). However, in contrast to the appearance of the allograft site at this time, the xenograft infiltrate was dominated by granulocytes, mainly eosinophils with few neutrophils (Fig. 5.7 b). Some mast cells were also present, particularly around the edge of the site. MNCs including macrophages and some multinucleate giant cells were also present. The phenotype of the cells showed that T cells, both CD4+ve and CD8+ve were present as well as many F4/80+ve macrophages and many plasma cells. The presence of ductal and islet cells was still evident at 7-9 days in most specimens and even by 11 days some islet cells and ducts were sometimes present (Fig. 5.7 c).

In contrast to the allograft site the xenograft site remained large and infiltrated for a prolonged period and was readily recognisable macroscopically even 6-8 weeks after transplantation as a large dense white swollen nodule in contrast to the thin and usually inconspicuous scar that characterised the allograft site. When examined microscopically, the xenograft site usually showed a large central acellular necrotic zone where there were often ghosts of cells present surrounded by a dense thick infiltrate of mixed MNC and granulocytes (Fig 5.7 d). In addition, there was a good deal of fibrosis with large amounts of collagen and elastin detected by the GAF stain. The infiltrate persisted for weeks after all trace of a graft had disappeared and the infiltrating cells included may eosinophils and some conspicuous multinucleate giant cells. Thus, in contrast to the allograft where rapid resolution occurred, in

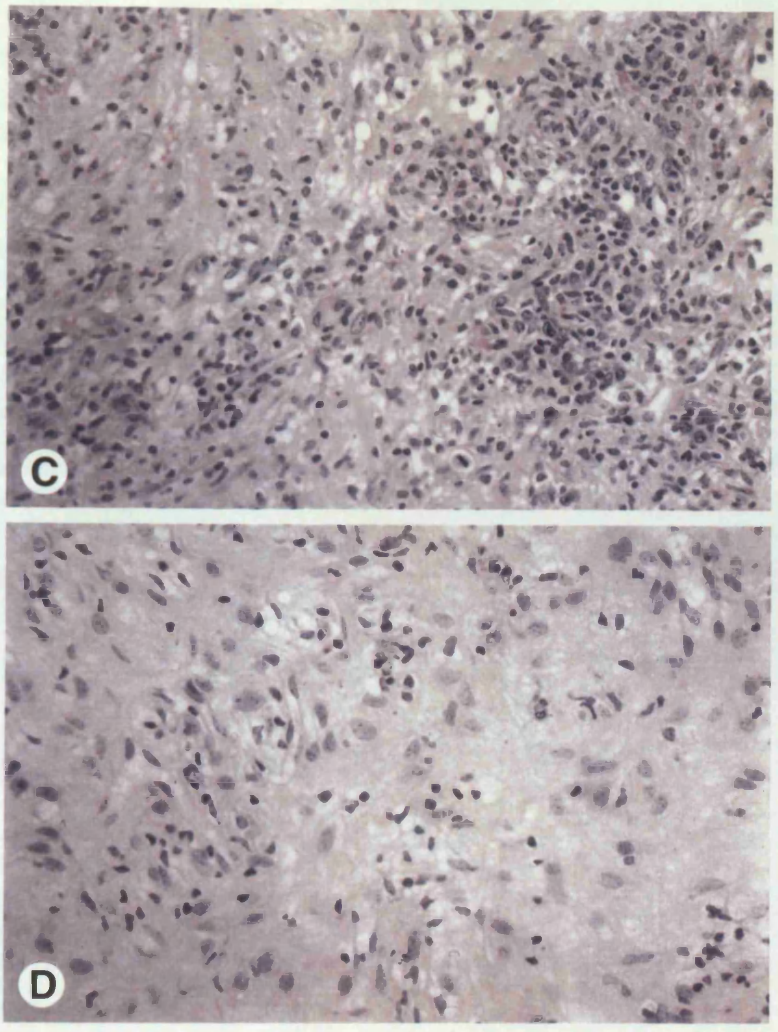


Fig. 5.6: Allografts of foetal mouse pancreas transplanted into NOD/Lt mice. **(c)** 14 day post-transplantation there is no evidence of graft only heavy MNC infiltrate is present (H&E, x250). **(d)** 28 day post-transplantation, the allograft site showing a thin acellular scar (H&E, x250).

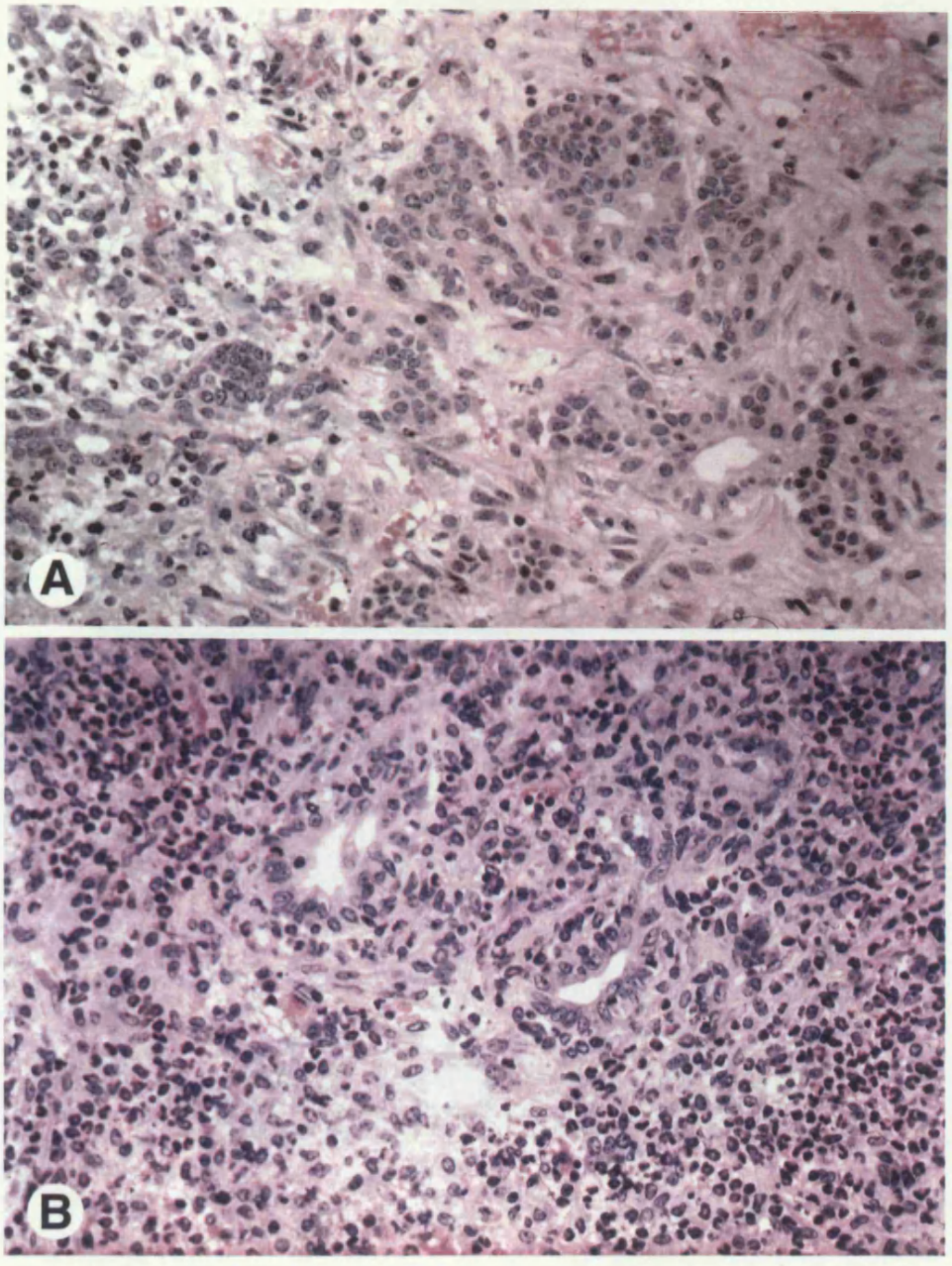


Fig. 5.7: Xenografts of foetal pig pancreas transplanted into NOD/Lt mice.
(a) 5 day post-transplantation the islets are well preserved with some peri-islet infiltrate (H&E, x250).
(b) 7 day post-transplantation some islets and ducts are still present. The infiltrate is dominated by granulocytes mainly eosinophils (H&E, x250).

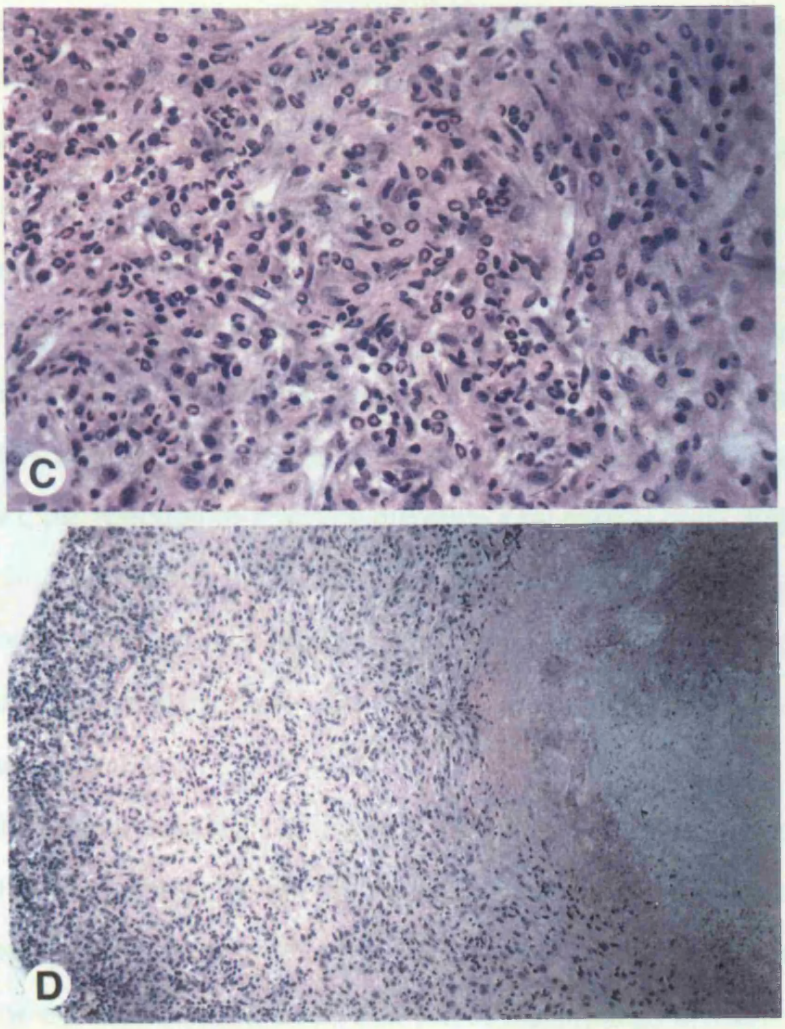


Fig. 5.7: Xenografts of foetal pig pancreas transplanted into NOD/Lt mice.
(c) 11 day post-transplantation the islets destroyed and only granulocytes are present, mainly eosinophils and neutrophils (H&E, x250).
(d) 28 day post-transplantation, the xenograft site showing a large central necrotic zone, surrounded by a dense infiltrate of mixed MNCs and granulocytes (H&E, x100).

the xenograft site there was a persistent infiltrate that differed both quantitatively and qualitatively from the former and a persistent granuloma remained.

5.4 DISCUSSION

There are obvious differences between the appearance of the inflammatory reactions in the 3 transplant situations described above. Recurrent autoimmune disease is seen in isografts in diabetic and pre-diabetic recipients and its severity mimics the severity of insulitis in the recipient's pancreas. Thus, in non-immunosuppressed diabetic mice there is rapid β cell destruction whereas in pre-diabetic mice there is peri-islet infiltration without intra-islet invasion and with good survival of β cells. Rejection of MHC-mismatched allografts occurs rapidly with a predominantly MNC infiltrate of many activated cells but apparently viable graft tissue is still present for about 8-10 days. Once graft rejection has occurred there is rapid resolution of the infiltrate. In contrast, the rejection of a widely disparate xenograft is quite different from the allograft response in that it is somewhat slower, there is a predominance of eosinophils, a relative paucity of lymphoid cells and a persistance of the reaction for periods far longer than in allografts. Thus, in all 3 instances there was a marked cellular response but this differed qualitatively and quantitatively.

In recurrent autoimmunity the infiltrate was entirely mononuclear and included both T cells and macrophages that resulted in specific β cell destruction if the recipient was already diabetic but did not effect pre-diabetic hosts. The mechanism of β cell damage is still controversial but the consensus seems to be that initial macrophage invasion results in the recognition and processing of autoantigenic epitopes, presentation of these to CD4+ve T cells with subsequent activation of CD8+ve effector cells (Kay *et al,* 1991; Thivolet *et al,* 1991).

In pre-diabetic NOD mice the peri-graft invasion by MNCs resembled closely that present in the recipient's own pancreas suggesting that there was a recapitulation of the disease process in the graft. In diabetic recipients specific β cell damage occurs rapidly but the other endocrine cells are spared. Thus, in an isograft there is selective damage to β cells only and its onset is rapid when the diabetic recipient is not immunosuppressed. Similar graft pathology has been reported in humans with pancreas grafts from an identical twin (non-diabetic) donor and also in immunosuppressed recipients of an HLA-matched sibling pancreas graft (Sibley *et al,*

1985; Sibley and Sutherland 1987; Sutherland *et al,* 1989) and the major infiltrating cells appear to be CD8+ve T cells.

In an MHC-mismatched allograft there was again a rapid invasion of the graft site by MNCs but in contrast to isografts, in the allograft all of the grafted cells were destroyed including α and δ cells as well as β cells and finally also the ducts that probably contain the precursors of the differentiated endocrine cells (Bonner-Weir *et al,* 1993). It is not clear why the ducts are spared for longer than the differentiated cells. However, the inflammatory infiltrate rapidly cleared when the transplanted tissue had been destroyed and there was a rapid resolution of the inflammation with the formation of a thin relatively acellular scar.

The striking feature of the xenograft response was the presence of large numbers of eosinophils that usually dominated the infiltrate. Eosinophils are not usually a major feature of allograft rejection but have been described in a variety of settings particularly when severe rejection occurs (Weir *et al,* 1986; Foster *et al,* 1989; and Wang *et al,* 1994). However, in this study the xenogeneic tissue survived for at least as long as the allografts and indeed the appearance of a response to the xenograft was even slightly delayed. Thus, initial severity of rejection *per se* seems not to be the sole reason for the presence of these cells.

Other studies with this and similar models have shown that treatment of the host with anti-T cell MAbs against the CD3 and/or CD4 epitopes is relatively effective in preventing acute xenograft destruction (Simeonovic *et al,* 1990b) and when rejection does occur it corresponds to the appearance of MNCs and eosinophils. The dependence of eosinophils on a prior CD4+ T cell mediated response is well documented in other models (Silberstein and David, 1987) and IL-5 (Hom and Estridge, 1994) as well as other cytokines (Weller, 1992; Iwamoto *et al,* 1993; Sanz *et al,* 1995) are also implicated in eosinophil recruitment.

CHAPTER 6 THE EFFECT OF PERI-TRANSPLANT ANTI-CD4 MONOCLONAL ANTIBODY AND GRAFT IMMUNOMODIFICATION ON FOETAL PIG XENOGRAFT SURVIVAL: LACK OF GAL(α1-3)GAL EXPRESSION ON ENDOCRINE CELLS.

6.1 INTRODUCTION

Hyperacute rejection (HAR) is the primary, and presently essentially untreatable, barrier to discordant-donor xenotransplantation of solid organs (Paul, 1991). It is believed to be due to the activation of donor endothelial cells by the interaction of cross-reactive natural antibodies (Nabs), predominantly IgM, with Gal(α1-3)Gal determinants on the surface of host microvascular endothelial cells (Bach *et al,* 1994; Sandrin *et al,* 1993) resulting in thrombosis and graft infarction (Platt and Bach, 1991). It has been shown in humans, higher apes and Old World monkeys that the major target of xeno natura antibodies (XNA) is the carbohydrate epitope, Gal(α1-3)Gal since these species lack the appropriate galactosyltransferase and are not tolerant to this epitope (Cooper *et al,* 1994; Galili *et al,* 1988).

McKenzie and colleagues (1994 and 1995) have shown that endocrine cells in the islets from the pig pancreas are Gal(α1-3)Gal negative in adult, and foetal pancreas before and after organ culture and after transplantation into athymic (nude) mice, where the foetal tissue can undergo further differentiation after transplantation but without generating an immune response that may upregulate antigen expression.

In this chapter, the fate of grafts of organ cultured foetal pig pancreas in Non-Obese Diabetic (NOD) mice that have had a brief peri-transplant treatment with the depleting anti-CD4 monoclonal antibody (MAb), GK1.5 was studied. Whether graft survival was affected by alteration ("immunomodification") by culture in 90%O_2 either with or without p/t immunosuppression with anti-CD4 MAb was also tested. The lack of expression of Gal(α1-3)Gal on all differentiated (ie hormone-containing) endocrine cells and also the ductal cells in the graft was demonstrated by immunoperoxidase histochemistry.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Xenograft recipients were pre-diabetic NOD/Lt females aged 60-80 days. At this age NOD/Lt mice are euglycaemic but have histopathologic changes in their pancreas with moderately severe insulitis. Normally they start to develop spontaneous overt diabetes after 100 days of age (Baxter and Mandel, 1991). Pre-diabetic mice were used because they are already "primed" to develop overt diabetes but are much healthier and easier to maintain in good health than overtly diabetic animals. Foetal pancreas was obtained at a gestational age of about 100 days from a pregnant sow that had been killed specifically to obtain the foetal tissue. The foetuses were removed within 5 minutes of death and immediately placed in plastic bags into an ice/water slurry for transport to the laboratory where all further procedures were performed. The pancreases were isolated under sterile conditions, cut into approximately $1mm^3$ pieces and placed in organ culture (chapter 2).

Two sets of culture conditions were used: "conventional culture" (CC) in which the tissue pieces were maintained in $10\%CO_2/90\%$ air for 6 days, or "immunomodifying" conditions where they were maintained for 2 days in a gas phase of $90\%O_2/10\%CO_2$ followed by 4 days in CC. Previous studies have shown that 2 days in $90\%O_2/10\%CO_2$ eliminates non-endocrine, non-ductal cells from the cultures (Kovarik *et al,* 1995). The organ cultured pieces were then transplanted bilaterally under the renal capsule of the NOD recipients (Mandel, 1994).

Four treatment groups, each of 25 animals, were studied: Group 1, mice received 0.3mg GK1.5 MAb; group 2, control mice received 0.3ml MTPBS intra-peritoneally on days -1, 0 and +1 (ie immunosuppressed versus non-suppressed); group 3, mice with grafts that had been cultured under conventional; and group 4, mice with grafts that had been cultured in high O_2 ("immunomodifying") conditions. Five mice from each of the four treatment groups were sampled at 2, 4, 7, 14 and 28 days after transplantation. The recipient pancreas was also removed on days 14 and 28 and scored for the severity of insulitis to determine whether the peri-transplant treatment with GK1.5 had any effect on its islets.

When the kidney bearing the graft was removed it was immediately placed in Bouin's fixative and the graft site was excised and embedded in paraffin. The tissue blocks were coded,

serial sections cut at 4-5 μm thickness and alternate slides stained with haematoxylin and eosin for a general assessment of graft site histology and by the Gomori's aldehyde fuchsin (GAF) method in an attempt to demonstrate differentiated β cells. Every third slide was left unstained so that immunohistology could be performed for the three major hormones (insulin, glucagon and somatostatin) and cytochemistry for the presence of Gal(α1-3)Gal using the biotinylated lectin IB4, as previously described in chapter 2.

Coded histologic sections were examined for graft survival and intensity of infiltration. Grafts were scored on a semi-quantitative basis for the presence of a graft on a 0-4+ grading as previously described in chapter 2.

6.3 RESULTS

6.3.1 Morphology of the organ cultured foetal pancreas

The late gestation foetal pig pancreas contained predominantly moderately well differentiated acinar tissue embedded in sheets of loose connective tissue. Islet cells were scattered throughout the pancreas and were frequent as determined by immunocytochemistry for the three major hormones (ie insulin, glucagon and somatostatin) but distinct and well developed islets clearly demarcated from the mass of acinar tissue, as seen in a mature pancreas, were uncommon. Rather, the endocrine cells appeared to be widely and loosely scattered throughout the tissue sometimes forming poorly defined islet-like masses. Gomori's aldehyde fuchsin staining was generally negative or at best weakly positive in the foetal pancreas (in contrast to strong staining in more mature tissue) but the immunoperoxidase method using anti-hormone antibodies stained α, β and δ cells strongly. In addition, there were obvious ducts associated with blood vessels and nerves and the lobules were separated by sheets of connective tissue.

After 6 days *in vitro* there was a striking change in the appearance of the tissue with a total loss of acinar cells but with good retention of ducts and well developed endocrine cells were detected by hormone immunocytochemistry but not by the GAF method. The major differences between the CC and HiO_2 cultured tissue were that there were many interstitial pyknotic cells throughout the fragments in the HiO_2 pieces but ductal and endocrine cells were

also well preserved, presumably reflecting the selective effect of O_2 toxicity, and there was no central ischaemic necrosis that is a feature of CC tissue (Fig. 6.1 a,b, c and d).

6.3.2 Rejection of grafts in non-immunosuppressed recipients

The semi-quantitative assessment of the graft sites is shown in Table 6.1. Clearly, graft destruction was occurring by day 4, was advanced by day 7, and essentially complete by day 14 in untreated recipients. There was a persistence of the infiltrate at the graft site at 28 days, well after all evidence of viable transplanted tissue had gone.

Grafts were first examined at 2 days when obvious graft tissue was present with prominent islets and ducts but without any evidence of an infiltrate. There were some free red cells as well a few large macrophage-like cells, presumably due to a response to operative trauma, but the graft appeared to be healthy with many cells in mitosis (Fig. 6.2 a). There was little or no evidence of vascularization at this stage. GAF staining was negative (Fig. 6.2 b) but immunoperoxidase staining showed that the three major endocrine cell types were present. No differences were seen between grafts that had been in CC throughout or in 90%O_2/10% air for two days.

By four days post-transplantation there was a slight mononuclear cell infiltration into the graft site with a few mostly small mononuclear cells (MNC) present that were still often within blood vessels. Vessels were clearly evident and were often dilated and sinusoidal with a margination of leucocytes. Islet cells and ducts were again obvious but were not being invaded (Fig. 6.2 c). There was no obvious difference between the two types of cultures used.

On day 7 there was a heavy infiltrate in the graft site which was large and swollen due to oedema and infiltrating cells that included many granulocytes, predominantly eosinophils, as well as a few neutrophils and mast cells and many large macrophage-like mononuclear cells (Fig. 6.3 a). Some multinucleate giant cells were also present. Lymphocyte-like mononuclear cells were also evident and previous experiments have shown that these are predominantly CD4+ve. The infiltrating cells now also invaded the islet cell clusters and there was obvious damage to the integrity of the transplanted tissue. However, despite the infiltration of the graft, endocrine and duct cells were still well preserved (Fig 6.3 b and c), particularly the ducts which are the last elements of the graft to be damaged and well defined ducts were usually still present even when most differentiated islet cells had been destroyed.

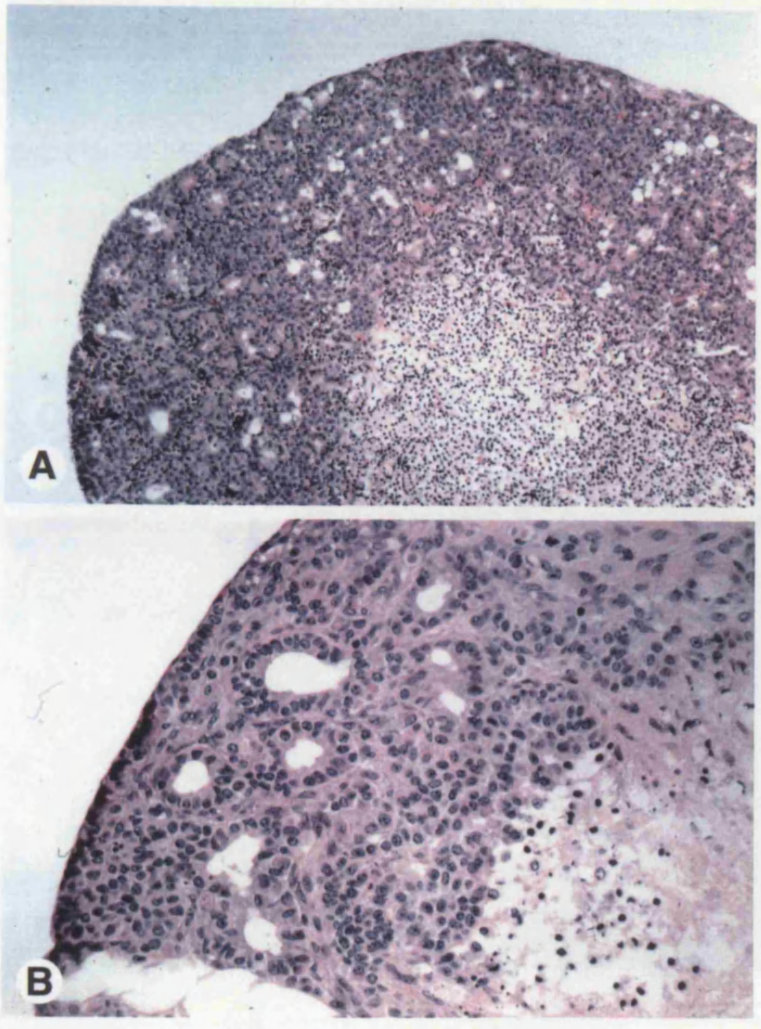


Fig. 6.1: (a) Foetal pig pancreas 2 days in CC showing central necrosis with viable cells around the edge H&E (x100).
(b) Foetal pig pancreas 2 days in CC showing viable endocrine tissue and ducts H&E (x250).

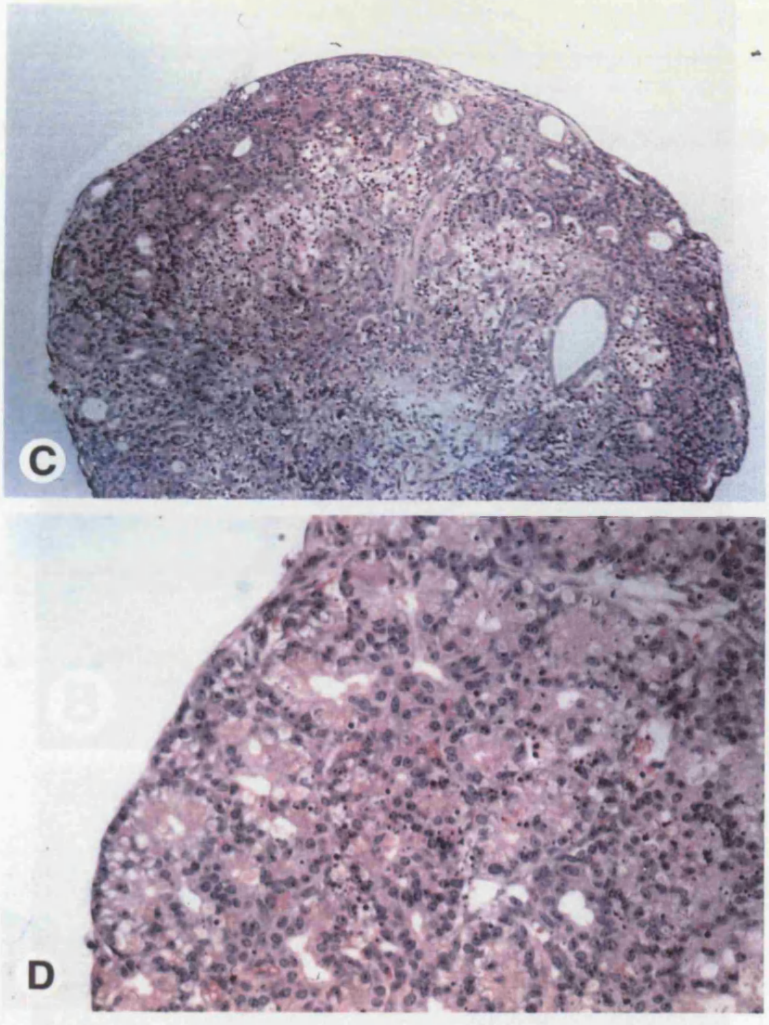


Fig. 6.1: (c) Foetal pig pancreas 2 days in HiO_2 showing viable cells with no central necrosis (H&E, x100).
(d) Foetal pig pancreas 2 days in HiO_2 showing viable endocrine tissue and ducts. Many pyknotic cells are seen due to the O_2 toxicity (H&E, x250).

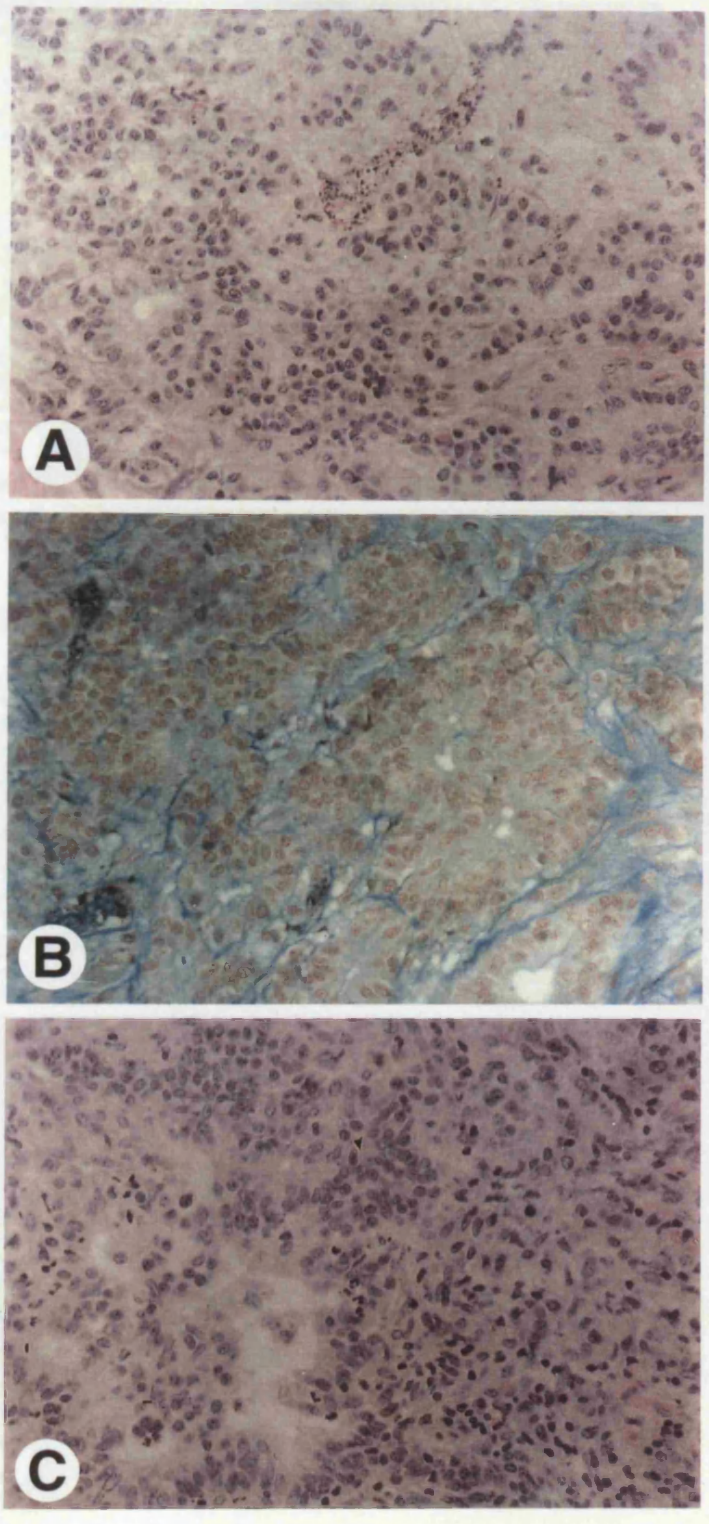


Fig. 6.2: **(a&b)** Foetal pig pancreas xenografts 2 days post-transplantation in control NOD/Lt mice.
(a) intact graft without infiltrate (H&E, x250).
(b) graft with β cells stained negative with GAF (GAF, x250).
(c) foetal pig pancreas xenografts 4 days post-transplantation in control NOD/Lt mice showing obvious infiltrate (H&E, x250).

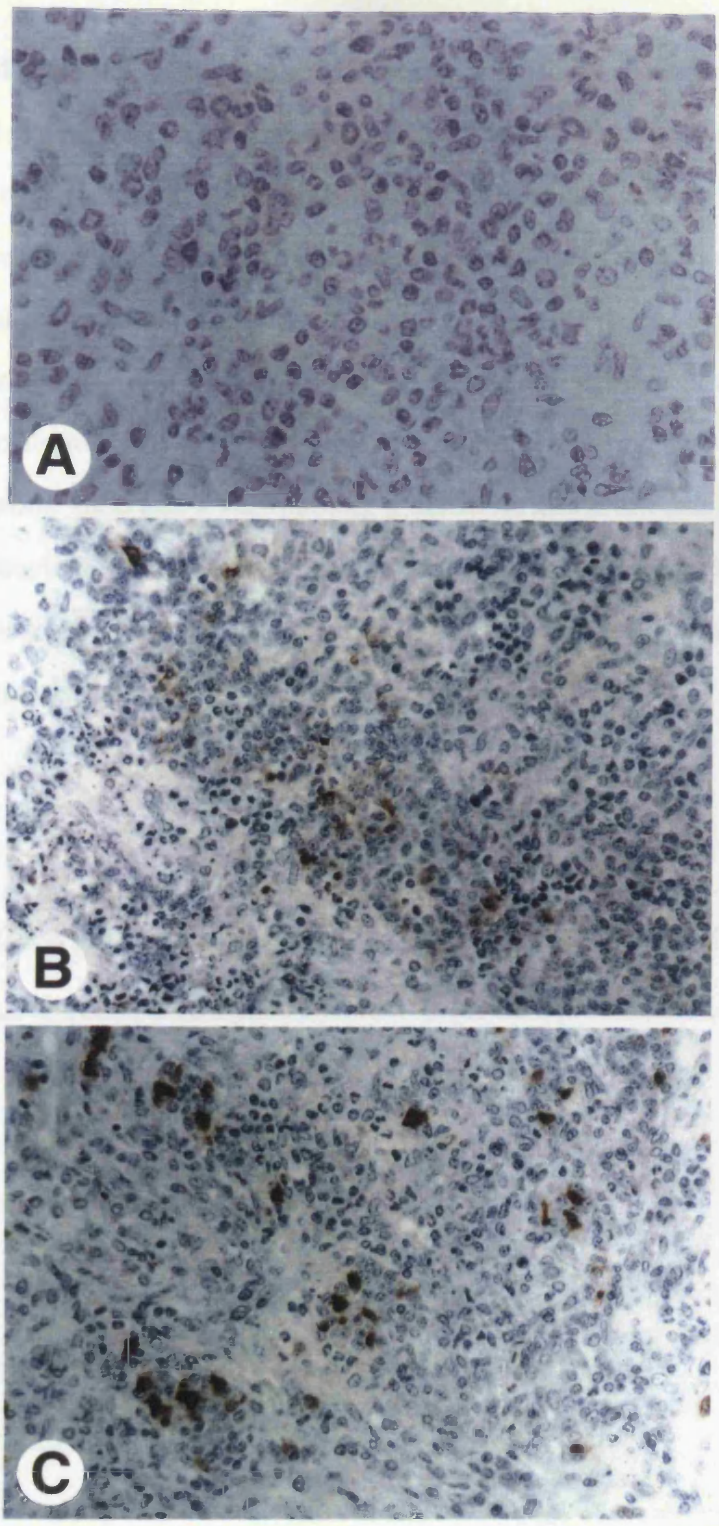


Fig. 6.3: Foetal pig pancreas xenografts 7 days post-transplantation in control NOD/Lt mice.
(a) graft site showing the infiltrate composed of eosinophils, neutrophils and MNCs (H&E, x400).
(b) IMP staining showing β cells within the infiltrate (insulin, x250).
(c) IMP staining showing δ cells (somatostatin, x250).

By day 14 there was complete graft destruction with no remaining transplanted cells identified on H&E staining but when immunocytochemistry was used scattered endocrine cells were sometimes still detected. There was a very dense mixed infiltrate which was again dominated by macrophages and eosinophils (Fig 6.4 a and b). A large central necrotic zone was present surrounded by a dense infiltrate suggestive of ischemic damage perhaps as a result of some late vascular obliteration. By day 28 there was total destruction of all transplanted cells and no endocrine cells were seen with immunoperoxidase cytochemistry but a large granuloma with a central necrotic zone and a surrounding a dense infiltrate of eosinophils, macrophages and smaller mononuclear cells was present (Fig 6.4 c). Macroscopically this appeared as a dull white nodule on the kidney surface. There was no difference in the response of the recipients to CC or HiO_2 cultured tissue.

Table 6.1.

Day	Conventional Culture		High O_2 Culture	
	Graft	**Infiltrate**	**Graft**	**Infiltrate**
2	4.0	0.0	3.9	0.1
4	3.1	2.7	3.1	2.5
7	1.2	3.9	1.1	3.8
14	0.0	3.6	0.0	3.9
28	0.0	1.8	0.0	2.2

Table 6.1: Graft and infiltrate scores in non-immunosuppressed recipients (average scores from 4-5 specimens per group and time point).

6.3.3 Effect of peri-transplant administration of GK1.5

The summary of the graft site histology is shown in Table 6.2. There was no evidence of rejection of the grafts on either days 2, 4 or 7. All grafts were intact with well developed islets and ducts. The β cells were GAF negative but were stained strongly positive by

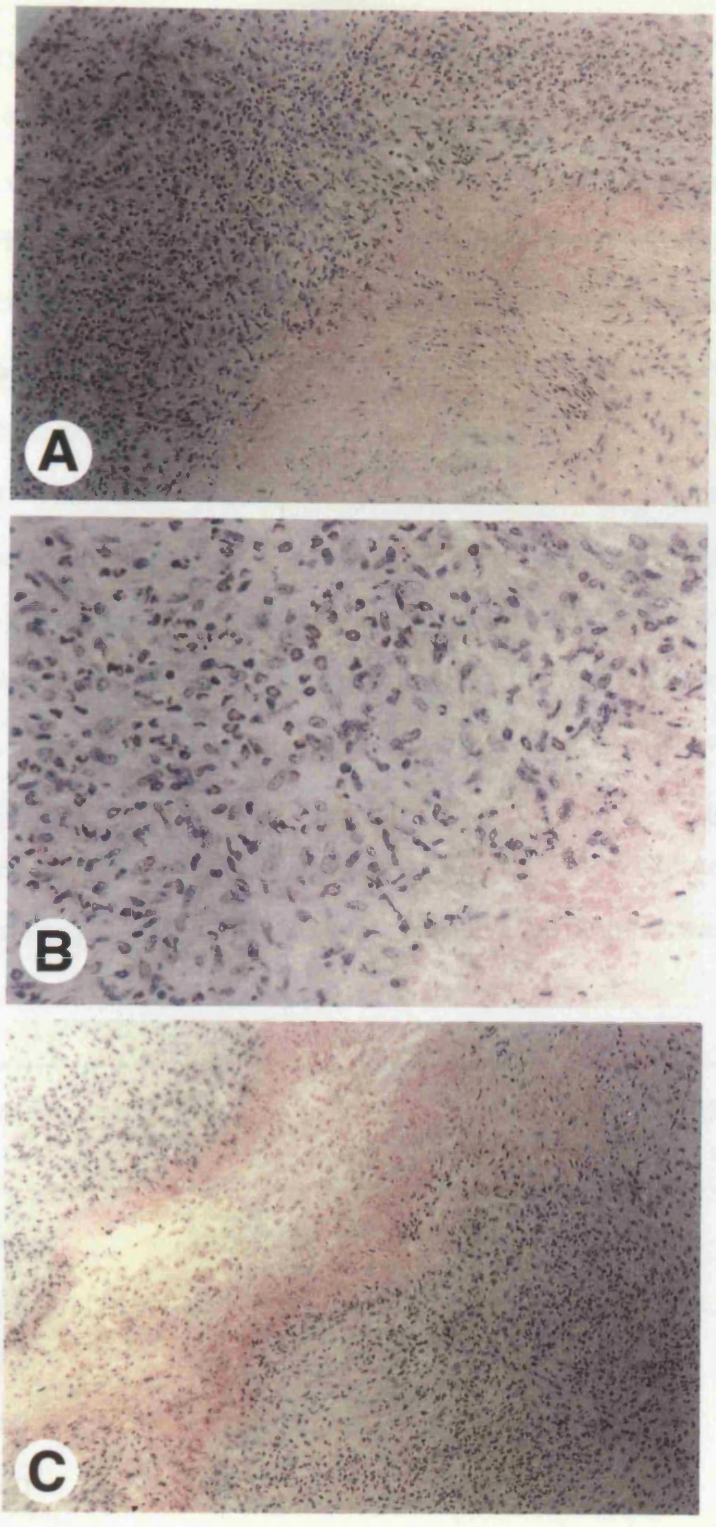


Fig. 6.4: (a&b) Foetal pig pancreas xenografts 14 days post-transplantation in control NOD/Lt mice.
(a) Heavy infiltrate with total graft rejection (H&E, x100).
(b) High magnification of the graft site showing details of infiltrate with eosinophils (H&E, x400).
(c) Foetal pig pancreas xenografts 28 days post-transplantation showing a central necrotic zone at graft site (H&E, x100).

immunocytochemistry, as were the α and δ cells (Fig. 6.5 a,b,c and d). By 14 days the grafts were still intact with well demarcated islets and ducts and some cells in mitosis, however there was now a slight but more obvious infiltrate in the peri-graft interstitial tissue but this was not invading the islets (Fig. 6.6 a). Immunoperoxidase staining showed well differentiated β cells and differentiated δ cells (Fig. 6.6 b and c). In marked contrast to the control animals, the GK1.5 treated mice had essentially intact grafts and the infiltrating cells were all mononuclear with a conspicuous absence of the eosinophils that dominated the control graft sites at this time.

By 28 days, when there was no longer evidence of any surviving tissue in the controls, there was moderately advanced rejection in the treated animals but obvious graft tissue remained (Fig. 6.7 a). Immunocytochemistry showed well stained endocrine cells and GAF positive β cells were also seen but the ducts which were still well preserved were hormone negative (Fig. 6.7 b). There was marked variation between individual mice within a group with some having well preserved grafts while others had advanced rejection. Since the graft sites were serially sectioned this was not due to sampling error. The infiltrate contained many eosinophils as well as many macrophages and a few conspicuous multinucleate giant cells.

Table 6.2

Day	Conventional Culture		High O_2 Culture	
	Graft	**Infiltrate**	**Graft**	**Infiltrate**
2	4.0	0.0	4.0	0.0
4	4.0	0.2	4.0	0.1
7	4.0	0.4	4.0	0.4
14	4.0	0.8	4.0	0.3
28	0.7	3.5	1.8	3.8

Table 6.2: Graft and infiltrate scores in immunosuppressed recipients that had been given 3 doses of GK1.5 (0.3mg/dose) on days -1, 0 and +1 (average scores from 4-5 specimens per group and time point).

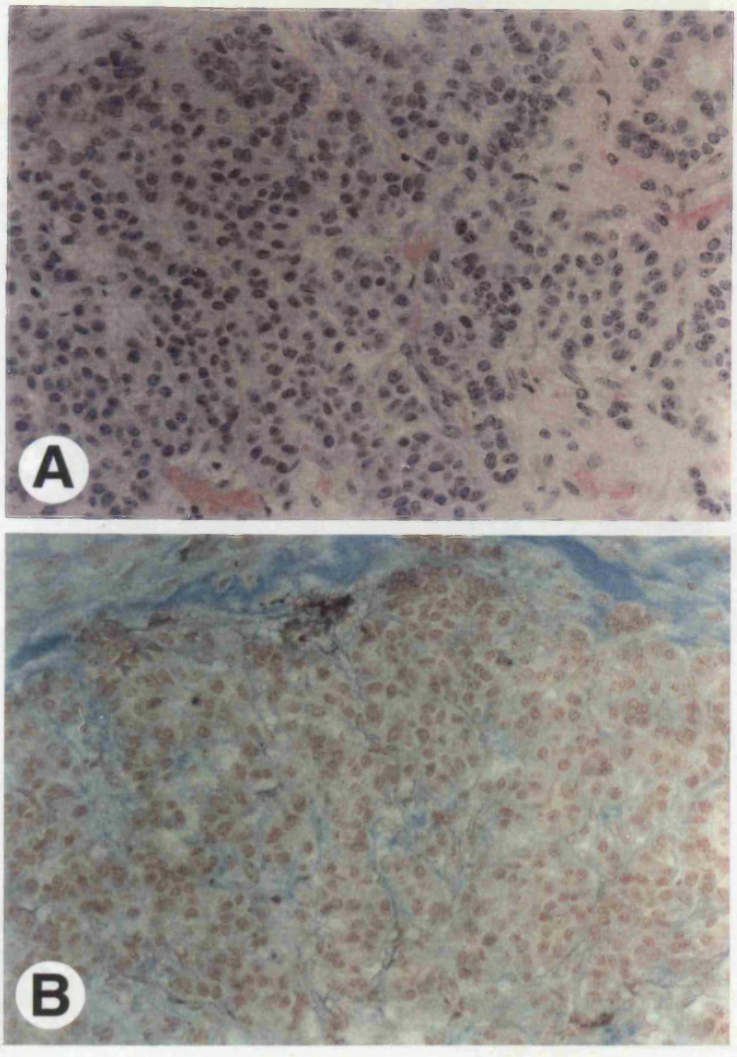


Fig. 6.5: Foetal pig pancreas xenografts 4 days post-transplantation in GK1.5 treated NOD/Lt mice.

(a) Intact graft with no evidence of infiltrate (H&E, x250).

(b) The graft is GAF negative for β cells (GAF, x250).

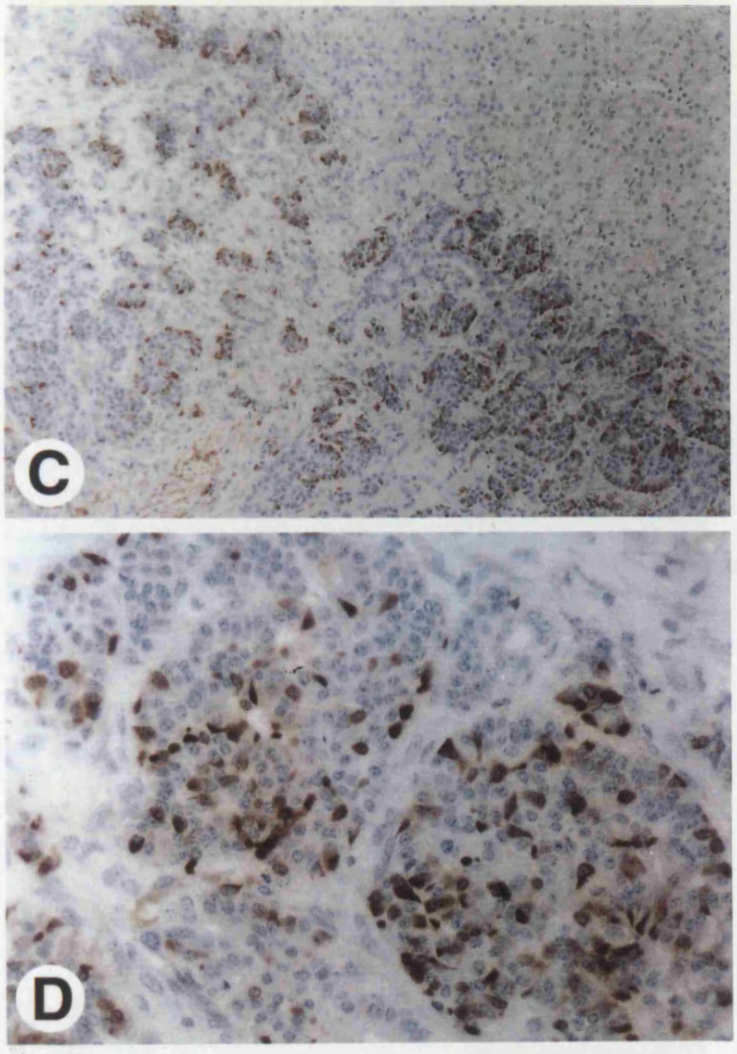


Fig. 6.5: Foetal pig pancreas xenografts 4 days post-transplantation in GK1.5 treated NOD/Lt mice.
(**c**) IMP staining showing well differentiated α cells (x250).
(**d**) IMP staining showing well differentiated δ cells (x250).

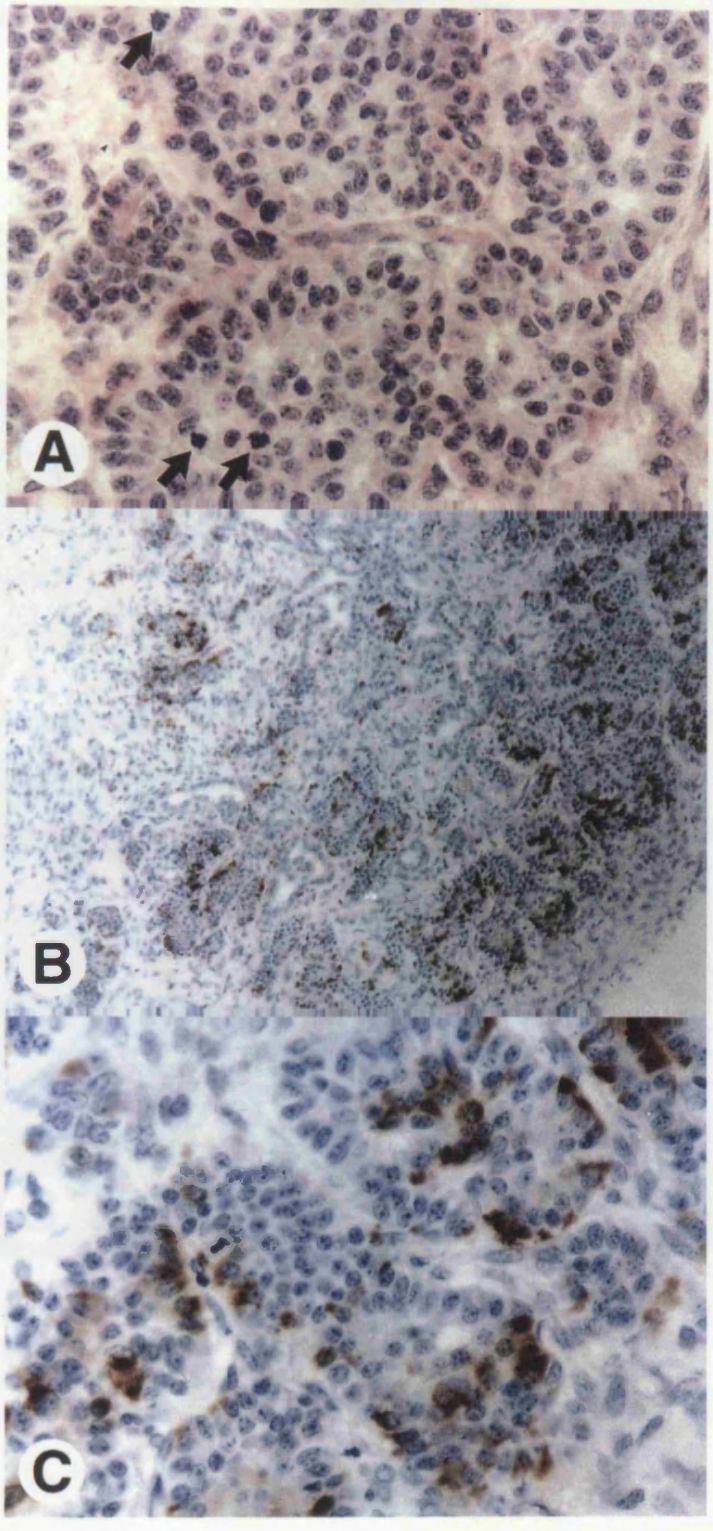


Fig. 6.6: Foetal pig pancreas xenografts 14 days post-transplantation in GK1.5 treated NOD/Lt mice.

(a) Well developed graft with some cells in mitosis (arrows) (H&E, x400).

(b) IMP staining showing well differentiated β cells (x100).

(c) IMP staining showing well differentiated δ cells (x400).

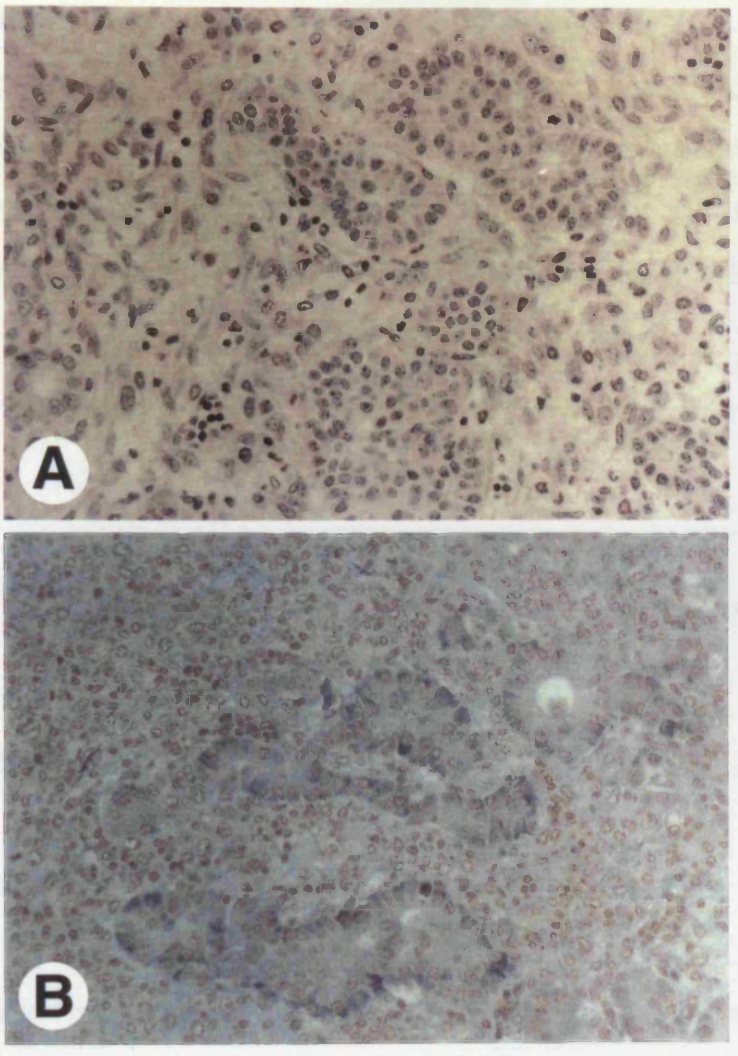


Fig. 6.7: Foetal pig pancreas xenografts 28 days post-transplantation in GK1.5 treated NOD/Lt mice.
(a) The graft is still present with moderate infiltrate (H&E, x250).
(b) Graft showing well stained GAF positive β cells surrounded by infiltrate (GAF, x250).

6.3.4. Effect of anti-CD4 treatment on insulitis

All mice remained euglycaemic during the course of this study regardless of treatment but there was a difference in severity of insulitis between the groups with animals that had been treated with the MAb having reduced insulitis severity on day 14 but this difference was no longer apparent at the end of the experiment. However, there was a wide variation in the severity of insulitis between individual mice. The difference in mean insulitis severity between pooled GK1.5-treated and untreated animals was statistically significant at 14 days ($p = 0.014$; Mann Whitney test) but not at 28 days. The data are summarised in Table 6.3.

Table 6.3

Culture type:	**CC**	**HiO_2**	**CC**	**HiO_2**
GK1.5:	+	+	-	-
Day				
14	29.0 ± 9.7	28.4 ± 11.7	62.8 ± 11.3	39.8 ± 9.3
28	24.6 ±4.3	33.6 ± 6.7	51.8 ± 12.0	17.2 ± 7.0

Table 6.3: Insulitis scores in the pancreas of NOD/Lt mice that had been transplanted with organ cultured foetal pig pancreas and either treated (+) or not treated (-) with peri-transplant GK1.5 monoclonal antibody (mean ± SEM; n = 5). Pooled values of GK1.5-treated versus non-treated animals showed that at 14 days there was a significant difference ($p = 0.014$) but this was not seen on day 28 (Mann-Whitney test).

6.3.5. Expression of Gal(α1-3)Gal

Gal(α1-3)Gal expression was studied in sections of grafts from all experimental animals except untreated controls at days 14 and 28 since all the transplanted tissue had been rejected in these. It was evident that differentiated endocrine cells, ie those that stained strongly for glucagon, insulin and somatostatin, were all Gal(α1-3)Gal negative (Fig. 6.8 a,b and c). However, there was strong staining for Gal(α1-3)Gal on the luminal surfaces of the ducts (Fig.

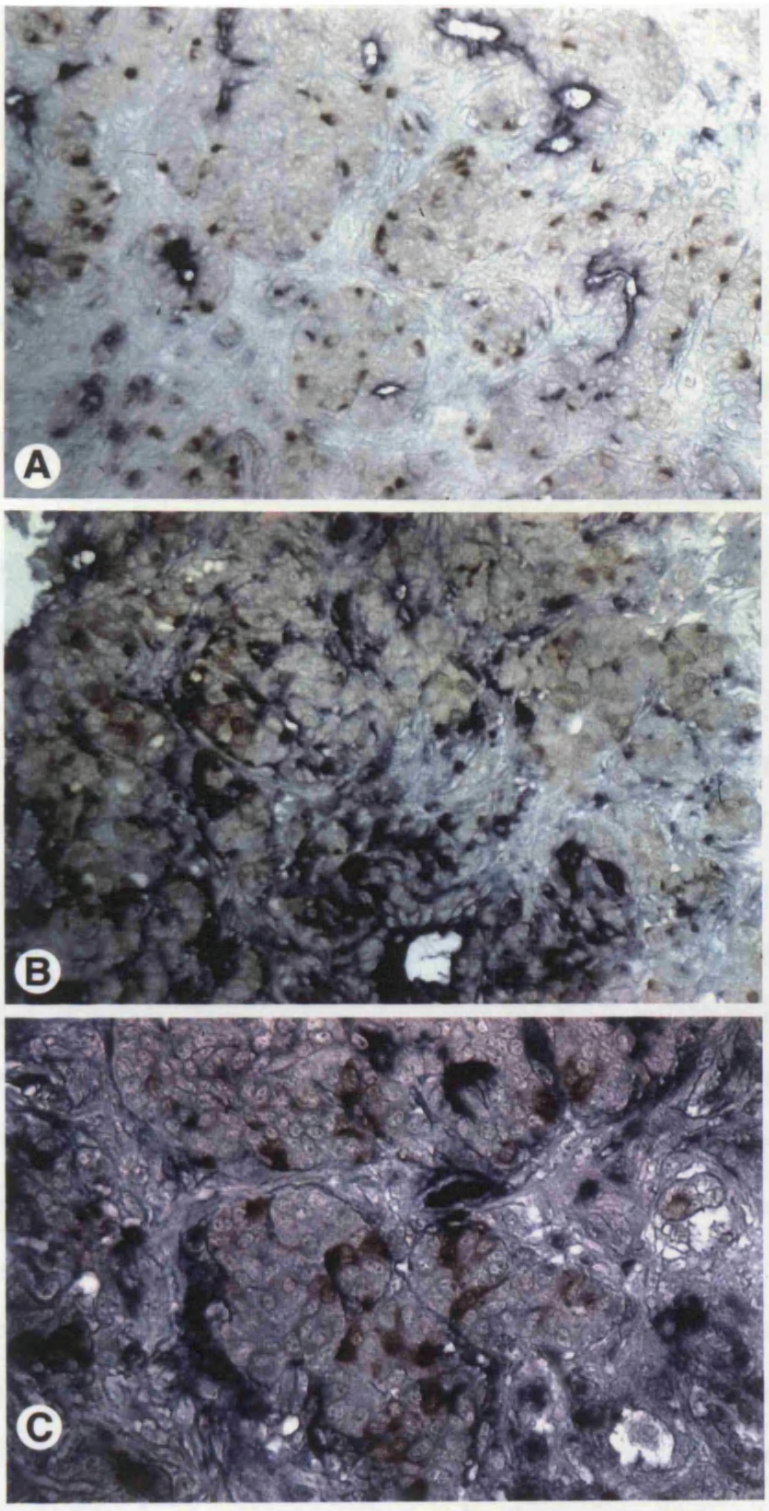


Fig. 6.8: Foetal pig pancreas xenografts 14 days post-transplantation in GK1.4 treated NOD/Lt mice. Two colour IMP staining showing endocrine cells stained brown and Gal(α1-3)Gal stained blue.
(a) IMP staining showing well stained α cells that are clearly Gal(α1-3)Gal negative (x400).
(b) IMP staining showing well stained β cells that are clearly Gal(α1-3)Gal negative but luminal surface of the ducts is strongly positive (x400).
(c) IMP staining showing δ cells that are clearly Gal(α1-3)Gal negative (x400).

6.8 b) and also on interstitial cells in the graft site but the identity of these cells is uncertain. It is possible that at least some are endothelial while others have the appearance of dendritic cells and macrophages. However, there was no staining at all in the underlying kidney and specifically none in its rich endothelial network. Gal(α1-3)Gal expression was reduced in the interstitial cells in the graft site as the graft matured but in rejecting grafts there was again strong staining in areas where infiltrating cells were present.

6.4 DISCUSSION

Transplantation of organ cultured foetal pig pancreas into non-immunosuppressed and immunologically highly responsive NOD mice (Leijon *et al,* 1994) results in rapid but not hyperacute rejection. The first evidence of a cellular host response occurred after day 2 and this represents acute cellular rather than HAR. In this study, at 2 days there was no microscopic evidence of any pathologic change and certainly nothing resembling the strong vascular response characteristic of HAR, ie, haemorrhage, thrombosis, platelet aggregation and graft infarction. Mice are, however, often not good models for HAR unless passive transfer of complement and antibody is used since many strains are complement deficient and NOD mice in particular are known to lack C5 (Baxter and Cooke, 1993). While it may be possible to avoid HAR simply by removing the primary target for NAbs, ie donor endothelial cells, this may be inadequate to prevent graft destruction by other mechanisms.

The histologic appearance of the graft site in control mice was characterised by a massive pleomorphic infiltrate. The invading cells included large numbers of eosinophils with smaller numbers of neutrophils, many macrophages including some multinucleate giant cells, as well as smaller numbers of other mononuclear cells that other studies have shown are predominantly CD4+ve T cells with smaller numbers of CD8+ve T cells as well as NK and other lymphocyte-like cells (Auchincloss, 1988).

The role of eosinophils is also still poorly understood, however they have also been described in rejecting allografts and their presence correlated with poor graft survival (Weir *et al,* 1986; Kormendi and Amend, 1988; Forsre *et al,* 1989; Wang *et al,* 1994).

It has also been shown that the anti-xenograft response is highly CD4+ve T cell dependent (Pierson *et al,* 1989). In this study, I have shown that even a brief treatment with a

depleting anti-CD4 MAb greatly altered the host response and extended, but not permanent, graft survival was seen. Whether these T cells are the effectors in graft damage is not clear but it is apparent that CD4+ve cells do play a major role in attracting other potential effector cells including eosinophils (Nakajima *et al,* 1992; Iwamoto *et al,* 1993) and perhaps also macrophages that can be potent cytotoxic cells. It is still not understood what the mechanism of xenograft killing is but it does appear to differ, qualitatively and perhaps also quantitatively, from lethal damage to allogeneic cells.

Pre-transplant treatment of a graft to eliminate from it highly immunogenic cells (ie "passenger leucocytes") has been widely used in experimental islet allografts in rodents of many strains with marked success (Lacy, 1994). Other studies have shown that immunomodified xenografts survive better than non-modified grafts in anti-T cell MAb-treated recipients but in those studies extended immunosuppression was used (Mandel and Koulmanda, 1993a and b). In this study the lack of an effect may be due to the relatively minor role that immunomodification may play when only brief peri-transplant immunosuppression is used, as in this study, and a small beneficial effect may not be apparent. In any case, NOD mice in contrast to other strains, also fail to accept immunomodified allografts and they may be a difficult model in which to test for small effects (Mandel and Koulmanda, unpublished results) as they are an immunologically highly responsive strain (Leijon *et al,* 1994).

In the present study in pre-diabetic NOD mice there was a suggestion that peri-transplant treatment with anti-CD4 MAb reduced the severity but did not eliminate, the endogenous autoimmunity as assessed by quantitative scoring of the native pancreas. Charlton and Mandel (1989), have reported that anti-CD4 treatment of NOD mice can decrease the extent of insulitis and reduce the prevalence of diabetes and in this study the severity of insulitis was significantly reduced but not eliminated in the GK1.5 treated mice at 14 days but not at 28 days post-treatment.

In this study it was shown that HAR does not occur, at least in this model, and that Gal(α1-3)Gal expression does not appear on differentiated islet cells but does on the ductal cells that contain the endocrine cell precursors that may also need to be transplanted. I have also demonstrated that even a brief treatment with a specific anti-T cell MAb has a profound effect on the host's immune response but this effect may be indirect by targeting cells that are not the

final damage-mediating population. Whether these findings have relevance to more appropriate models for pre-clinical evaluation remains to be determined and experiments are in progress to study this in foetal pig pancreas grafts in Old World primates.

CHAPTER 7 THE EFFECT OF ANTI-CD4 MONOCLONAL ANTIBODY ON T AND B CELL SUBSETS AND FOETAL PIG ISLET XENOGRAFT SURVIVAL IN VARIOUS STRAINS AND SEX OF MICE

7.1 INTRODUCTION

The use of anti-CD4 MAbs has been of particular interest in transplantation because CD4+ve T cells play an important role in the generation of anti-graft responses, particularly against xenografts (Pierson *et al,* 1989). Long-term survival of discordant and concordant islet xenografts has been reported in several strains of mice (Lacy *et al,* 1989 and Goss *et al,* 1994). However, the effect of anti-T cell MAb immunosuppression and the prolongation of graft survival is usually strain specific and often "permissive" recipient strains are chosen for study because they are good responders to the treatment and allow grafts to survive for a long period of time.

In this chapter the effect of a single dose of GK1.5 MAb (0.3 mg) on the T cells of different strains of inbred mice was studied; C57BL/6, CBA, BALB/c and NOD/Lt. Not only did these 4 inbred strains vary markedly in the number and proportion of lymphocytes present in their peripheral blood and lymphoid organs (spleen and lymph node), with C57BL/6 and NOD/Lt mice representing two extremes, but they also showed differences in their response to GK1.5.

Also the survival of organ cultured foetal pig pancreas transplanted into female NOD/Lt, BALB/c and CBA mice, and NOD/Wehi and CBA male mice was studied and have shown that graft survival differs between these strains and even between the sexes in the same strain after peri-transplant immunosuppression with the anti-CD4 MAb, GK1.5.

The various strains of mice were chosen because C57BL/6, CBA and BALB/c mice are widely used in experimental transplantation, and tolerance to allografts and xenografts has been demonstrated in each under specific circumstances (Faustman and Coe 1991; Simeonovic *et al,* 1990a; Goss *et al,* 1994; Lacy *et al,* 1989). NOD/Lt mice cannot be tolerised to pig proislet xenografts by methods that are effective in CBA mice (Simeonovic and Wilson, 1992) and therefore may be considered immunologically hyper-responsive (Leijon *et al,* 1994).

7.2 MATERIALS AND METHODS

Young adult C57BL/6, CBA, BALB/c, and NOD/Lt female mice aged approximately 80 days at the start of the experiment were used. The MAb used in this experiment was GK1.5 MAb used as ascites raised in pristane-primed nude (athymic) mice and diluted with MTPBS to contain 0.3 mg/dose. The antibody content of the ascites was assessed by ELISA and the concentration adjusted to 1 mg/ml.

7.2.1 Transplantation

For transplantation, groups of NOD/Lt, CBA and BALB/c female mice and CBA and NOD/Wehi male mice were transplanted bilaterally under the renal capsule with organ cultured foetal pig pancreas (chapter 2). The donor tissue was from a litter of pigs of about 90 days gestation. The pancreas was removed under sterile conditions, diced into small pieces ($<1mm^3$) and placed in organ culture for 7 days in DME medium supplemented with 5% foetal calf serum in humidified 90% O_2/10%CO_2 for 2 days followed by 5 days in 10%CO_2/90% air at 37°C, as previously described in chapter 2. The recipients were anaesthetised with Methoxyfluorane and each kidney was exposed though a separate flank incision. The grafts, each consisting of a single piece of tissue, were placed under the renal capsule and milked away from the capsular incision so that they were less likely to be dislodged when the kidney was replaced in the abdominal cavity. The skin wounds were closed with metal clips. Non-immunosuppressed mice were not transplanted in this experiment because many previous studies in our laboratory, and results in chapter 5 and 6, show that the xenografts are invariably fully rejected within 7-10 days in mice of all strains and both sexes.

For immunosuppression the mice were given 0.3 mg of the MAb intra-peritoneally on days -1, 0 and +1. All mice (4-8 per group) were nephrectomized on day 14 (chapter 2) and the excised kidney was fixed in Bouin's fluid. The graft sites were located, cut out, coded and processed for light microscopic examination. Serial sections 4 μm thick were stained with H&E for general examination of the graft and infiltrate and with GAF for islet β cells. In some instances tissue was also processed for immunoperoxidase staining for the presence of α, β, and δ cells to confirm that the tissue present was indeed islet tissue since GAF stain is usually negative on immature foetal islets while the immunoperoxidase stain is invariably strongly positive.

Three animals from each group were bled weekly, and additionally on day 28 a piece of spleen was taken from the same animals for flow cytometric analysis of T and B cells. A single cell suspension was made as described in chapter 2 and 10^4 cells per sample were used for flow cytometric analysis by staining for Thy 1.2 and CD3 to identify T cells, CD4 and CD8 to detect the two major T cell subsets, and for surface Ig (sIg) to mark B cells using the FACScan flow cytometer.

7.2.2 The response of various strains of mice to transient anti-CD4 immunosuppression

In an attempt to analyse in more detail the response of various strains of mice to transient anti-CD4 immunosuppression and to study the recovery of T cells, a separate experiment was performed using groups of 8 mice, all young adult females. The strains used were C57BL/6, CBA, BALB/c and NOD/Lt. Three mice of each strain were given 0.3 mg of the MAb as a single dose while 2 mice (control) were given MTPBS. Blood samples were taken at weekly intervals from the peri-orbital plexus for 6 weeks post-treatment. The blood leucocytes were stained for Thy 1.2 and CD3 to identify T cells, CD4 and CD8 to detect the two major T cell subsets, and for surface Ig (sIg) to mark B cells by flow cytometric analysis using the FACScan. A 5 µl blood sample was also taken for cell counts using a Coulter Counter to determine the absolute cell numbers of the various subsets of cells. Three mice from each of the 4 strains were also sampled for their spleen and pooled lymph node (axillary, inguinal cervical and mesenteric) lymphocyte profiles and in this instance expression of B220, a B cell marker, was also assessed.

7.3 RESULTS

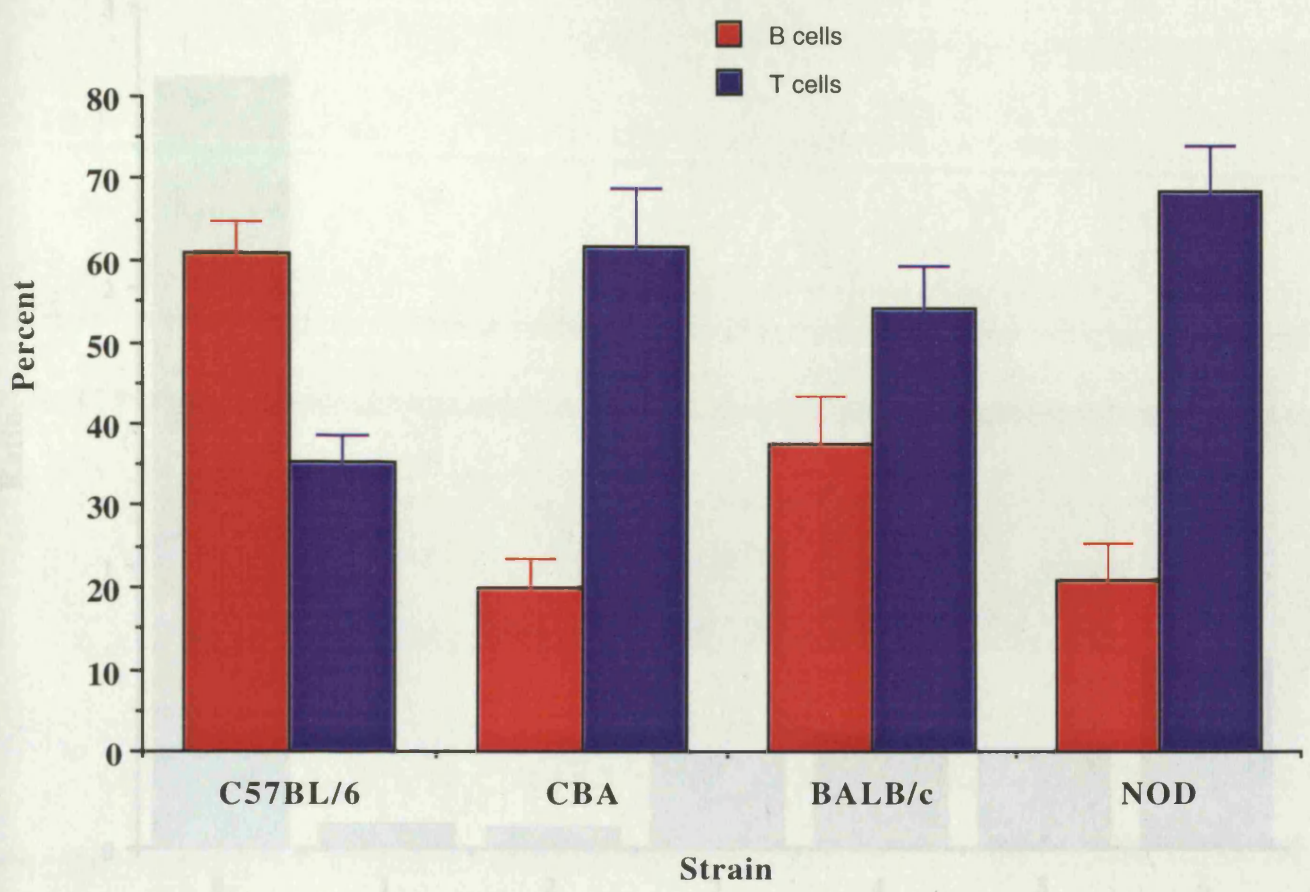
7.3.1 T and B cell subsets in different strains of normal (untreated) mice

Total leucocyte counts and T cells (Thy 1.2+ve/CD3+ve), T cell subsets (CD4+ve/CD8+ve) and B cells (sIg+ve) were obtained from the flow cytometry contour plots on 10^4 cells per sample. T cells were defined as cells that were both CD3 and Thy 1.2 positive and the 2 major subsets (CD4+ve and CD8+ve) were separately identified. B cells were taken as small mononuclear cells that were sIg positive. There was a good correlation between the sum of CD4+ve and CD8+ve cells and the number of Thy 1.2/CD3 double positive cells. Also

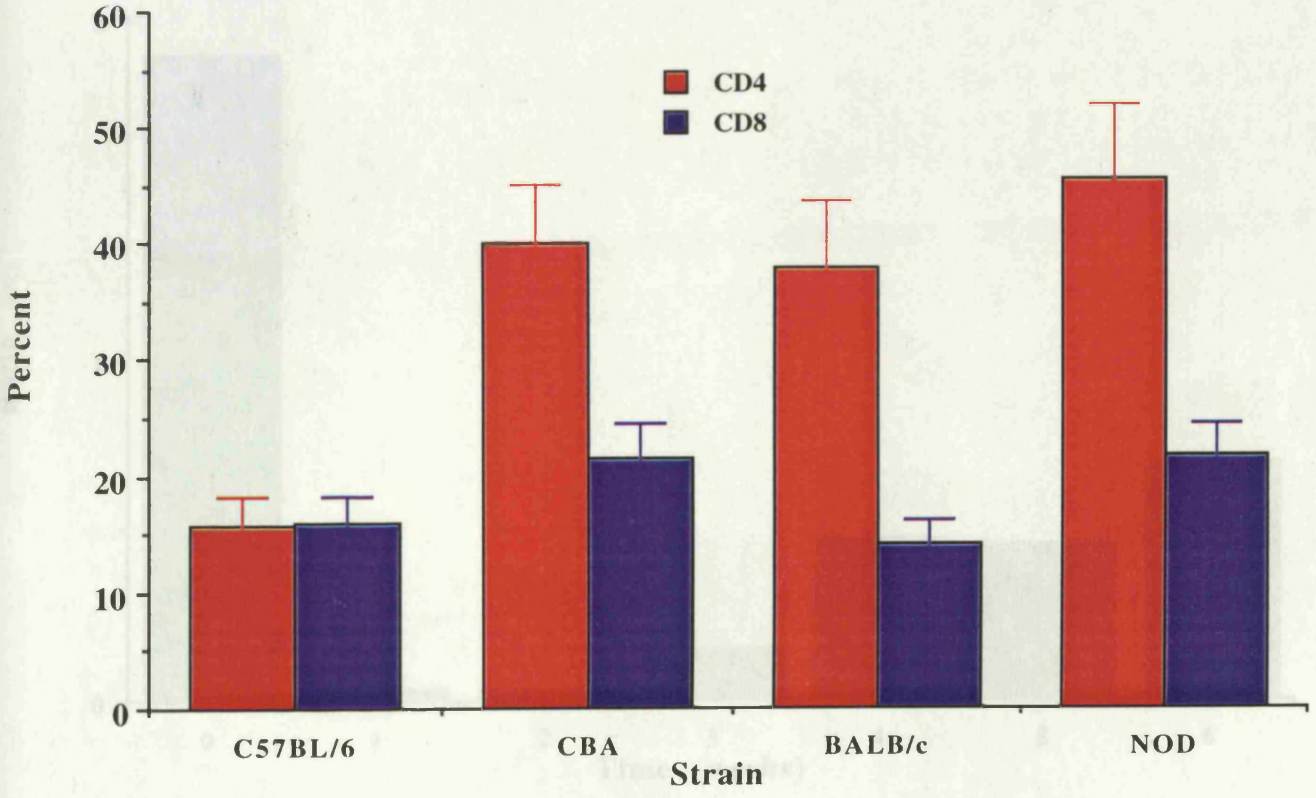
a good correlation was seen between B220 and sIg expression in the spleen and lymph node cells in this study.

There was marked variation in the total peripheral blood cell counts in the untreated animals, even in individual mice sampled in weekly intervals, but overall the range of values was usually between 5-10 x 10^6/ml in all 4 strains and there was no systematic variation between the strains. However, there was a much more consistent pattern in the percentage of lymphocyte subsets between the different strains. In particular, C57BL/6 mice had a very high proportion of B cells (60.8±4.0%) and a correspondingly low percentage of T cells (35.5±3.3%) while their CD4 to CD8 ratio was 1.00±0.28. The other strains had more T cells and less B cells (Graph 7.1) with NOD/Lt and CBA mice having the lowest percentage of B cells (both had approximately 20%) and BALB/c mice were intermediate with almost 40% B cells. T cells were correspondingly increased in these strains and non-lymphoid cells were a minority in all 4 strains. In all the strains except C57BL/6 the CD4:CD8 ratio was around 2 (Graph 7.3 a,b,c and d). Hence, there were striking differences in the normal population of peripheral blood lymphoid cells with C57BL/6 mice standing out as being quite different from the others in proportions and numbers of lymphocytes as well as the ratio of B to T cells and in the CD4/CD8 ratio. C57BL/6 mice also have low absolute numbers of circulating T cells and particularly low numbers of CD4+ve cells; while they have low numbers of CD4+ve cells and a low CD4:CD8 ratio their number of CD8+ve cells is similar to those of the CBA, BALB/c and NOD/Lt mice (Graph 7.2).

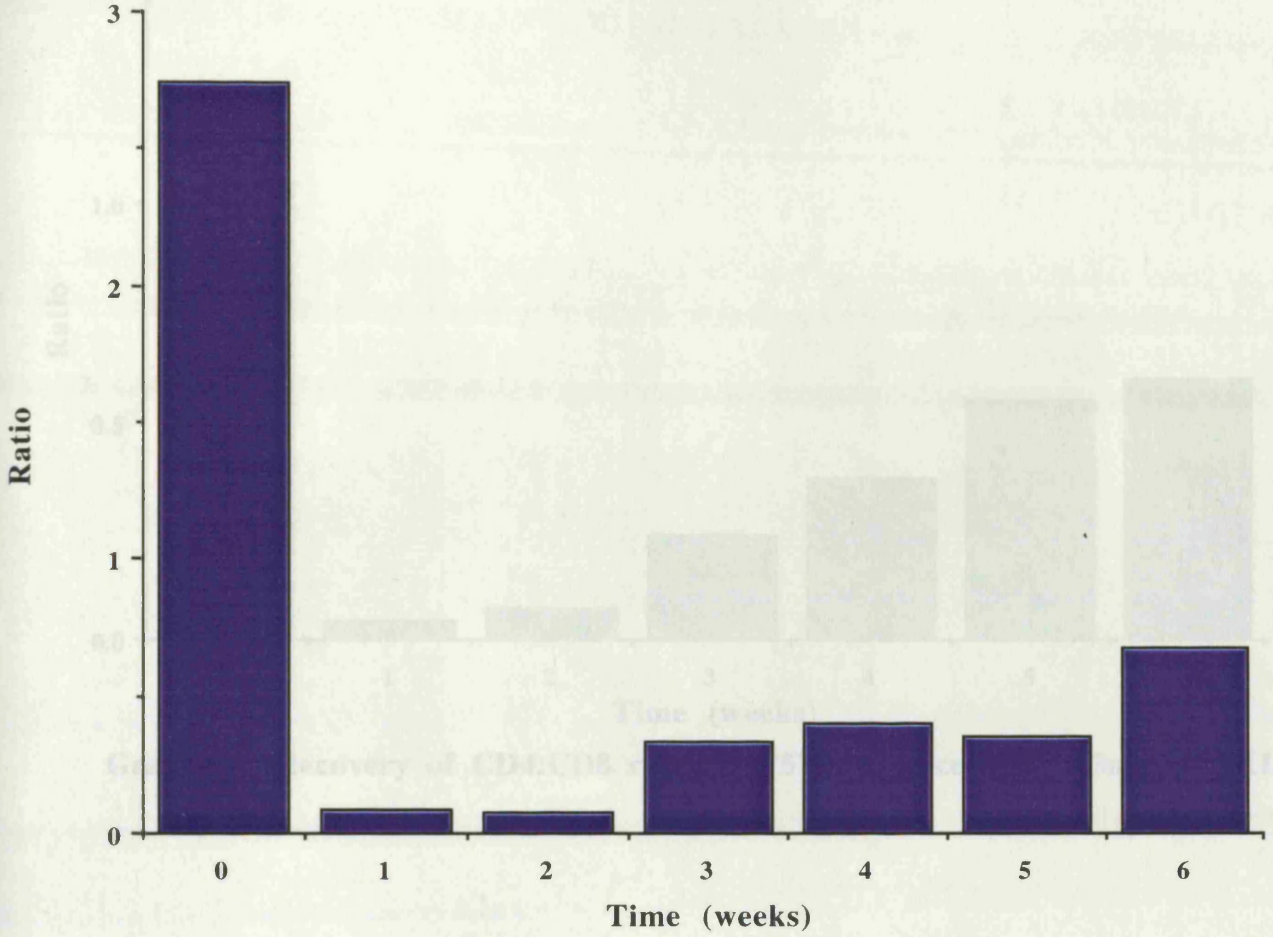
Spleen and pooled LN profiles were assessed in normal young mice as shown in Graph 7.4 a, b, c and d. There was good correlation between sIg^+ and $B220^+$ cells, and also between $CD3^+$/$Thy1^+$ and the sum of $CD4^+$ and $CD8^+$ cells. As for blood, the lymphocyte profiles in C57BL/6 mice differed from the other strains with both spleen and LN having a relative dearth of T cells and a lower proportion of $CD4^+$ T cells with a correspondingly low CD4:CD8 ratio. Hence, the difference in T cells was present in lymphoid organs as well as peripheral blood in this strain but the variation was not as marked as in the peripheral blood.



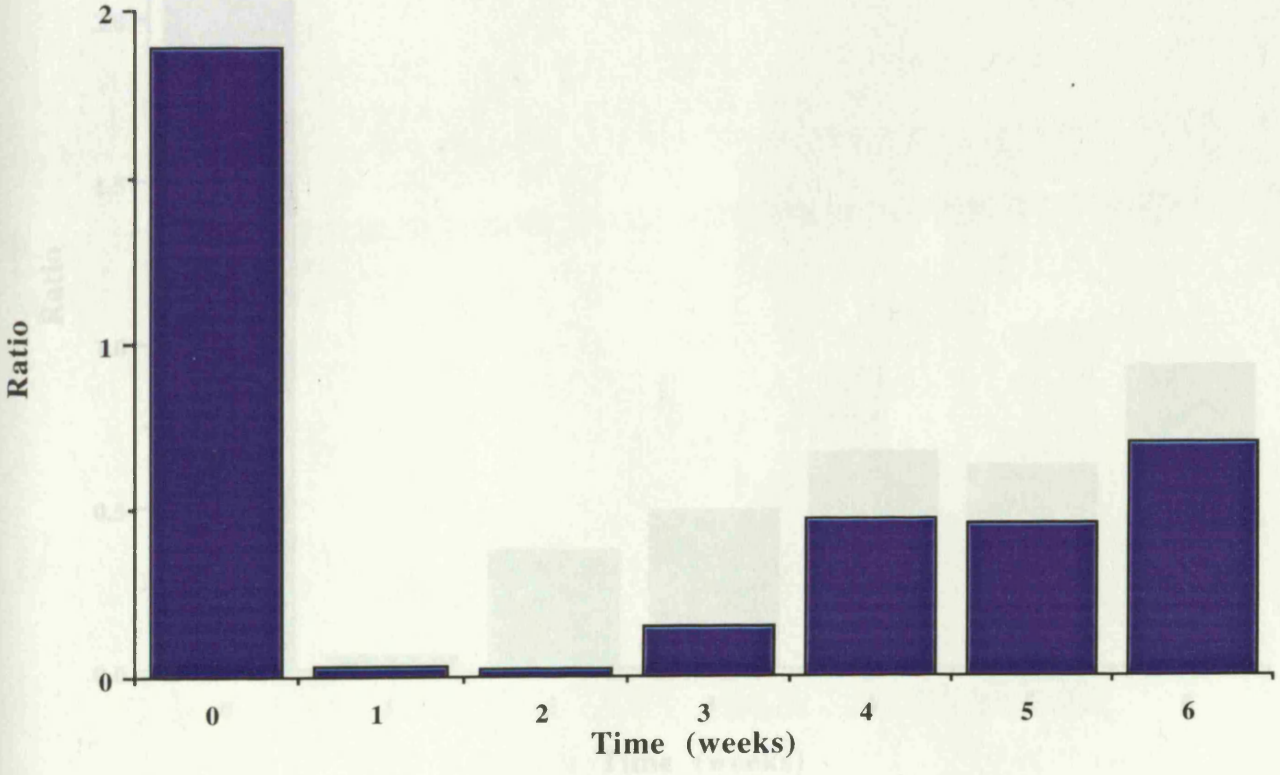
Graph 1: Percent of T and B cells in peripheral blood of normal mice



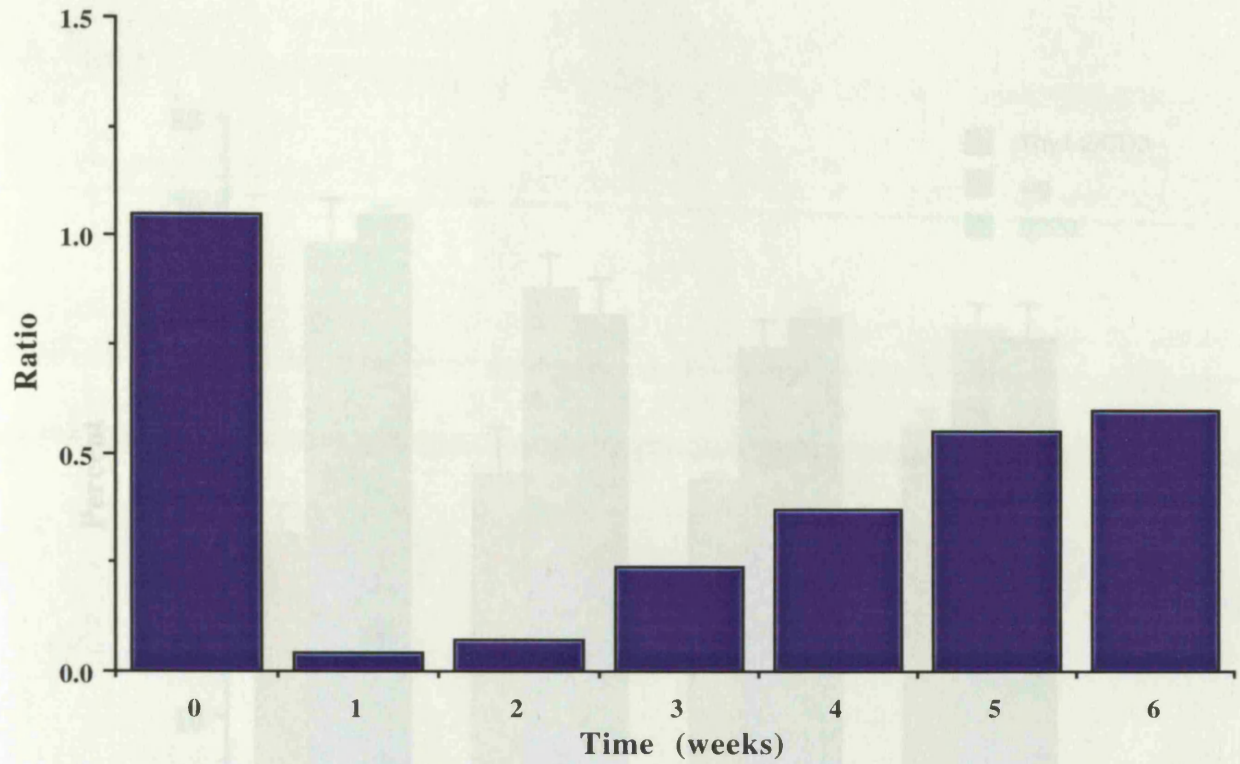
Graph 2: Percent CD4 and CD8+ve cells in peripheral blood of normal mice



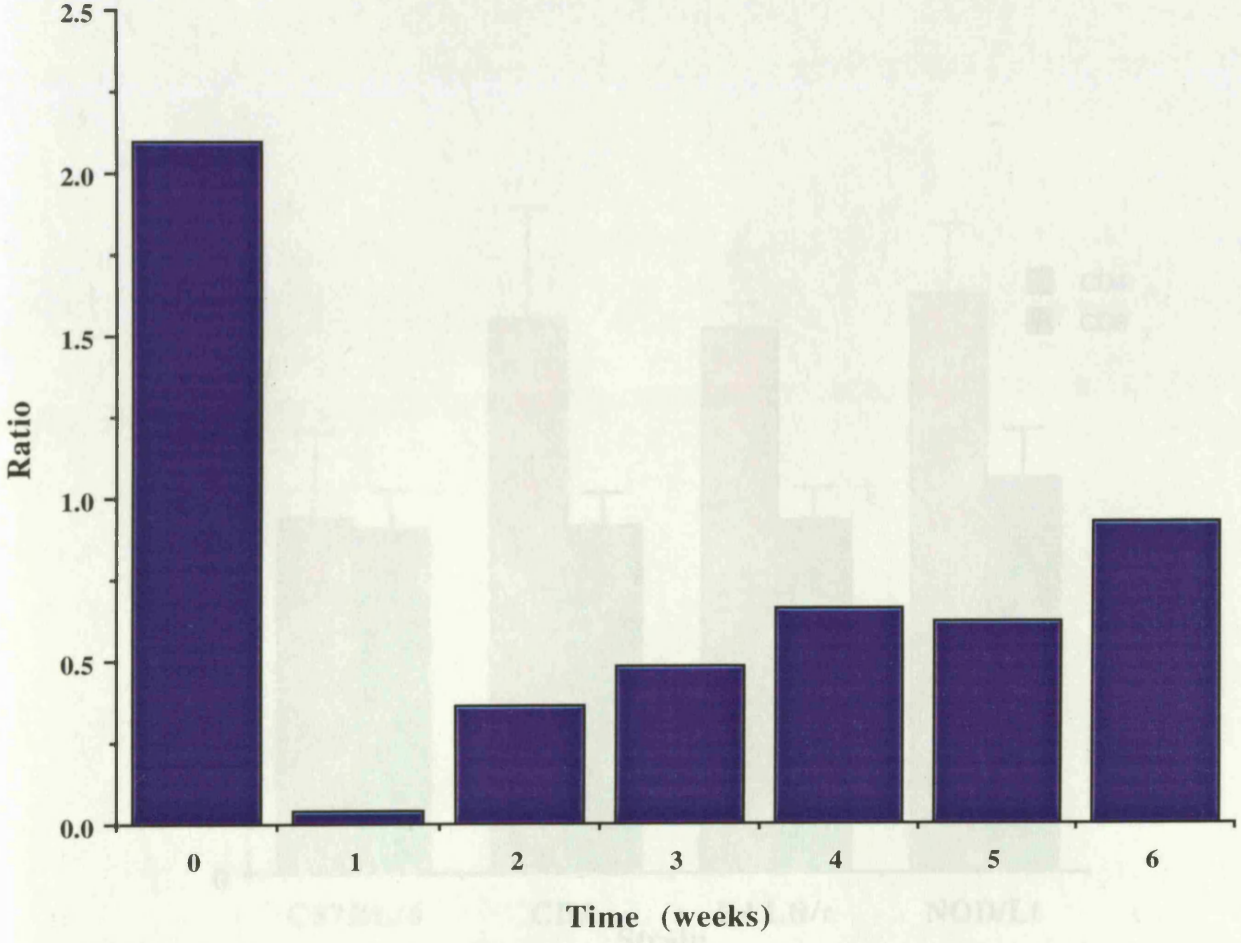
Graph 3a: Recovery of CD4:CD8 ratio in BALB/c mice after 0.3mg of GK1.5



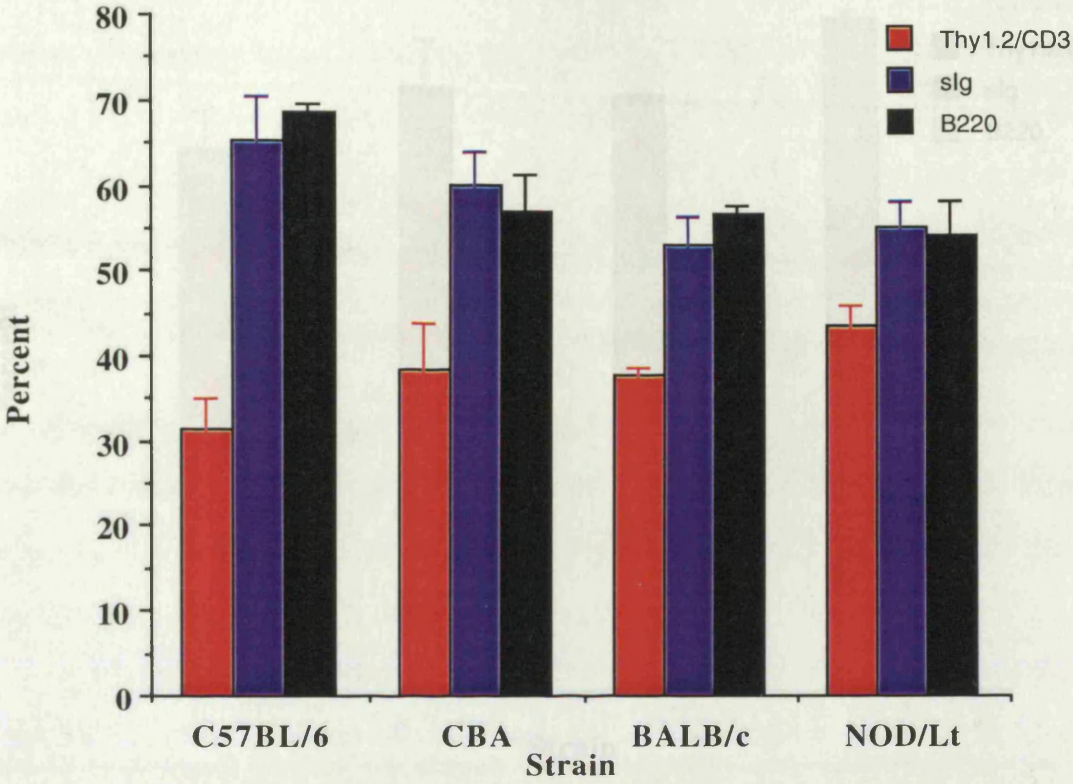
Graph 3b: Recovery of CD4:CD8 ratio in CBA mice after 0.3mg of GK1.5.



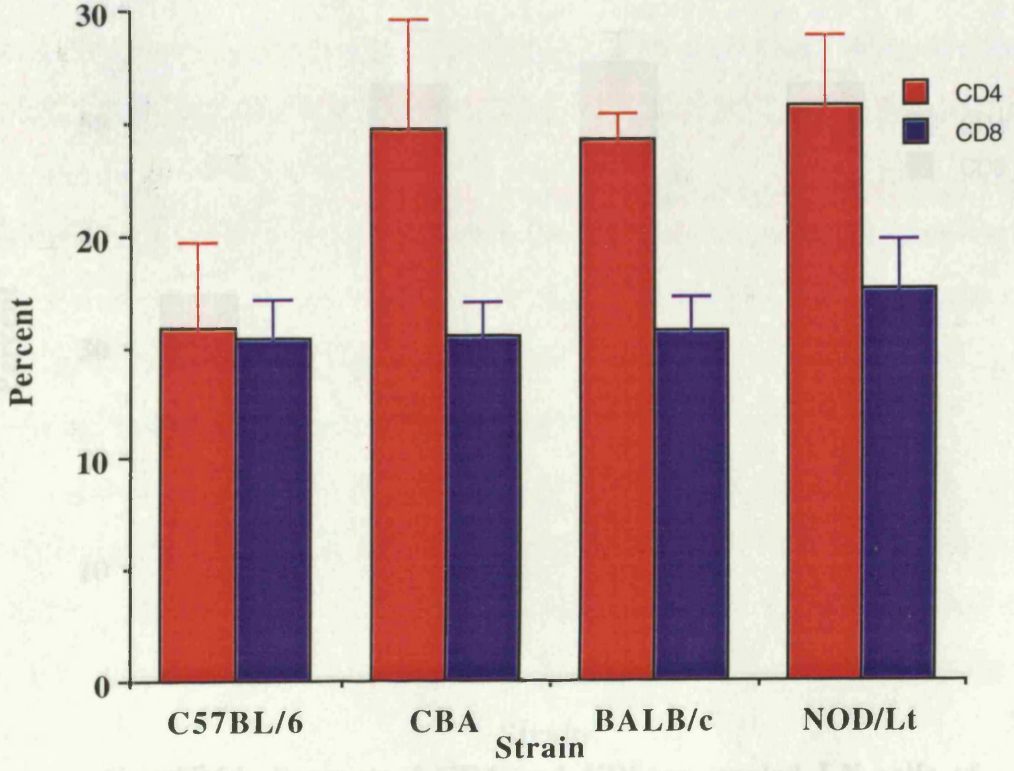
Graph 3c: Recovery of CD4:CD8 ratio in C57BL/6 mice after 0.3mg of GK1.5.



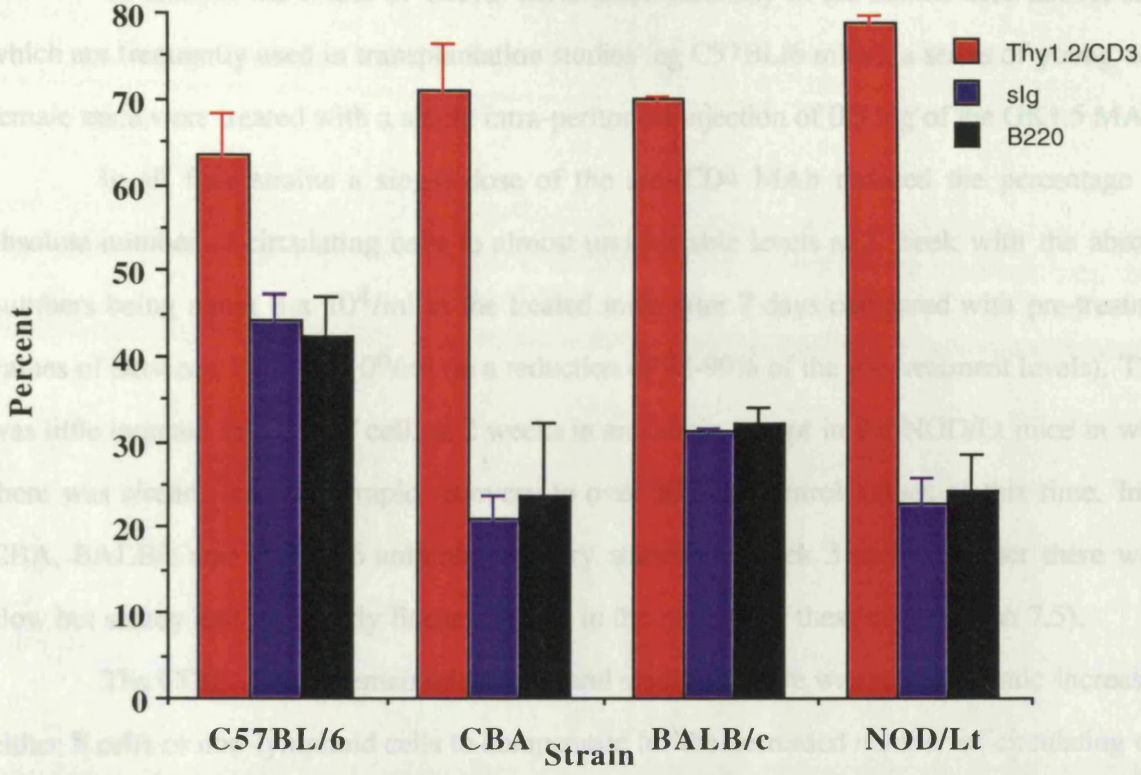
Graph 3d: Recovery of CD4:CD8 ratio in NOD/Lt mice after 0.3mg of GK1.5.



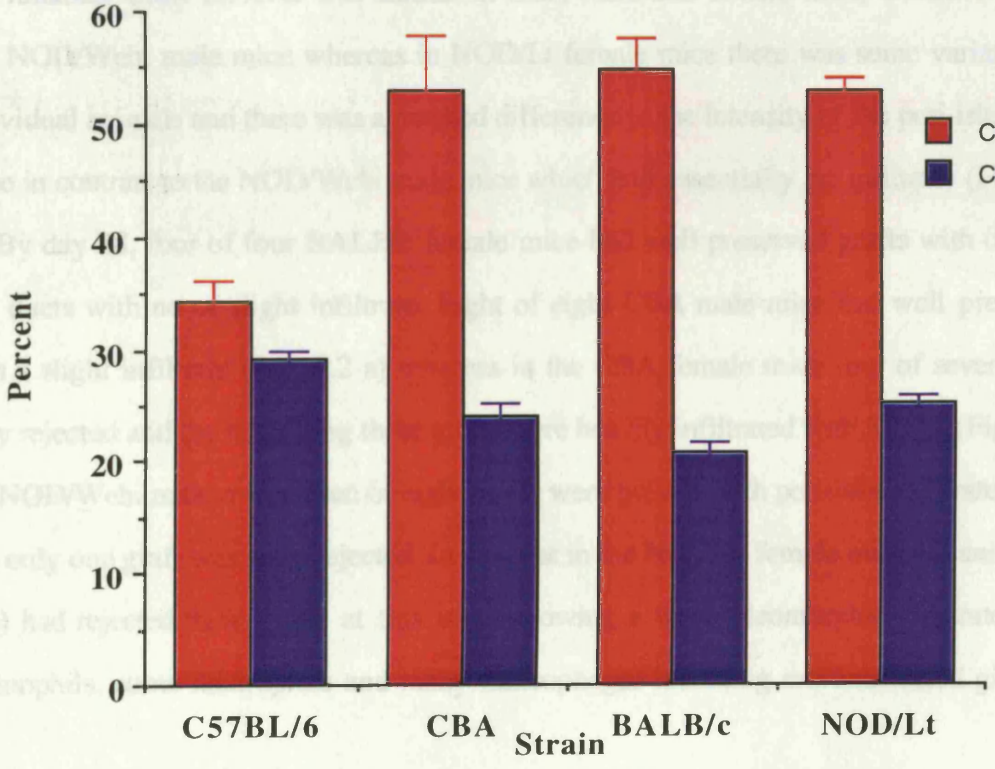
Graph 4a: Percent of Thy1.2+/CD3+, sIg+ and B220+ spleen cells of normal mice



Graph 4b: Percent of CD4 and CD8+ve spleen cells of normal mice



Graph 7.4c: Percent of Thy1.2+/CD3+, sIg+ and B220+ pooled LN cells of normal mice



Graph 7.4d: Percent of CD4 and CD8+ve pooled LN cells of normal mice

7.3.2 The effect of treatment with GK1.5 on T and B cell subsets

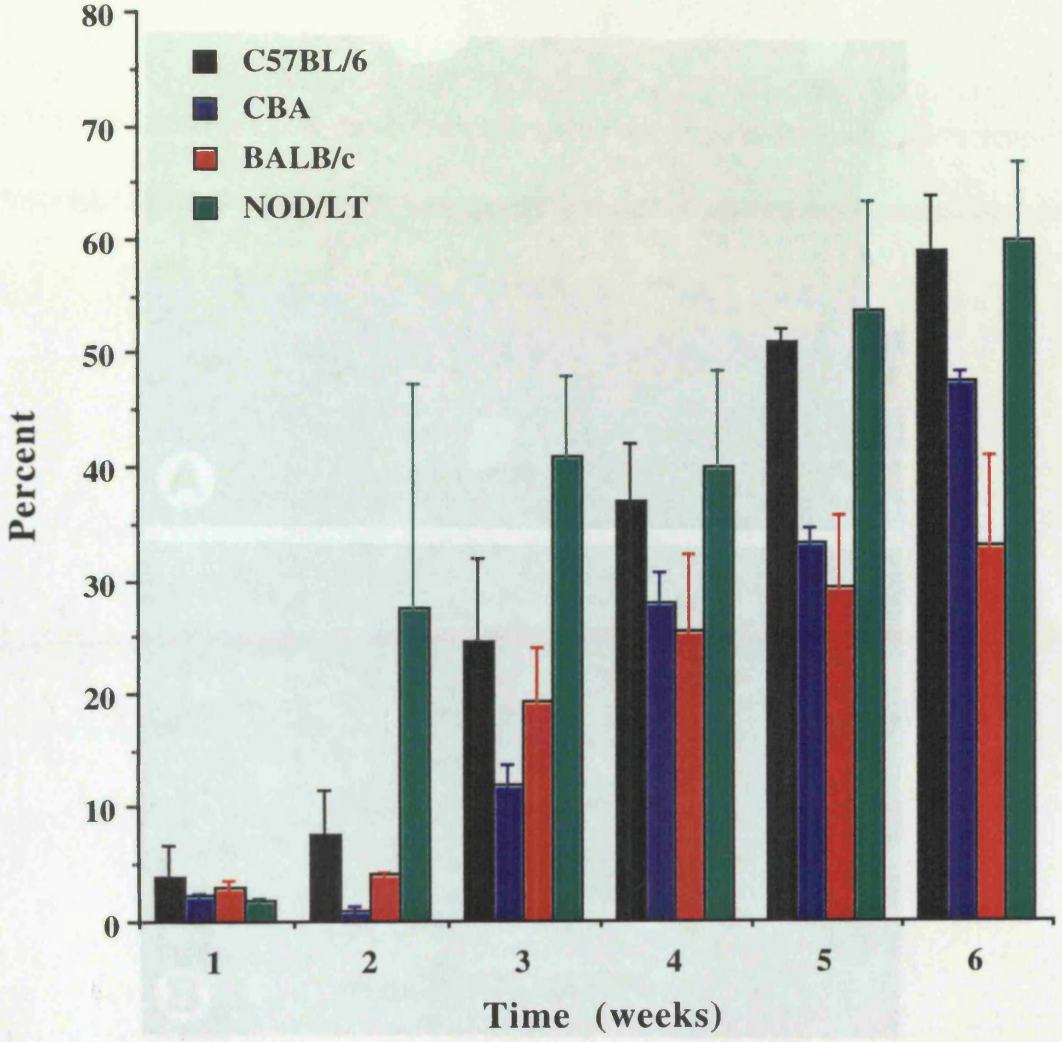
To analyse the effect of GK1.5 MAb more carefully in the strains used above, some which are frequently used in transplantation studies (eg C57BL/6 mice), a series of young adult female mice were treated with a single intra-peritoneal injection of 0.3 mg of the GK1.5 MAb.

In all four strains a single dose of the anti-CD4 MAb reduced the percentage and absolute number of circulating cells to almost undetectable levels at 1 week with the absolute numbers being about 3 x 10^4/ml in the treated mice after 7 days compared with pre-treatment values of between 1 - 2.5 x 10^6/ml (ie a reduction of 98-99% of the pre-treatment levels). There was little increase in CD4+ T cells at 2 weeks in any strain except in the NOD/Lt mice in which there was already a marked rapid recovery to over 30% of control values at this time. In the CBA, BALB/c and C57BL/6 animals recovery started by week 3 and thereafter there was a slow but steady and apparently linear increase in the number of these cells (Graph 7.5).

The $CD8^+$ T cells remained normal, and similarly, there was no systematic increase in either B cells or non-lymphoid cells to compensate for the decreased number of circulating cells after CD4 depletion.

7.3.3 The effect of peri-transplant GK1.5 on graft survival

The first time point the grafts were examined was on day 14 post-transplantation, and well developed grafts were seen in CBA, BALB/c, NOD/Wehi and NOD/Lt mice in both males and females. Graft survival was similar in CBA male and female mice, BALB/c female mice and NOD/Wehi male mice whereas in NOD/Lt female mice there was some variation between individual animals and there was a marked difference in the intensity of the peri-islet infiltrate in these in contrast to the NOD/Wehi male mice which had essentially no infiltrate (Fig. 7.1 a and b). By day 28, four of four BALB/c female mice had well preserved grafts with obvious islets and ducts with no or slight infiltrate. Eight of eight CBA male mice had well preserved islets with a slight infiltrate (Fig. 7.2 a) whereas in the CBA female mice four of seven grafts were fully rejected and the remaining three grafts were heavily infiltrated with MNCs (Fig. 7.2 b). In the NOD/Wehi male mice seven of eight grafts were present with peri-islet infiltrate (Fig. 7.2 c) and only one graft was fully rejected. In contrast in the NOD/Lt female mice, all animals (five of five) had rejected their grafts at this stage showing a large pleomorphic infiltrate with many eosinophils, some neutrophils and many macrophages including multinucleated giant cells, as



Graph 7.5: Recovery of CD4+ve T cells in various strains of mice that had been given a single intra-peritoneal injection of 0.3mg of GK1.5 MAB (mean±SD; n=6).

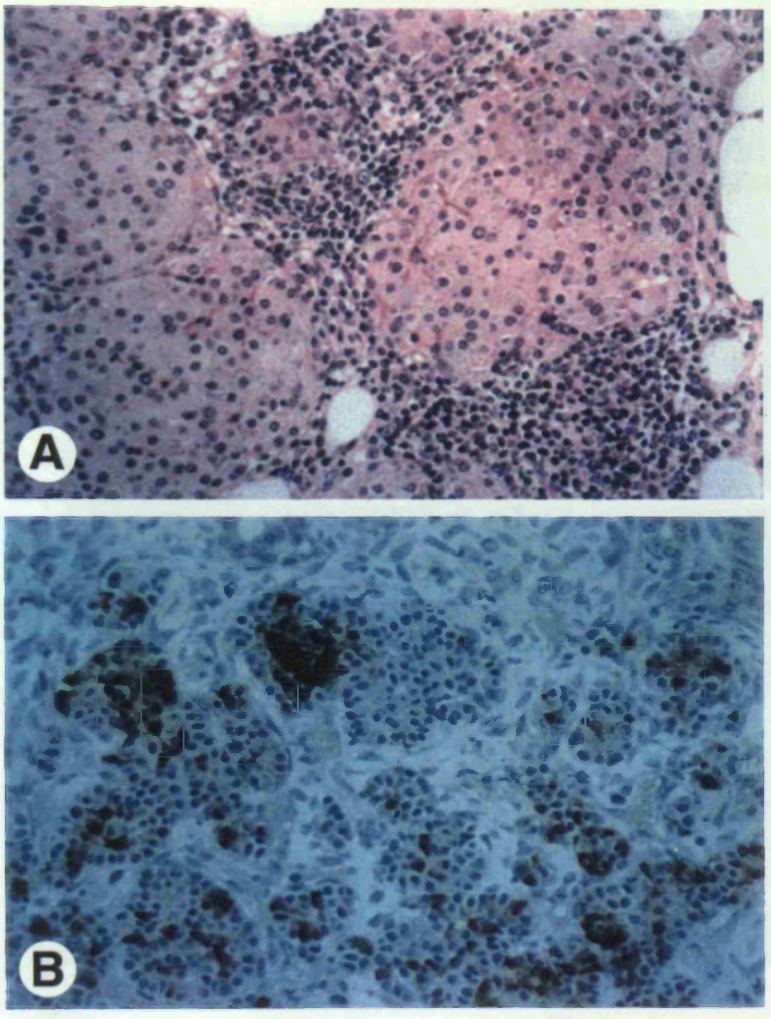


Fig. 7.1: Foetal pig pancreas xenografts 14 days post-transplantation in NOD mice immunosuppressed with GK1.5 peri-transplant.
(a) Xenograft from a NOD/Lt female mouse showing intact islets with peri-islet infiltrate (H&E, x250).
(b) Xenograft from a NOD/Wehi male stained with IMP showing intact islets with no infiltrate (Insulin, x250).

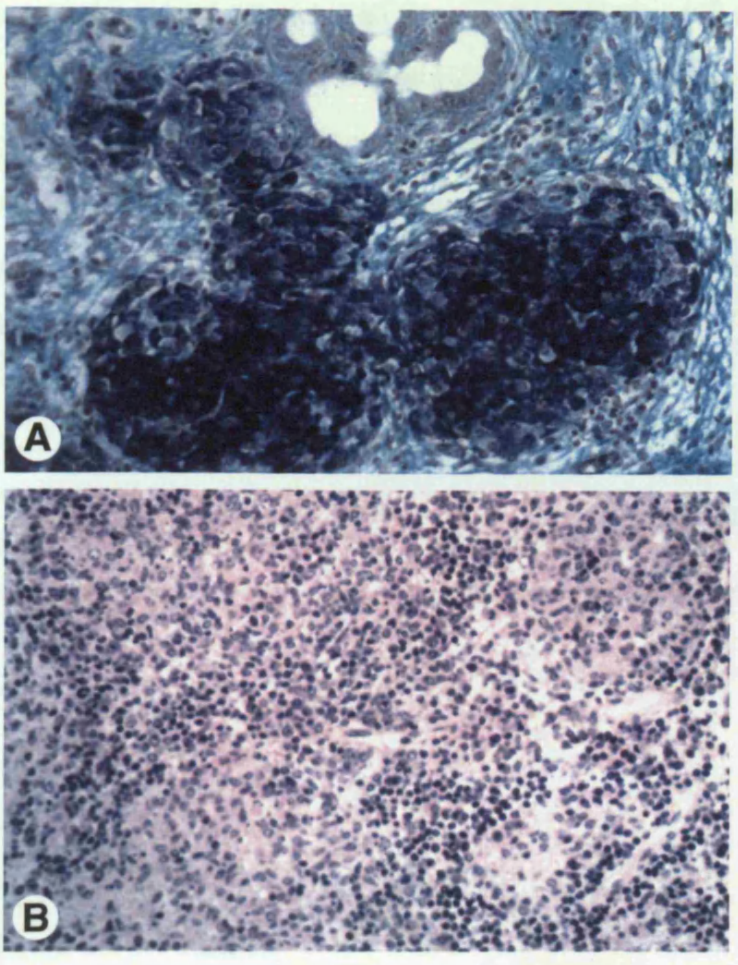


Fig. 7.2: Foetal pig pancreas xenografts 28 days post-transplantation in CBA mice immunosuppressed with GK1.5 peri-transplant.
(a) Xenograft from a CBA male showing well developed islets and ducts, the β cells are stained positive for GAF and no infiltrate of the graft site (GAF, x250).
(b) Xenograft from a CBA female mouse showing islets heavily infiltrated with MNCs(H&E, x250).

well as smaller mononuclear cells being present at the graft site (Fig. 7.2 d). The results of graft and infiltrate scores are summarised in Table 7.1.

The grafts present in the BALB/c females CBA males and NOD/Wehi male mice were clearly composed of islet cells, (despite their negative reaction to the GAF stain) as confirmed by immunoperoxidase staining, clearly showing all three major endocrine cell types (α, β and δ cells). On occasions when a graft was not obvious on H&E staining, as in the case of the CBA grafts for female mice on day 28, isolated cells or small clusters of hormone positive cells were still seen by immunocytochemistry. In grafts that were being rejected there was a heavy accumulation of granulocytes, particularly eosinophils, but these cells appeared after the initial infiltration with mononuclear cells.

Table 7.1

Strains		Day	Grafts present/total	>2+/total	Infiltrate mean
BALB/c	(F)	14	5/5	5/5	0.0
		28	4/4	4/4	1.3
CBA	(F)	14	7/7	7/7	0.0
		28	3/7	1/7	2.8
	(M)	14	8/8	8/8	0.0
		28	8/8	8/8	1.8
NOD/Lt	(F)	14	4/4	4/4	1.8
		28	0/5	0/5	3.5
NOD/Wehi	(M)	14	8/8	8/8	0.0
		28	7/8	7/8	2.2

Table 7.1: The graft and infiltration scores in BALB/c, CBA, NOD/Wehi and NOD/Lt mice on days 14 and 28 post-transplantation.

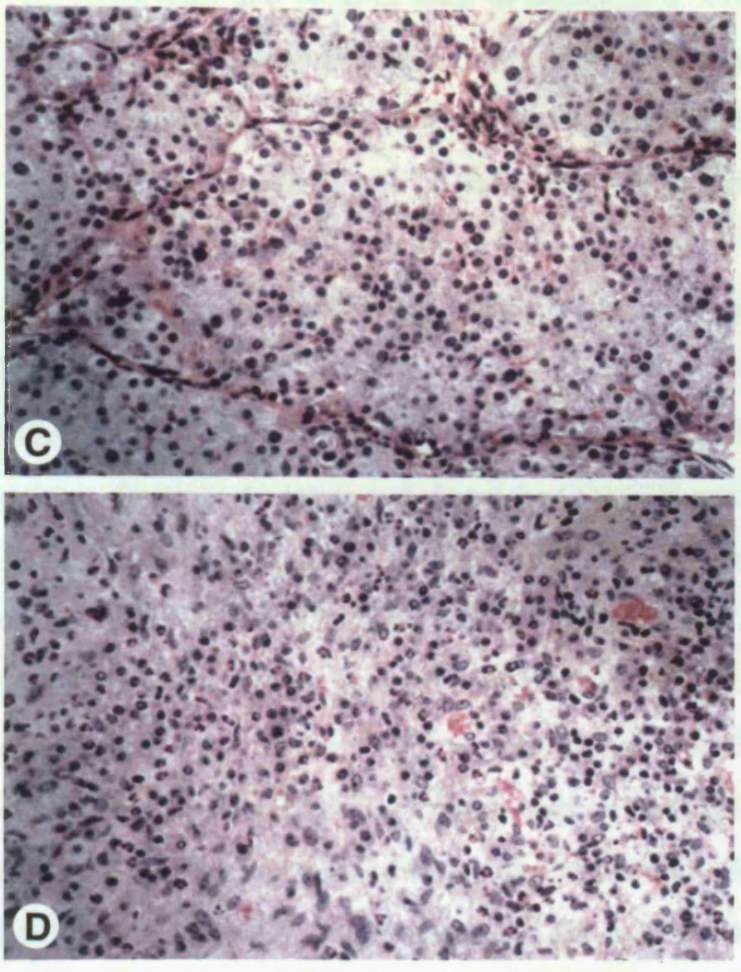


Fig. 7.2: Foetal pig pancreas xenografts 28 days post-transplantation in NOD mice immunosuppressed with GK1.5 peri-transplant.
(c) Xenograft from a NOD/Wehi male mouse showing intact islets with minimal peri-islet infiltrate (H&E, x250).
(d) Rejected xenograft from a NOD/Lt female mouse showing a large pleomorphic infiltrate with many eosinophils and some neutrophils (H&E, x250).

7.3.4 The effect of peri-transplant GK1.5 on PBL, spleen and LN cells

7.3.4.1 PBL

Anti-CD4 peri-transplant treatment greatly reduced the numbers of CD4+ve T cells in the CBA, BALB/c, NOD/Wehi and NOD/Lt mice to undetectable levels at week 1 post-treatment. The rate of recovery of these cells varied in the different strains (but not in different sexes), with the most rapid recovery in the NOD/Lt mice, as previously seen in section 7.3.2. At 28 days (27 days after the last dose), the NOD/Lt mice had a substantial recovery, to approximately 50% of control levels, compared to the slow recovery of CD4+ve cells in the CBA and BALB/c mice. The B cells and CD8+ve cells remained within normal range.

7.3.4.2 Spleen

The administration of 3 doses of GK1.5 in the peri-transplant period markedly reduced the proportion of CD4+ T cells in the spleen and altered the CD4/CD8 ratio from a mean of over 2 (2.05 in CBA, 2.15 in BALB/c and 2.35 in NOD/Lt) in the control mice at 13 days to 0.01 in CBA, 0.04 in BALB/c mice and 0.34 in NOD/Lt mice. However, by day 27 after the last dose of GK1.5 the CD4/CD8 ratio had started to recover, especially in NOD/Lt mice, but there was no good correlation between T cell recovery and graft survival since both the CBA and BALB/c mice had a similar degree of recovery of their splenic cells but big differences in their graft survival. A feature of the T cell recovery was the rapid regeneration of splenic CD4+ve cells in NOD/Lt mice which showed that there was about a 35% recovery by day 27 in comparison with less than 20% recovery in the CBA and BALB/c mice.

7.4 DISCUSSION

There are consistent differences seen in the numbers and proportions of T and B cells in the CBA, BALB/c, C57BL/6 and NOD/Lt mice. C57BL/6 mice are frequently used for transplantation experiments and in this study they clearly differed from other strains examined, both in the predominant circulating leucocytes in the blood, in their lymphoid organs and in containing the least number of T cells of all strains. However, C57BL/6 also differed from the other strains in that they had low relative and absolute numbers of T cells and particularly low numbers of $CD4^+$ T cells. The blood CD4/CD8 ratio is generally around 2 in most mouse strains whereas in C57BL/6 mice it is usually around 1. While $CD4^+$ T cells are relatively

sparse the number of $CD8^+$ T cells is similar to that seen in the other strains. This status is not confined to the circulating cells; the same was shown when the spleen and LN profiles were examined and the C57BL/6 mice were again different from the other strains although the differences were not as marked as in the blood. It may be that the small absolute number of CD4+ve T cells as well as their relatively low percentage is responsible for the ability of C57BL/6 mice to react poorly to a xenograft and respond well to anti-T cell immunosuppressants. While the percentage recovery of $CD4^+$ T cells in the blood relative to the number of these cells present in untreated controls after a single dose of MAb was as rapid as in other strains, by 6 weeks the absolute number of these cells was still quite low. By contrast, NOD/Lt mice that have been reported to be immunologically high responders (Leijon *et al,* 1994), have high numbers of CD4+ve cells and regenerate these much more rapidly despite the excellent initial depletion produced by the MAb. The early CD4+ve cell recovery, is still incomplete even 6 weeks after treatment, but by this time there are as many $CD4^+$ T cells in the blood of NOD/Lt mice as there are in non-immunosuppressed C57BL/6 mice. Perhaps this accounts, in part at least, for the rapid recovery of immune responsiveness in this strain. Spleen cells also showed a similar initial depletion and rapid recovery in NOD/Lt mice.

I have shown in this study that there are consistent differences both in the numbers and proportions of circulating lymphocytes in various strains of normal mice and that the mice differ in their response to the effects of an anti-T cell MAb both as determined by xenograft survival and recovery of splenic and circulating CD4+ve cells. Also I have shown that temporary immunosuppression with GK1.5 prolongs xenograft survival in NOD, CBA and BALB/c in both male and female mice, however rejection occurs in most mice with a marked variation in its rate. There are reports in the literature on the effects of immunosuppression on graft survival showing that MHC-disparate allografts can often be accepted, even with the development of tolerance, after treatment with a variety of immunosuppressive agents, including anti-T cell MAbs (Wood, 1993). There are also reports of apparently prolonged discordant xenograft survival in certain strains of mice without subsequent rejection when further treatment was withheld.

Results from this study show a strong strain and sex effect on the survival of xenografts. This variation in xenograft survival in inbred mice may have parallels in the innate

variation of immune responsiveness of individuals in outbred populations. Tests of their ability to respond to a challenge may need to be assessed prior to transplantation, and perhaps *in vitro* assays of the ability of T cells to respond against potential graft cells may give a guide. This could be done in islet transplantation since stimulator cells from the foetal donor would be available while the pancreas was being cultured.

CHAPTER 8 THE IMMUNOMODULATION OF GRAFTS AND THE EFFECT OF PERI-TRANSPLANT AND CONTINUOUS ANTI-CD4 OR ANTI-CD3 MONOCLONAL ANTIBODY THERAPY ON FOETAL PIG PANCREAS XENOGRAFTS IN NOD MICE

8.1 INTRODUCTION

It has previously shown that foetal pig pancreas is less susceptible to rejection than allogeneic pancreas in immunosuppressed NOD/Lt female mice (Mandel *et al,* 1989; Mandel *et al,* 1990a; Mandel *et al,* 1990b) and may also be resistant to disease recurrence in recipients treated with peri-transplant anti-CD4 monoclonal antibody. However, the results of this study have not been able to repeat the encouraging results of long-term survival and tolerance of foetal pig proislets grafts in CBA mice reported by Simeonovic *et al,* (1990a). Reports published by that group have suggested that tolerance to xenogeneic (foetal pig) proislets is possible in mice made diabetic with streptozotocin and immunosuppressed with anti-CD4 MAb. The reasons for failure may be because less anti-CD4 MAb was used and may also relate to inherent differences in the ability of different strains of mice to mount an anti-graft immune response as reported in chapter 7.

Anti-CD3 MAbs are powerful immunosuppressants in humans but a number of problems are associated with their use. These include toxic reactions presumably due to the release of large amounts of cytokines, and the development of antibodies against the foreign immunoglobulins. There have been few studies in rodents on the effect of anti-CD3 treatment in transplantation and most have used a hamster MAb, 145-2C11 that is effective in prolonging skin allograft survival (Henrickson *et al,* 1995) and is also very effective in preventing diabetes in NOD mice (Hayward and Shriber, 1992). However, this MAb has side effects similar to OKT3, and may be lethal in mice.

In this study the role of "immunomodification" of the foetal pig islets using 90%O_2/10%CO_2 (HiO_2) cultures were compared to that of foetal pig islets grown in "conventional culture" (CC) 10%CO_2/90% air, also graft survival using either peri-transplant or peri-transplant and continuous treatment with anti-CD4 was investigated. In a separate study the survival of organ cultured foetal pig pancreas grown either in HiO_2 or CC in NOD/Lt

female mice immunosuppressed with peri-transplant or peri-transplant and weekly treatment using an anti-CD3 MAb, KT3 (Tomonari, 1988) was investigated.

8.2 MATERIALS AND METHODS

8.2.1 Mice

In the first part of the experiment I used NOD/Lt female mice (about 100 days old) as recipients; they were immunosuppressed with anti-CD4 MAb (GK1.5) on days -1, 0, 1, 3, 7, 10, 14 and 21 (0.3mg/dose). Control mice were given MTPBS in place of GK1.5. In the second part of the experiment I used NOD/Lt female mice (about 100 days old) as recipients and they were immunosuppressed with either peri-transplant or peri-transplant and weekly doses of KT3 (2.5 mg/dose).

8.2.2 Foetal Pancreas

Foetal pigs were obtained from a local abattoir and gestational age was estimated from foetal weight and crown-rump length as being around 70-90 days. There was a significant warm ischemia time since the foetuses were obtained only when the sows were eviscerated with an interval of 15-30 min between the death of the sow and the placement of the foetal pigs on ice. The pancreas was removed under sterile conditions, diced into pieces approximately of $1mm^3$ and placed in organ culture as previously described in chapter 2. The pig foetal pancreas were cultured either at 10%CO_2/90% air (CC) for 5 days or 90%O_2/10CO_2 (HiO_2) for 2 days followed by 3 days in 10%CO_2/90% air when graft "immunomodification" was used.

8.2.3 Transplantation

GK1.5 treated and control mice were transplanted under the left renal capsule with a single piece of foetal pig pancreas whereas KT3 treated mice were transplanted bilaterally with one piece of foetal pig pancreas per kidney. The graft was placed under the kidney capsule and milked away from the incision. The kidneys were carefully placed back into the abdominal cavity so that the grafts were not dislodged. The skin incisions were closed with clips. In the GK1.5 treated and the control animals the grafts were removed on day 40 post-transplant, ie 19 days after the last dose of GK1.5. The left kidney (including the attached adrenal gland) and the host pancreas were placed in Bouin's fixative. The spleen was excised for FACS analysis of lymphocyte subsets using MAbs for T and B cells. The graft sites were identified, carefully cut

out and placed in individually numbered vials for processing for histological assessement by light microscopy. Graft sites were serially sectioned at 4 μm and alternate slides, each with 4-6 serial sections, were stained with H&E for assessment of infiltration, with GAF for assessment of β cell survival, and by immunocytochemistry for insulin and other hormones. The latter was of particular use with foetal pig grafts since these usually stained either poorly or not at all with the GAF stain, but stained well with the immunoperoxidase method. The slides were coded and examined in random order. The graft sites were assessed for the presence of a graft and scored on a 0-4+ ranking; 0 representing no graft while 4+ represented a large well-differentiated graft. Infiltration was also scored on a 4 grade scale; 0 representing no infiltration and 4+ being an extensive pleomorphic infiltrate in a large swollen graft site.

In the animals treated with KT3 p/t or p/t and weekly the grafts were removed on days 37 or day 70 by left nephrectomy and with the adrenal gland attached placed in Bouin's fixative and processed for light microscopy. Usually the lower pole of the spleen was excised for FACS analysis of lymphocyte subsets. At the end of the experiment, the animals were killed (day 84 post-transplantation) and the right kidney and host pancreas processed for light microscopy as above. The rest of the spleen was removed and dispersed into a single cell suspension for FACS.

8.2.4 Flow cytometry analysis

For FACS spleen and PB cells were made into a single cell suspension (chapter 2). T cells were detected with anti-Thy 1.2 and anti-CD3 MAbs, T cell subsets with anti-CD4 and anti-CD8 MAbs, and B lymphocytes by surface Ig expression.

8.3 RESULTS

8.3.1 Flow cytometry results

GK1.5 peri-transplant treatment resulted in an acute depletion of CD4+ve cells in the PB and spleen and continuous treatment maintained CD4+ve cells at reduced but not absent levels (Fig. 8.1). In contrast, KT3 treatment only blocked the expression of CD3 and many Thy 1.2+ve/CD3-ve cells were seen (Fig. 8.2). There was, however, some loss of T cells as the total CD4 and CD8 T cell number was reduced compared to the numbers present in the untreated mice.

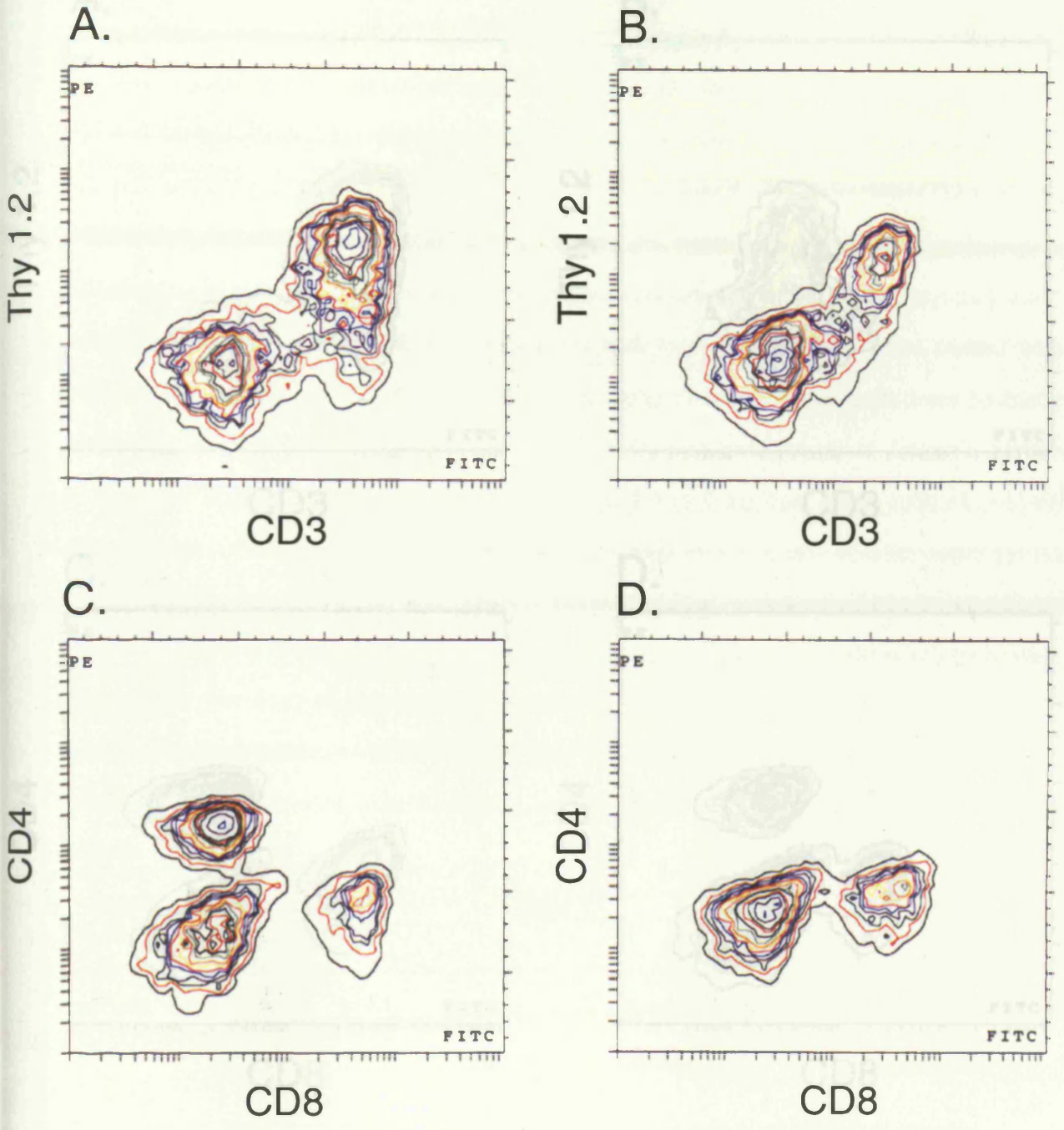


Fig. 8.1: Peripheral blood samples of control and anti-CD4 treated NOD/Lt female mice.
(A) Control, stained for Thy 1.2 (PE) and CD3 (FITC)
(B) GK1.5-treated, stained for Thy 1.2 (PE) and CD3 (FITC)
(C) Control, stained for CD4 (PE) and CD8 (FITC)
(D) GK1.5-treated, stained for CD4 (PE) and CD8 (FITC)

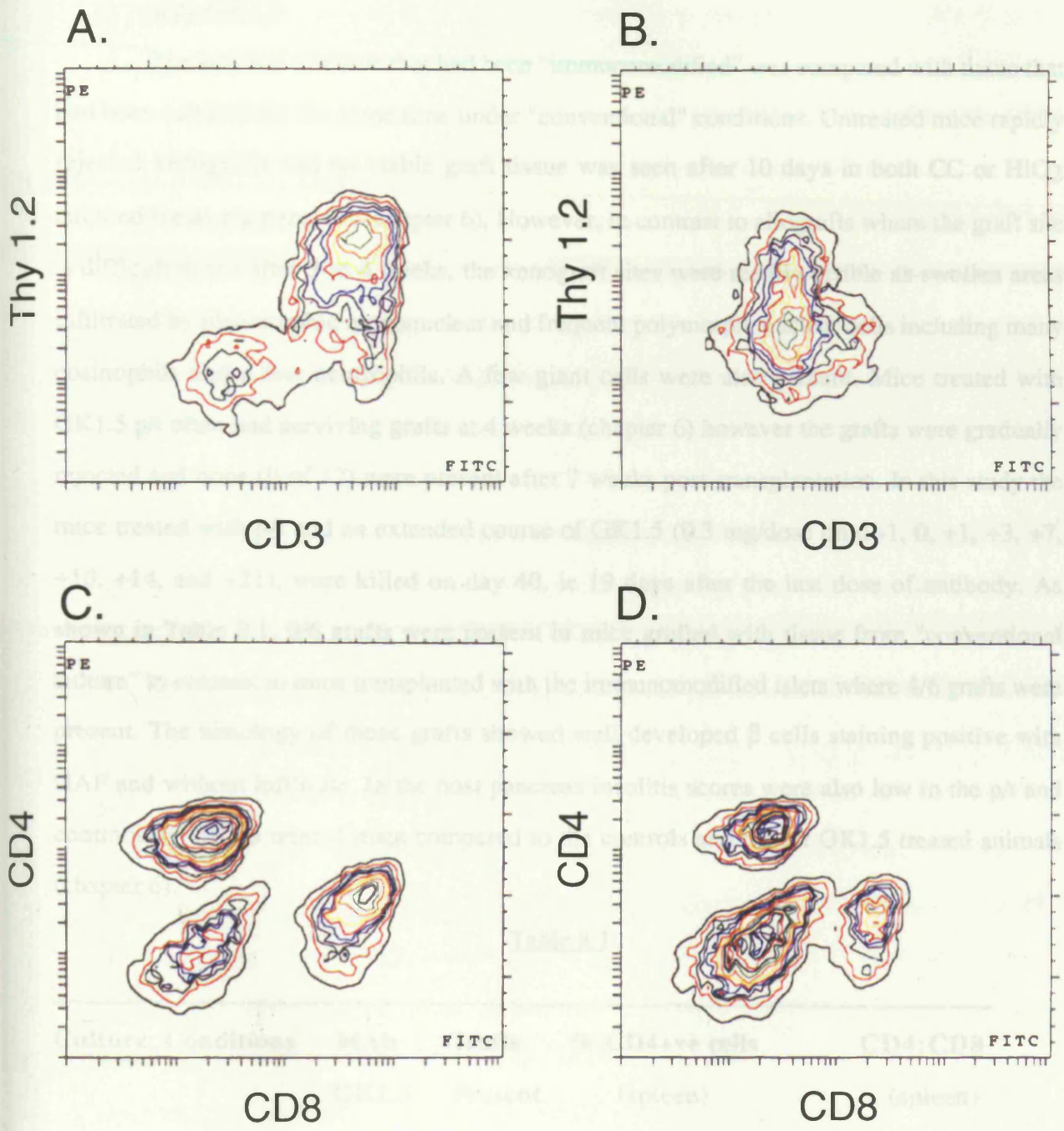


Fig. 8.2: Peripheral blood samples of control and anti-CD3 treated NOD/Lt female mice.

(A) Control, stained for Thy 1.2 (PE) and CD3 (FITC)
(B) KT3-treated, stained for Thy 1.2 (PE) and CD3 (FITC)
(C) Control, stained for CD4 (PE) and CD8 (FITC)
(D) KT3-treated, stained for CD4 (PE) and CD8 (FITC)

8.3.2 Effect of organ culture in 90% O_2 on xenografts in NOD/Lt female mice treated with GK1.5

The survival of tissue that had been "immunomodified" was compared with tissue that had been cultured for the same time under "conventional" conditions. Untreated mice rapidly rejected xenografts and no viable graft tissue was seen after 10 days in both CC or HiO_2 cultured foetal pig pancreas (chapter 6). However, in contrast to allografts where the graft site is difficult to see after 3 to 4 weeks, the xenograft sites were readily visible as swollen areas infiltrated by pleomorphic mononuclear and frequent polymorphonuclear cells including many eosinophils and a few neutrophils. A few giant cells were also present. Mice treated with GK1.5 p/t often had surviving grafts at 4 weeks (chapter 6) however the grafts were gradually rejected and none (0 of 12) were present after 7 weeks post-transplantation. In this study the mice treated with p/t and an extended course of GK1.5 (0.3 mg/dose on d -1, 0, +1, +3, +7, +10, +14, and +21), were killed on day 40, ie 19 days after the last dose of antibody. As shown in Table 8.1, 0/6 grafts were present in mice grafted with tissue from "conventional culture" in contrast to mice transplanted with the immunomodified islets where 4/6 grafts were present. The histology of those grafts showed well developed β cells staining positive with GAF and without infiltrate. In the host pancreas insulitis scores were also low in the p/t and continously GK1.5 treated mice compared to the controls and the p/t GK1.5 treated animals (chapter 6).

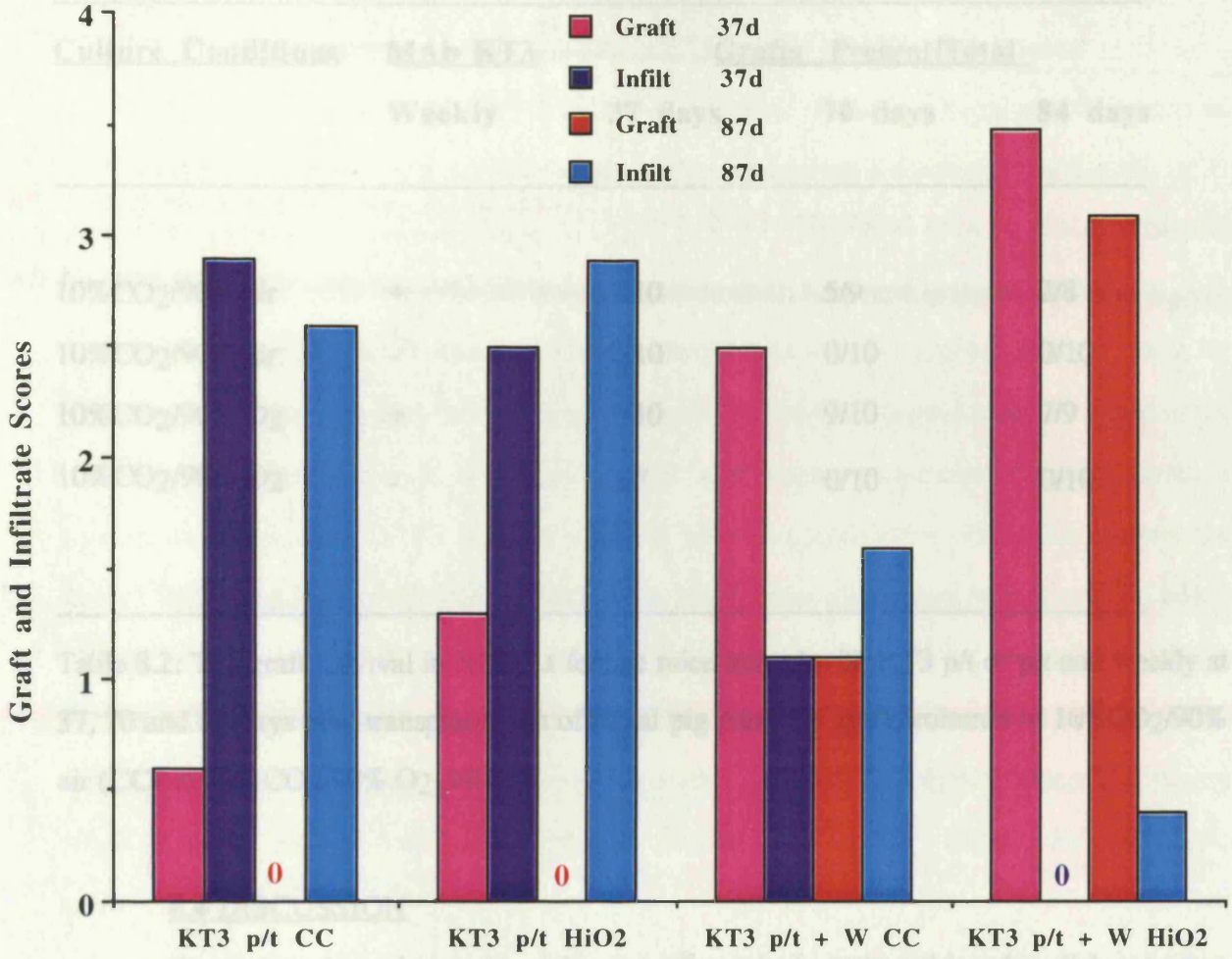
Table 8.1

Culture Conditions	**MAb GK1.5**	**Grafts Present**	**% CD4+ve cells (spleen)**	**CD4:CD8 (spleen)**
10%CO_2/90% air	+	0/6	6.9	0.41
10%CO_2/90% air	-	0/6	23.4	1.74
10%CO_2/90% O_2	+	4/6	9.4	0.54
10%CO_2/90% O_2	-	0/6	25.9	1.72

Table 8.1: The effect on immunomodification of foetal pig pancreas (90%O_2) and anti-CD4 MAb treatment (on days -1, 0, 1, 3, 7, 10, 14 and 21) on xenograft survival at day 40 post-transplantation.

8.3.3 Effect of organ culture in 90% O_2 on xenografts in NOD/Lt female mice treated with KT3 p/t or p/t and weekly.

Peri-transplant treatment was used with the addition of a weekly dose of KT3 (anti-CD3 MAb, at 2.5 mg/dose) in NOD/Lt female mice transplanted with foetal pig pancreas cultured in either CC or HiO_2. At day 37 post-transplantation KT3 p/t treated mice had most grafts rejected in contrast to animals with KT3 p/t and weekly treatment where most of the grafts were still present. Grafts cultured in HiO_2 appeared to have better graft survival than CC grafts (9/10 excellent grafts in HiO_2 vs 7/10 in CC). Graft survival and infiltrate score at day 37 and 87 after transplantation is shown in Graph 8.1. All mice treated with KT3 p/t at days 70 post-transplantation had completely rejected their grafts and there was intense infiltration at the site with no difference seen between CC and HiO_2 cultured grafts. In contrast, mice treated with p/t and weekly KT3 had excellent graft survival, particularly in mice grafted with HiO_2 cultures: intact and non-infiltrated grafts were present in 9/10 mice and the grafts contained well stained α, β and δ cells. The survival of CC grafts in p/t and weekly KT3 treated mice was less impressive; 2/9 had totally rejected their grafts, 2/9 had residual grafts with heavy MNC infitrate and the remaining 5/9 had essentially intact grafts with little infiltrate. On day 84 post-transplantation the only grafts present were from animals treated with KT3 p/t and weekly: most of the grafts were rejected in the conventionally cultured pancreas (only 2/8 were present) whereas in the HiO_2 cultured pancreas most of the grafts were present with hardly any infiltrate. The graft survival in CC vs HiO_2 and p/t vs p/t and weekly KT3 treatment is summarised in Table 8.2.



Graph 8.1: Graft and infiltrate scores in female NOD/Lt mice 37 and 87 days post-transplantation

Table 8.2

Culture Conditions	MAb KT3	Grafts Present/Total		
	Weekly	37 days	70 days	84 days
10%CO_2/90% air	+	7/10	5/9	2/8
10%CO_2/90% air	-	2/10	0/10	0/10
10%CO_2/90% O_2	+	9/10	9/10	7/9
10%CO_2/90% O_2	-	3/7	0/10	0/10

Table 8.2: The graft survival in NOD/Lt female mice treated with KT3 p/t or p/t and weekly at 37, 70 and 84 days post-transplantation of foetal pig pancreas grafts cultured in 10%CO_2/90% air (CC) vs 10%CO_2/90% O_2 (HiO_2).

8.4 DISCUSSION

These data show that prolonged xenograft survival can be achieved in diabetes-prone mice, with the MAb therapy used in this study, however indefinite survival was not achieved and there was no evidence of tolerance. This study shows that, under some circumstances, islet xenografts from a discordant donor species show surprisingly good survival in animals that are immunosuppressed with p/t and continuous GK1.5, anti-CD4 MAb that depletes CD4+ve cells and excellent xenograft survival if the pancreas is cultured in HiO_2. The inability to fully deplete CD4+ve cells could mean that the few CD4+ve cells remaining may be sufficient to initiate rejection. The rejection of the xenografts seems largely to depend on the presence of CD4+ve T helper cells, as suggested by others (Simeonovic *et al,* 1990a; Simeonovic *et al,* 1990b) and continuous treatment with anti-CD4 MAb may block the function of the effector cells that may escape initial anti-CD4 treatment.

In these experiments I was not successful in producing indefinite xenograft survival although extended survival was seen in mice continuously treated with GK1.5 and transplanted

with HiO_2 cultured grafts although the grafts appeared to be undergoing slow rejection. Although anti-CD4 treatment is very effective in less responsive strains of mice (eg CBA, BALB/c), it is a less effective in highly responsive strains like NOD/Lt female mice.

On the other hand, anti-CD3 treatment is effective in allowing foetal pig xenografts to survive essentially intact, even in mice that are highly immunologically responsive like the NOD mice. The immunosuppressive effect of anti-CD3 MAb occurs despite the presence of relatively large numbers of T cells, but requires continuous treatment. Excellent graft survival was seen in the continuously KT3 treated mice and HiO_2 cultured grafts with 7/9 grafts scoring 4+ with no infiltrate compared with only 2/8 grafts seen in continuous KT3 treated and CC grafts at 12 weeks post-transplantation. It is interesting that such treatment remains effective, since in humans treatment with OKT3 is often nullified by the appearance of antibodies against the foreign immunoglobulins (Chatenoud and Bach, 1990). Also of interest is the apparent safety of KT3; this contrasts with the often lethal effect of another frequently used anti-CD3 MAb (145-2C11) in mice when they are treated with this MAb alone. I saw no deleterious effects with KT3, either acutely or after chronic treatment. Thus, KT3 treatment seems to be very effective and it may be useful to combine it with anti-CD4 to see whether tolerance can be achieved.

Pre-transplant treatment of a graft to eliminate highly immunogenic cells (ie "passenger leucocytes") has been widely used in experimental islet allografts in rodents of many strains with marked success (Lacy, 1994). The rationale behind immunomodification is that by selective removal from the graft of antigen presenting cells (APC) by whatever means (eg exposure to high concentrations of O_2, low temperature culture or ultraviolet light), the capacity of the graft to present alloantigens directly, ie by donor APC, is eliminated and recognition of the graft is via indirect antigen presentation alone. Whether direct antigen presentation plays any role in xenotransplantation remains controversial but there are studies that support this concept (Linsley *et al,* 1992; Lucas, 1990) although the effect may be highly species or even strain dependent.

CHAPTER 9 REVERSAL OF DIABETES IN SPONTANEOUSLY DIABETIC NOD/LT MICE USING FOETAL PANCREAS ISO-, ALLO- (CBA) AND XENOGRAFTS (PIG)

9.1 INTRODUCTION

Due to the serious side effects of currently used immunosuppressants, pancreas transplantation for the treatment of IDDM is generally restricted to patients that are in end-stage renal failure (ESRF) and require a renal allograft (Sutherland, 1992; Robertson, 1991). Patients with IDDM in ESRF usually have severe long-term complications of chronic diabetes including retinopathy, neuropathy and often macrovascular diseases. Most of these advanced complications, once established, appear to be irreversible, and while neuropathy may be improved (Kennedy *et al,* 1990), retinopathy may, at best, be stabilised (Ramsey *et al,* 1988). However, studies in experimental animals indicate that islet transplantation can prevent the development of early diabetic complications and therefore prevention may also be possible if successful islet transplants are performed in the early stage of IDDM. Britland *et al,* (1991) showed that islet transplantation can prevent neuropathy and nephropathy in spontaneously diabetic BB rats transplanted with islets (Woehrle *et al,* 1990).

The cure for IDDM will require some form of islet replacement by either transplantation of insulin producing allogeneic, xenogeneic, or genetically engineered cells, or possibly by the regeneration of β cells from preserved progenitors in the diabetic pancreas. The latter approch was used by Bonner-Weir and colleagues (1993) who have demonstrated regeneration of islet cells in the host pancreas in partial pancreatectomized young adult rats.

Previously it has been shown that organ cultured foetal pig pancreas can survive for more than 5 months in prediabetic NOD/Lt mice immunosuppressed with a combination of anti-CD3 and anti-CD4 monoclonal antibodies (MAbs), if immunomodified tissue is used (Mandel and Koulmanda, 1992b; Mandel and Koulmanda, 1993a). In this chapter I have studied the development and differentiation of discordant foetal pig pancreas xenografts and their ability to reverse chronic diabetes in spontaneously diabetic NOD/Lt female mice. Foetal mouse pancreas iso- and allografts were also be used to study their ability to reverse chronic spontaneous diabetes in NOD/Lt female mice immunosuppressed with anti-CD4 and anti-CD3 MAb therapy

which has previously been shown to be effective in maintaining foetal pig islet xenografts. Earlier, when (STZ) induced diabetic animals (chapter 4) were used, I showed that foetal islet isografts were able to reverse acute and chronic diabetes and maintain euglycaemia and (as expected) when the grafts were removed by nephrectomy the mice reverted to hyperglycaemia. In contrast, as shown in this chapter, the removal of a successfully established xenograft from previously diabetic NOD/Lt mice did not lead to hyperglycaemia suggesting possible regeneration of β cells in the host pancreas. Animals transplanted with either iso- or allografts, as expected, once the graft was removed became hyperglycaemic.

9.2 MATERIALS AND METHODS

9.2.1 Foetal pig pancreases

Foetal pigs were obtained from a local abattoir and their gestational age was estimated from foetal weight and crown-rump length. Since the foetal pigs were obtained in the course of routine operations of the abattoir and since a range of commercial pig strains was used, gestational ages were approximate, estimated generally to be between 90-100 days. There was a significant warm ischaemia time since the foetuses were obtained only when the sows were eviscerated with an interval of 15-30 min between the death of the sow and the placement of the foetal pigs on ice. The pancreases were isolated under sterile conditions and placed in organ culture and maintained in the gas phase of 90%O_2/10%CO_2 for two days followed by 3 days in 10%CO_2/90% air. The organ cultured pieces were transplanted under the renal capsule of the NOD/Lt diabetic recipients as previously described in chapter 2.

9.2.2 Foetal mouse pancreas

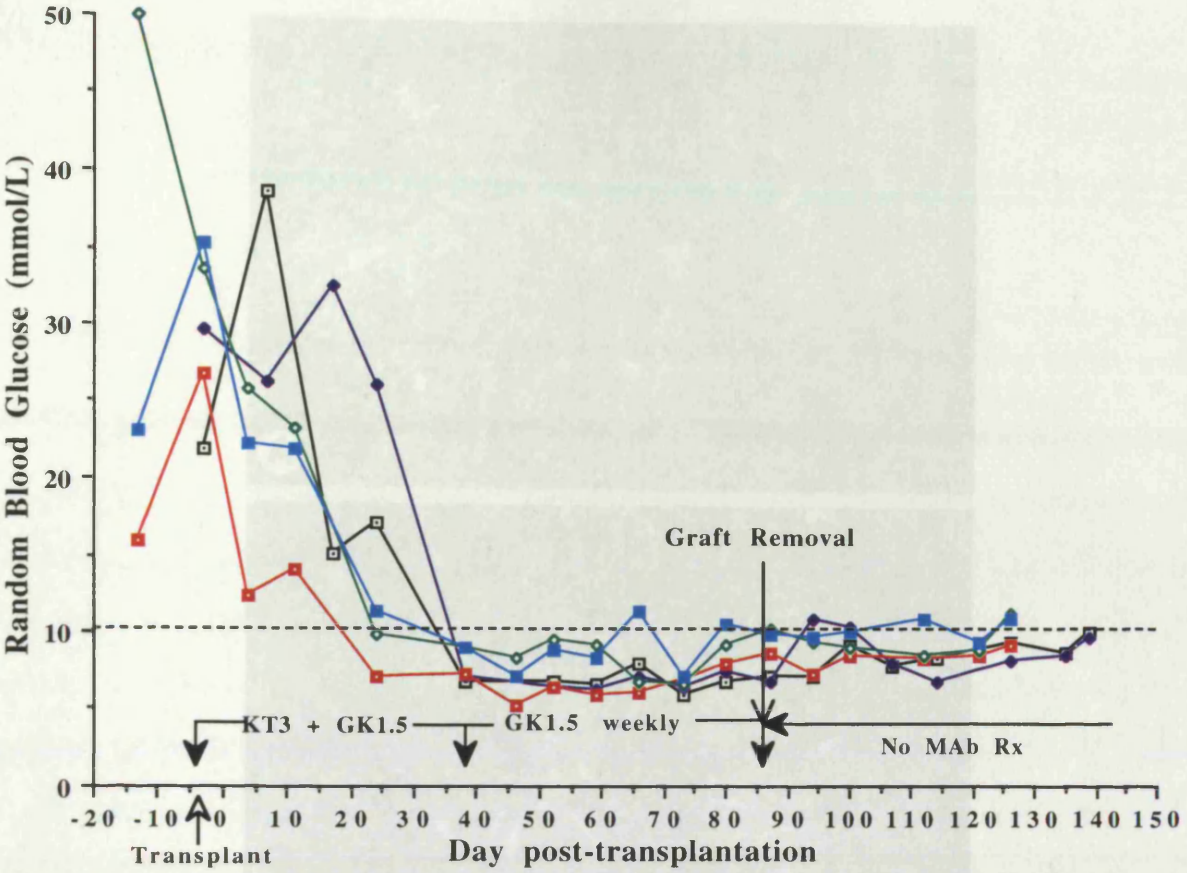
Foetal 17-18 day NOD/Lt pancreas were used for isografts and foetal 17-18 day CBA pancreas were used as major mismatch allografts. Each foetus was aseptically removed from the uterus and the pancreas was isolated under sterile conditions using a dissecting microscope and placed in organ culture and maintained in gas phase of 10%CO_2/90% air for 10 days. The organ culture technique is described in chapter 2. At the time of transplantation one foetal pancreas was placed in Bouin's fixative and processed for histological assessment. The organ cultured pieces were transplanted under the renal capsule of the NOD/Lt diabetic recipients as previously described in chapter 2.

9.2.3 Recipients

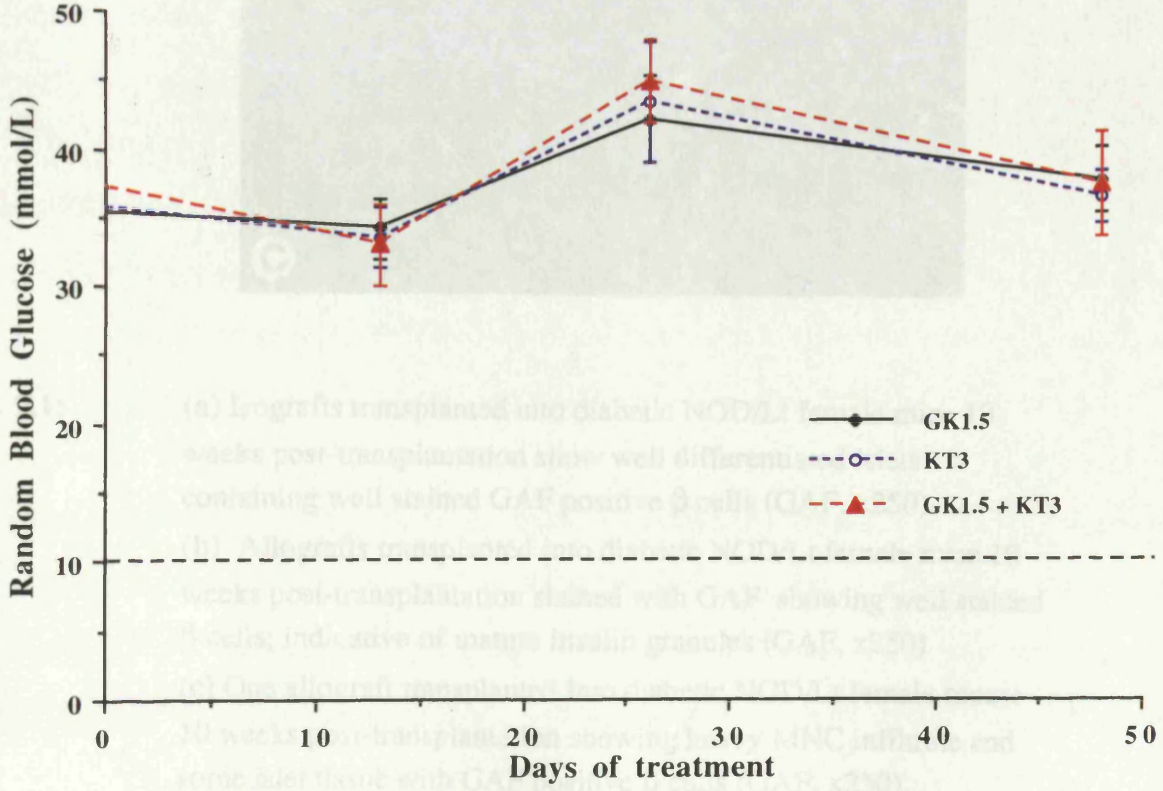
Inbred NOD/Lt female mice of various ages (>120 days) with spontaneous diabetes of more than 14 days duration were used. Mice were regarded diabetic if they had at least two random blood glucose (RBG) levels more than 20 mmol/L at least 1 week apart. The mice were treated daily with parenteral insulin (2 to 4 U ultralente) pre- and post-transplantation.

Groups of animals that were going to be transplanted with iso-, allo- or xenografts were immunosupressed with anti-CD3 MAb (KT3) at 1 mg per dose and anti-CD4 MAb (GK1.5) at 0.3 mg per dose peri-transplant (on days -1, 0, +1), then weekly for 6 weeks with both MAbs at the above doses, followed by GK1.5 MAb weekly until the graft was removed by a left nephrectomy. In a separate experiment, the effect of immunosuppression and or insulin on the reversal of spontaneous diabetes in the NOD/Lt female mice was tested in four groups of 10-12 mice per group. Group 1; animals received GK1.5 only at 0.3 mg per dose, group 2; animals received KT3 only at 1 mg per dose, group 3; the animals received KT3 at 1 mg per dose and GK1.5 at 0.3 mg per dose at days -1, 0, +1, then weekly for 6 weeks with both MAbs at the same dose, followed by GK1.5 MAb weekly and group 4; diabetic non-immunosuppressed NOD/Lt mice treated with insulin only.

The mice were bled for random blood glucose (RBG) and body weights (BW) were measured at weekly intervals. Mice were taken off parenteral insulin when RBG readings were less than 15 mmol/L. The graft was removed by left nephrectomy (chapter 2) at either 77 or 90 days post-transplantation and placed in Bouin's fixative. The graft sites were identified, carefully cut out and placed in individually numbered vials for processing for light microscopy. Graft sites were serially sectioned at 4 μm and alternate slides, each with 4-6 serial sections, were stained with H&E for assessment of infiltration, with GAF for assessment of β cell survival, and by immunocytochemistry for insulin and other hormones. The slides were coded and examined in random order. The graft sites were assessed for the presence of a graft and scored on a 0-4+, infiltration was also scored on a 0-4+ grade scale. The animals were monitored (RBG and BW) but with no further immunosuppression; at the end of the experiment the mice were killed and the host pancreas removed for histological studies.



Graph 3: Reversal of spontaneous diabetes in NOD/Lt mice with foetal pig pancreas



Graph 4: Spontaneous diabetic NOD/Lt mice treated with MAbs.
(n = 10, mean ± SD)

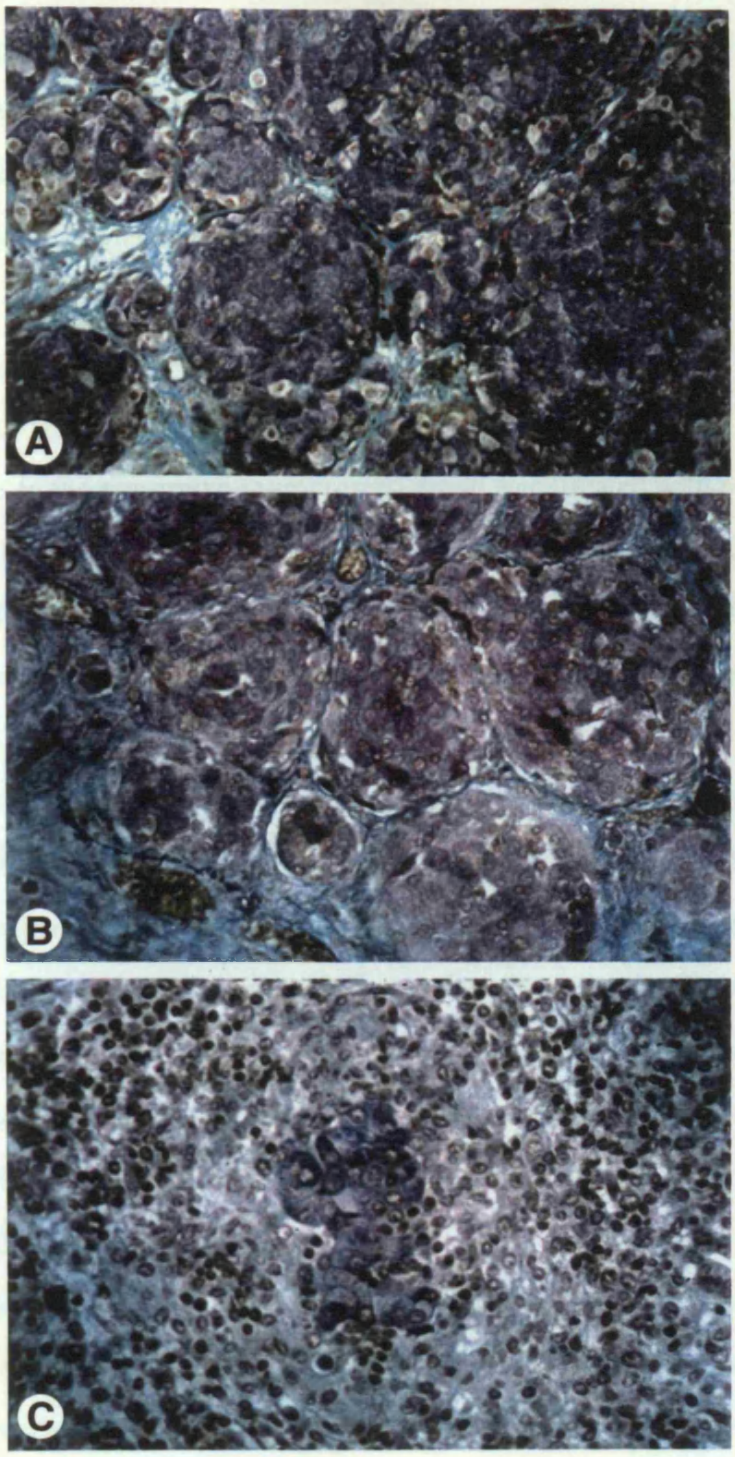


Fig. 9.1: (a) Isografts transplanted into diabetic NOD/Lt female mice 10 weeks post-transplantation show well differentiated islets containing well stained GAF positive β cells (GAF, x250).
(b) Allografts transplanted into diabetic NOD/Lt female mice 10 weeks post-transplantation stained with GAF showing well stained β cells; indicative of mature insulin granules (GAF, x250)
(c) One allograft transplanted into diabetic NOD/Lt female mouse 10 weeks post-transplantation showing heavy MNC infiltrate and some islet tissue with GAF positive β cells (GAF, x250).

9.2.4 Flow cytometry analysis

Peripheral blood leucocyte profiles were assessed by FACS (chapter 2). Total T cells were detected with anti-Thy 1.2 and anti-CD3 MAbs, T cell subsets with anti-CD4 and anti-CD8 MAbs, and B lymphocytes by surface Ig expression.

9.3 RESULTS

9.3.1 Non-transplanted diabetic NOD/Lt mice

The animals in the non-immunosuppressed group were treated with parenteral insulin 2 to 4 U per day. All twelve animals in this group remained hyperglycaemic and some died during the course of the study. In this study using spontaneously diabetic NOD/Lt mice, the animals showed no evidence of β cell regeneration and remained hyperglycaemic during the course of the study.

Animals in the immunosuppressed groups given GK1.5 or KT3 or both MAbs stayed hyperglycaemic during the course of the experiment (Graph 4). The histology of the host pancreas of non-transplanted mice with or without immunosuppression showed atrophic islets lacking β cells and in some cases the acinar pancreas was also atrophic. The insulitis score of those animals was around 100. Thus, in this study the spontaneously diabetic NOD/Lt mice showed no functional or histological evidence of islet regeneration nor any evidence of residual β cells. This observation correlates with previous reports.

9.3.2 Foetal NOD/Lt isografts

Fifteen of sixteen immunosuppressed diabetic NOD/Lt female mice transplanted with foetal mouse isografts reversed diabetes within 35 days post-transplantation. The animals remained normoglycaemic until the grafts were removed by left nephrectomy on day 72 post-transplantation (Graph 1). All sixteen isografts were present at 10 weeks post-transplantation, 15 of the 16 grafts had well preserved and well differentiated islets containing well stained GAF positive β cells with some focal patches of peri-islet infiltrate mostly MNC (Fig. 9.1 a). One graft had heavily infiltrated islets with MNC infiltrate, the β cells were mostly depleted resembling recurrence of the disease. In this animal the histological appearance of the graft resembled that of its pancreas. This animal stayed hyperglycaemic during the course of the experiment (Table 9.1). When the grafts were removed the fifteen animals with functional

grafts reversed to hyperglycaemia within 2-5 days post-nephrectomy and the animals stayed hyperglycaemic until they were sacrificed. The host pancreas of all animals showed atrophic islets with no evidence of β cell staining, the insulitis score of those animals was around 100.

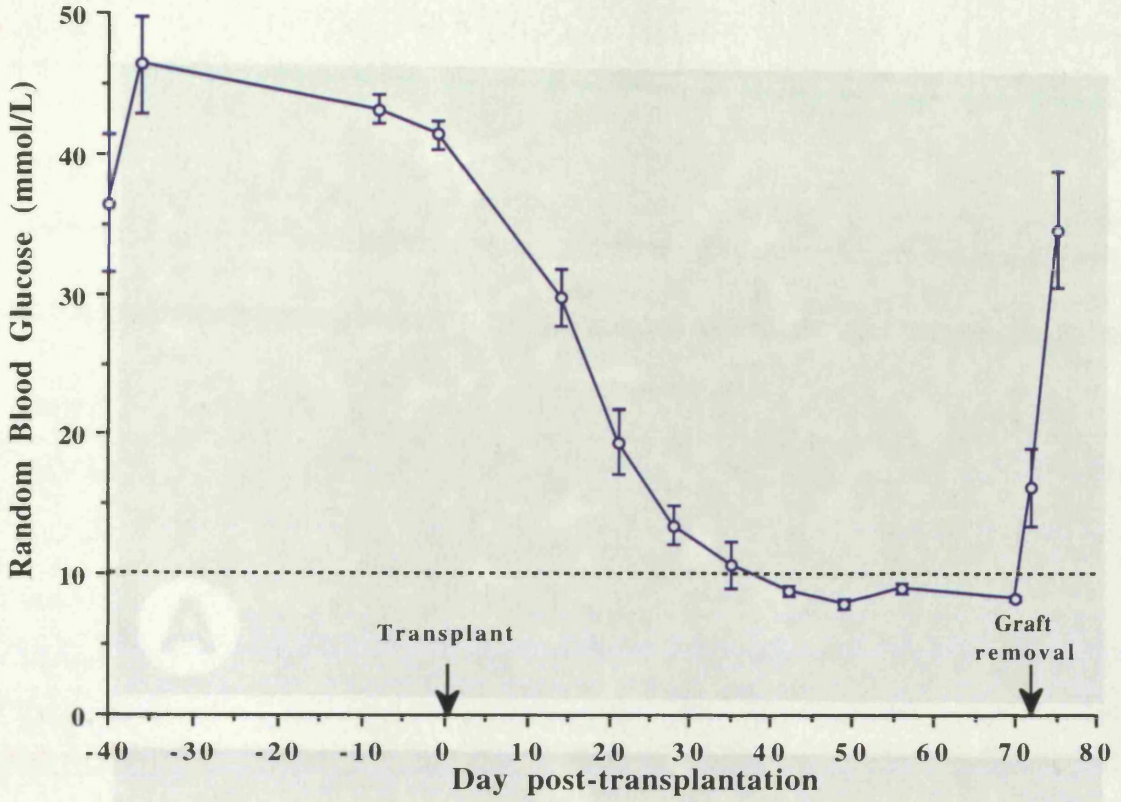
9.3.3 Foetal CBA mouse allografts

The allografts slowly reversed diabetes around the same time as the isografts, however their RBG fluctuated during the normoglycaemic period. Once normoglycaemia was reached the animals were taken off parenteral insulin. When the graft was removed the animals slowly reverted to hyperglycaemia (Graph 9.2). Eight of eleven mice at week 10 had large well differentiated allografts with many well stained GAF positive β cells with either none or minimal peri-islet infiltration of MNC (Fig. 9.1 b). One allograft had islets present with a heavy MNC infiltrate (Fig. 9.1 c), however there were no polymorphonuclear cells, giant cells or eosinophils present as commonly seen in xenograft rejection. Two allografts were totally rejected with minimal residual MNC infiltration at the graft site. The host pancreas of these animals resembled those of the isografted animals and those of the non-transplanted and non-immunosuppressed mice, showing atrophic islets with no evidence of β cell staining. The insulitis score of those animals was around 100.

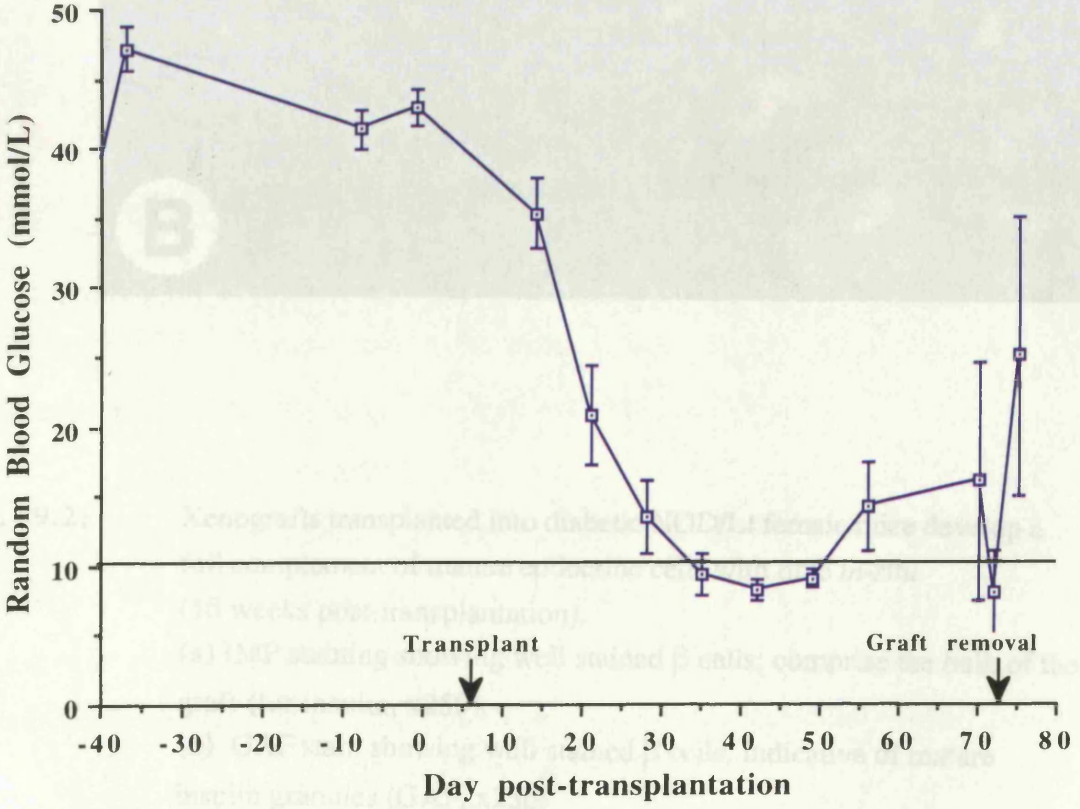
9.3.4 Foetal pig xenografts

Most of the diabetic animals (19 of 24 transplanted) died of severe diabetes before the graft began to function around day 21 post-transplantation. The grafts removed from these animals were all intact with no infiltration and well stained α, β, and δ cells by immunoperoxidase staining but negative by GAF staining. The host pancreas of these animals had a total absence of pancreatic β cells and their endogenous islets were small and atrophic with some α and δ cells present.

Five of the 24 mice that survived slowly reversed their diabetes, usually about 4-8 weeks post-transplantation. The RBG levels were within the normal range for pigs (5-6 mmol/L) rather than the NOD/Lt mouse range (7-10 mmol/L). Once normoglycaemia was reached the animals were taken off parenteral insulin. The grafts were removed by left nephrectomy after 77 or 90 days post-transplantation (Graph 9.3). Grafts from these animals had well differentiated islets containing insulin stained β cells some in mitosis and positive by GAF (Fig. 9.2 a) (Table 9.1). The grafts had no infiltrating cells either intra-islet or peri-islet



Graph 1: Reversal of spontaneous diabetes in NOD/Lt mice with foetal pancreas isografts (n = 15, mean ± SD)



Graph 2: Reversal of spontaneous diabetes in NOD/Lt female mice with foetal CBA pancreas (n = 10, mean ± SD)

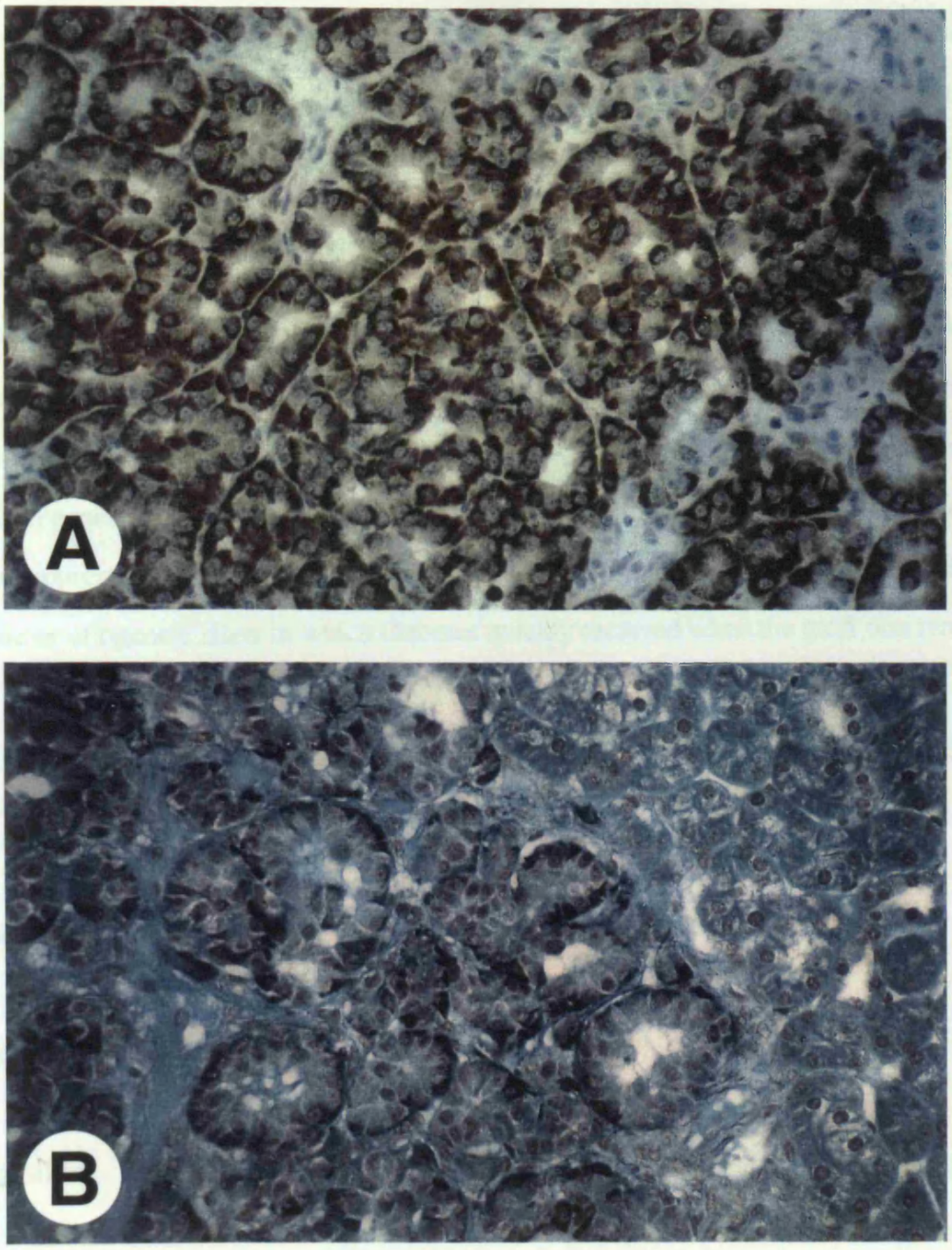


Fig. 9.2: Xenografts transplanted into diabetic NOD/Lt female mice develop a full complement of mature endocrine cells with time *in-situ* (10 weeks post-transplantation).
(a) IMP staining showing well stained β cells; comprise the bulk of the graft (for insulin, x250).
(b) GAF stain showing well stained β cells; indicative of mature insulin granules (GAF, x250)

and large numbers of ducts were also observed. Immunoperoxidase staining showed the presence of α, β and δ cells (Fig. 9.2 b,c,d). However, when the grafts were removed the mice did not revert to hyperglycaemia. The RBG levels rose 2-3 mmol/L into the normal range for NOD/Lt mice and stayed at that level until the end of the experiment. When the mice were killed 50 days after graft removal the host pancreas was removed for histological examination.

The histology of the pancreas showed two distinct types of islets, a) typical atrophic islets lacking β cells but with the normal appearance of α and δ cells typically present in chronically diabetic NOD/Lt mice (Fig. 9.3 a), and b) apparently regenerated islets with many β cells but with mild insulitis (Fig. 9.3 b). It appeared that the mice transplanted with foetal pig islets were the only animals to show regeneration of islets in the head of the pancreas. This apparent regeneration was not seen in non-transplanted diabetic NOD/Lt mice with or without immunosuppression or in NOD/Lt mice immunosuppressed and transplanted with either isogeneic or allogeneic islets in which diabetes quickly recurred when the graft was removed.

Table 9.1.

	Grafts		Infiltrate	
	present/total	>2+/total	score	mean
Isograft	16/16	15/16	0.0-4.0	1.38
Allografts	9/11	9/11	0.0-4.0	1.32
Xenografts	5/5	5/5	0.0	0.0

Table 9.1: The grafts and infiltration scores in the iso-, allo- and xenografted immunosuppressed recipients at week 10 post-transplantation.

9.3.5 Flow cytometry results

Anti-CD4 p/t therapy greatly reduced the number of CD4+ve cells in the peripheral blood and weekly treatment maintained CD4+ve cells at reduced but not absent levels. In a previous study I demonstrated (Koulmanda *et al,* 1991) that recovery of CD4 cells following anti-CD4 therapy is rapid in the NOD/Lt mice compared to other strains tested. By day 28

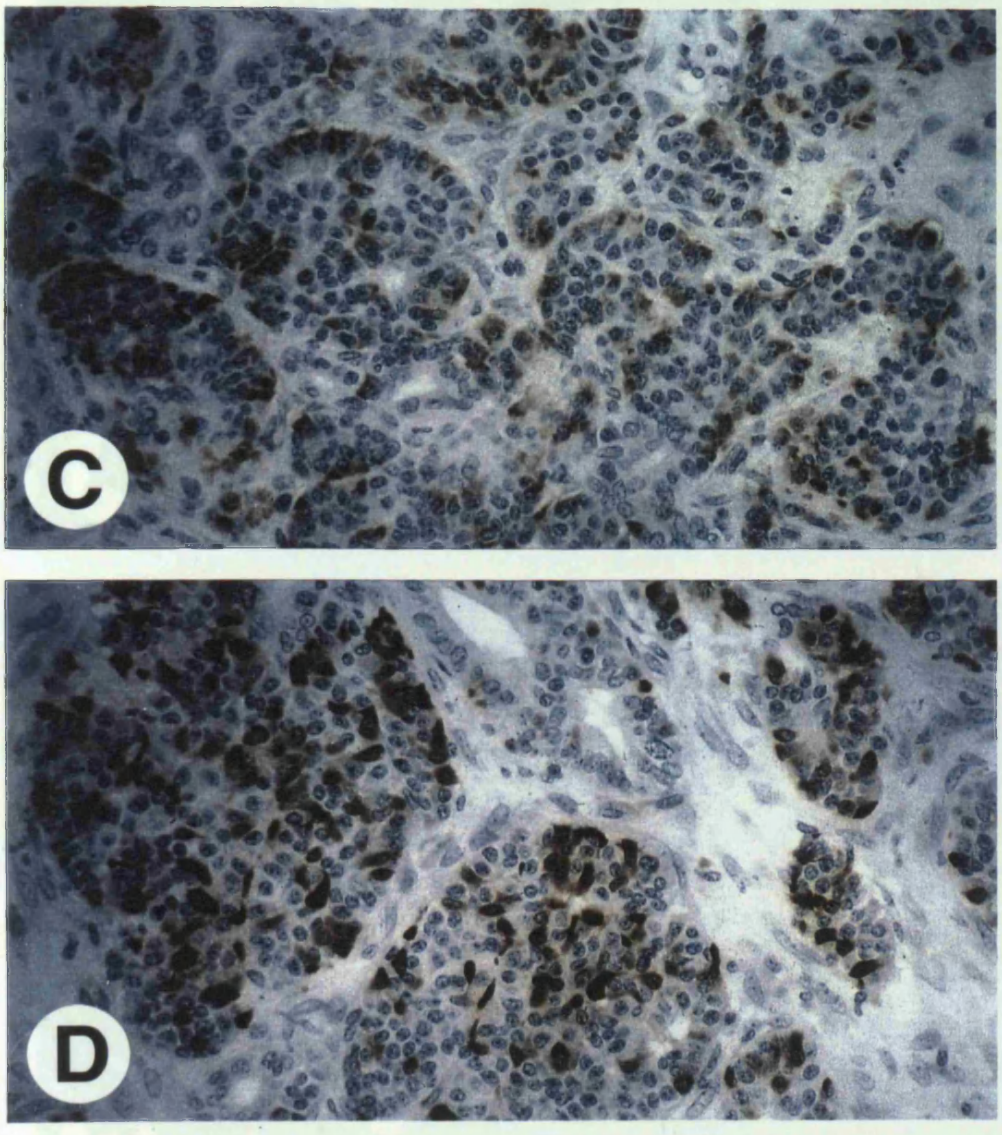


Fig. 9.2: Xenografts transplanted into diabetic NOD/Lt female mice develop a full complement of mature endocrine cells with time *in-situ*
(10 weeks post-transplantation).
(c) IMP staining showing positive δ cell (for glucagon x250).
(d) IMP staining showing positive α cell (for somatostatin x250).

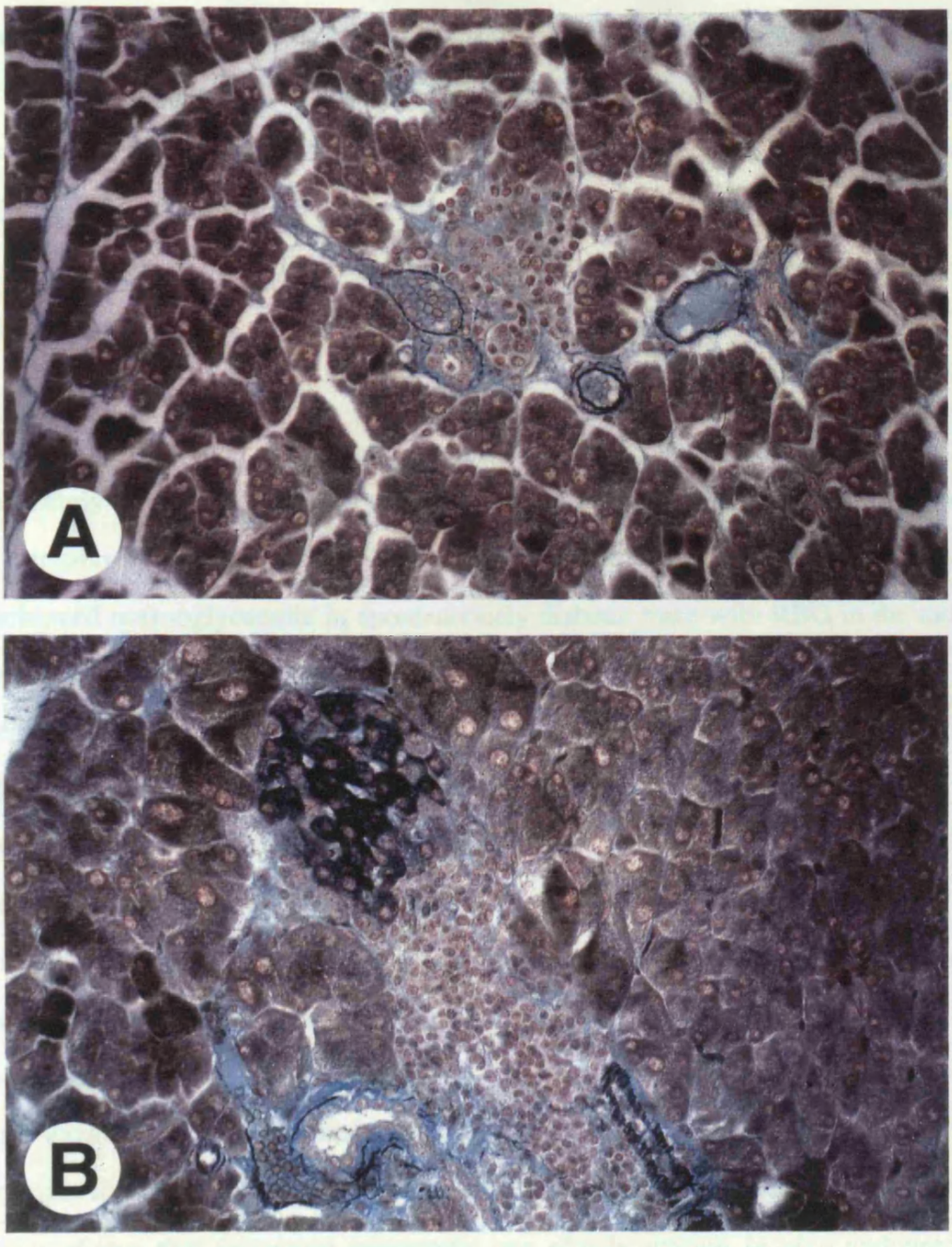


Fig. 9.3: Adult pancreas of chronically diabetic NOD/Lt female mice transplanted with foetal pig pancreas.
(a) atrophic islet (GAF, x250).
(b) regenerated islet with well stained β cells (positive for GAF) and mild insulitis (GAF, x250).

NOD/Lt animals have a substantial recovery, to approximately 50% of pre-treatment levels, compared with <20% in BALB/c and CBA mice. Results from this study showed similar recovery of CD4+ve cells to 50% of normal levels in all treated groups within 21-28 days after treatment was ceased. In contrast, anti-CD3 treatment blocked the expression of CD3 and large numbers of Thy 1.2+ve/CD3-ve cells were seen. Recovery of CD3 expression in all treated groups was rapid within 14 days after the last KT3 dose. The FACS profiles were similar in all treated animal groups.

9.4 DISCUSSION

It is clearly shown in this chapter that spontaneously diabetic NOD/Lt female mice with or without immunosuppression do not reverse diabetes without a transplant. Both iso- and allografts achieved normoglycaemia in spontaneously diabetic mice with RBG in the mouse normal range and once the graft was removed the animals reverted to hyperglycaemia. My findings are similar to those of others; Lake *et al,* (1988) showed reversal of diabetes in STZ treated nude rats after transplanting either allogeneic or xenogeneic (mouse) adult islets and graft removal resulted in subsequent hyperglycaemia in 22 of 23 animals. Lum *et al,* (1991) also showed reversal of diabetes in spontaneously diabetic NOD mice using adult rat islets, and when the islets were removed after 4-5 months post-transplantation the animals became hyperglycaemic showing no evidence of regeneration of islets in the host pancreas.

In the xenografted NOD/Lt mice, those animals that survived long enough to reverse their diabetes showed prolonged maintenance of normoglycaemia, generally at RBG levels that were appropriate to the pig rather than the normal mouse range. The reversal of diabetes in those animals shows that immature xenografts can slowly mature *in vivo* and produce functional islets with well developed β cells. It was anticipated that the animals would again become diabetic once the graft was removed and their failure to do this was unexpected. The reason(s) for this failure of reversion to hyperglycaemia is still unclear. The absence of β cell containing islets in the biopsied tail of the pancreas gland suggest that regeneration of cells within previously destroyed islets was not occuring. However, when the remaining pancreas was removed well developed islets were present and may have regenerated from ductal precursors, as suggested by Bonner-Weir *et al* (1993).

Other groups using cellophane wrapping of the pancreas have been able to induce progenitor cells to differentiate by stimulating the ductal cells (Rafaeloff *et al,* 1992). Insulin by itself did not induce regeneration of β cells in our spontaneously diabetic mice however studies from Tomioka and colleagues (1991) have shown that drug induced (STZ) diabetic hamsters without islet cell transplants can regenerate their β cells. This regeneration occurred mostly from undifferentiated cells within the islets and daily treatment with exogenous insulin was able to inhibit β cell differentiation from the precursor cells in the islets.

It is possible that the transplanted foetal pig islets that are undergoing rapid expansion may be producing growth factor(s) that can stimulate regeneration of endogenous β cell if there is adequate immunosuppression to prevent recurrent autoimmune damage. My results also suggest that growth factor(s) for islet or β cell precursor are being produced by the foetal pig pancreas and not by foetal mouse iso- or allogeneic pancreas grafts. Futhermore studies by others have shown that recipients of grafts of adult pig islets that reverse diabetes needed the presence of the grafts to maintain normoglycaemia (Tze *et al,* 1992) and graft removal resulted to hyperglycaemic levels indicating that regeneration of β cells might only work with foetal pig tissue.

The concept that β cell growth factor(s), presumably produced by the foetal pig pancreas, is at present being investigated in our laboratory.

CHAPTER 10 GENERAL DISCUSSION AND CONCLUSIONS

Islet transplantation may be the safest and most effective way of replacing destroyed β cells in IDDM. Foetal islets are a functionally immature source of tissue but, in contrast to adult islets, they can retain their capacity for proliferation if harvested and cultured in an appropriate manner. In chapter three it was shown that organ cultured foetal mouse pancreas of 13, 14, 15 and 17 days gestation and neonatal pancreas remain viable and secrete insulin into the culture medium for at least 3 weeks. During the first 2 weeks of culture, the tissue consists of up to 75% β cells and by day 14 the islets appear morphologically mature as the α, β, and δ cells gradually distribute into their characteristic adult islet arrangement. At the same time all acinar tissue degenerates and disappears usually during the first week of organ culture.

Previous work from our laboratory showed that isotransplantation of 1/2 of a cultured foetal mouse pancreas can reverse hyperglycaemia and maintain normoglycaemia in a drug-induced model of diabetes. In contrast, transplantation of uncultured foetal pancreas into diabetic animals was ineffective unless the disease was controlled by parenteral insulin while the graft matured (Hoffman *et al,* 1981). Brown *et al,* (1981) showed that the insulin content of one uncultured foetal rat islet graft was about 20% of a normal adult rat pancreas of the same sex. However, to achieve optimal function of the transplanted foetal pancreas ie. reverse diabetes, careful control of the blood glucose with insulin is necessary during the period of growth and development of the grafts. The mechanism by which insulin injection enhances the growth and function of foetal pancreas transplanted into diabetic animals is not clear but it maybe due to the prevention of toxic effects of high BG on the development of the foetal tissue. Poor regulation of blood glucose has indeed been described in infants born to diabetic mothers. In chapter four it was shown that chronic diabetes in the recipient mice in the pre-transplant period can impair the function and growth of foetal mouse pancreas isografts.

The dearth of suitable allogeneic organ donors is now the major limiting factor in organ replacement (First, 1992; Evans 1990) (e.g in USA there are 1 million patients with IDDM and only 6000 suitable cadaver donors per year). If animal organs (ie xenografts) could be used in humans without a need for severe immunosuppression, this limitation would

disappear but formidable problems still need to be solved before xenotransplantation can be considered a real option.

In chapter five, when iso-, allo- and xenografts were compared in non-immunosuppresed NOD/Lt mice, isografts were rapidly invaded by lymphoid cells and the graft pathology was similar to that in the host pancreas. In pre-diabetic NOD/Lt mice the graft site was invaded by small mononuclear cells (CD4 and CD8+ve T cells) and macrophages. In diabetic mice there was specific β cell destruction indicative of recurrent disease. The allografts were invaded and totally destroyed by mononuclear cells that included many blast cells. In the allograft sites the infiltrating cells soon disappeared and within 3 weeks only a scar remained. The xenografts, in contrast, were invaded by macrophages and eosinophils with some neutrophils and mast cells and multinucleated giant cells. Xenograft destruction also occurred but the site remained large and swollen with a central necrotic zone and massive fibrosis forming a large granuloma and the infiltrate persisted for many weeks. Hence, there are marked differences in the host response to a challenge with tissue that is prone to cell-specific autoimmune disease, to a graft of immunogenic allogeneic tissue and to a transplant of discordant xenogeneic islets and different immunosuppressive strategies may be needed to cope with the immuneresponse to these different grafts.

The application of transient treatment to produce graft survival with apparent tolerance in humans is a major goal in transplantation immunobiology but has so far not succeeded. It should be noted that this approach has only been successful in rodents when particular strains of recipients were used; possibly permissive recipients have features of their immune system that make them particularly susceptible to the effects of immunosuppression and they may represent models of the far less common situation in outbred populations, including humans, where innate low responsiveness may allow ready graft survival but is disadvantageous to the survival of the species. Studies with highly encouraging data have been presented suggesting that survival of xenografts, and perhaps even immune tolerance to such grafts, can be achieved with limited immunosuppression in some rodent strains. Faustman and Coe (1991) produced apparently indefinite survival of human islets that had been coated with highly purified high affinity anti-class I MHC $F(ab)_2$ antibodies. In that study BALB/c females mice were used and as shown in my study those mice were clearly "low responders" to xenografts compared to

other strains. In another approach, Linsley *et al,* (1992) showed that human islets were retained in C57BL/6 mice when the recipients were treated for a short time with CTLA4Ig. Simeonovic and Wilson showed that CBA male mice made diabetic with streptozotocin and given extensive anti-CD4 MAb treatment could, on some occasions, retain their graft even when treatment was ceased (Simeonovic *et al,* 1990a). Rejection could not be produced by challenge with donor spleen cells but was rapidly produced when hyperimmune serum was given (Simeonovic *et al,* 1990b). It is interesting that the mice that were used in their studies (CBA males) were similar to mice in which I also found relatively ready acceptance of xenografts. Falqui *et al,* (1991) showed that "graft immunomodification" using low-temperature culture and transient immunosuppression with ALS could produce prolonged xenograft survival in C57BL/6 mice. This study also showed a benefit of immunomodification using organ culture of foetal pig islets in high concentrations of O_2 (90%) which enhanced xenograft survival in NOD/Lt female mice if the animals were given an extended course of anti-CD4 or anti-CD3 MAb treatment. However, foetal pig islets that were not immunomodified were rapidly rejected in such recipients. Thus, despite the theoretical objection that immunomodification should have no effect in a situation where antigen presentation is mostly indirect, two independent studies using different forms of immunomodification have shown a benefit for procedures that have been strikingly effective in endocrine allografts in rodents. Perhaps the situation of antigen presentation in discordant combinations is not as clear-cut as theory would predict.

Inbred strains of mice show marked differences in their numbers of peripheral blood leucocytes and consistent strain-dependent differences in their response to a specific immunosuppressant that targets a pivotal T cell, ie the $CD4^+$ population. In NOD/Lt mice that showed heightened immune responses there is a markedly accelerated recovery of $CD4^+$ T cells and we have previously shown that these mice reject xenografts even when anti-CD4 MAb treatment is maintained (Mandel and Koulmanda, 1991) whereas strains such as CBA may even develop tolerance with such treatment (Simeonovic *et al,* 1990a). Thus, recipient immunosuppression, strain, sex and mode of pre-culture graft preparation all play a role in xenograft survival.

In this thesis, it has been shown that even a brief treatment with a depleting anti-CD4 MAb greatly altered the host response and extended, but not permanently, graft survival. The

effect of immunosuppression also depends on the strain and sex of recipients used for xenograft survival. Whether the CD4 T cells are the effectors in graft damage is not clear but it is apparent that CD4+ve cells do play a major role in attracting other potential effector cells, including eosinophils and perhaps also macrophages, that can be potent cytotoxic cells. It is still not understood what the mechanism of xenograft killing is but it does appear to differ, qualitatively and perhaps also quantitatively, from lethal damage to allogeneic cells. The precise role of eosinophils in xenograft destruction is still poorly understood but it is known that eosinophils can be potent cytotoxic cells via the production of a number of defined secreted products, ie major basic protein, eosinophil cationic protein, eosinophil peroxidase and eosinophil derived neurotoxin (de Groen *et al,* 1994) which could cause cell damage in transplantation (Hallgren *et al,* 1991; Martinez *et al,* 1993; and Foster et al, 1991) as well as in many other better known situations such as asthma and parasitic infestations (Sanderson, 1992; and Kroegel *et al,* 1994). The many functions of eosinophils in a range of disease states have recently been comprehensively reviewed (Wardlaw, 1994) but in neither extensive review was xenotransplantation mentioned. Eosinophils have also been reported to activate platelets (Rohrbach *et al,* 1990) and this too may exacerbate graft damage via platelet-derived factors. The persistance of the infiltrate and the formation of a large granuloma with evidence of central necrosis and extensive fibrosis suggests that perhaps a quite different form of rejection was occurring in the xenografts and eosinophils may play an important role in the rejection of these free cell transplants. Eosinophils have also been associated with inflammatory fibrosis as a consequence of the release of their granules (Noguchi *et al,* 1992) and this may be a reason for the extensive fibrosis of the xenograft sites. Finally, the important role of T cells in an anti-xenograft response is not being challenged by these data since it is well known that T cells are necessarty for xenograft rejection since such grafts survive well in T cell depleted nude mice (Thompson and Mandel, 1990b). However, what is being suggested is that T cells may act via a different final effector mechanism. Thus, it may be necessary to develop immunosuppressive protocols that are different from those that are generally very effective against allograft rejection. These may include the use of agents that act against eosinophils (eg anti-IL5), drugs that are anti-macrophage in their action (eg Deoxyspergualin) or interfere with the products of these cells such as NO (eg, NG-monomethyl-L-arginine), as well as more potent anti-B cell

reagents (eg cyclophosphamide, rapamycin) since Ab-mediated effects may also be important in xenograft rejection (Murase *et al,* 1993; Langer *et al,* 1993).

In islet transplantation in NOD mice there is also the problem of the underlying disease to consider. Even if it were possible to develop tolerance to the graft it may still be the case that recurrent autoimmune disease may develop despite tolerance to MHC coded differences. When xenograft survival in spontaneously diabetic NOD/Lt mice treated with anti-CD4 MAb was compared with recurrence of the disease in isografts, the xenograft rejection occured slowly, whereas isografts showed rapid and specific loss of β cells despite good depletion of CD4+ve T cells (unpublished observation). It appears that recurrent autoimmune disease is more difficult to control with anti-CD4 MAb treatment than is xenograft rejection. Of the three types of grafts used, the xenografts appeared to be resistant to autoimmune attack and were also rejected at a slower rate than the MHC-mismatched allografts in anti-CD4 treated recipients.

Hyperacute rejection is a major reason for the loss of discordant xenografts, but a free islet transplant, where the tissue develops a blood supply from the recipient may be one way of avoiding HAR. In chapter six it was shown that the differentiated pig islet cells are Gal(α1-3)Gal negative. Thus, they should not be recognised by anti-Gal(α1-3)Gal Nabs and be resistant to effector cell damage but this has yet to be clearly demonstrated. It is potentially worrisome that there is quite strong Gal(α1-3)Gal expression on the surface of at least the lumenal aspect of the ductal cells in the foetal pig grafts. The Nabs present in humans would recognise Gal(α1-3)Gal but apart from the immediate damage that they do to donor-derived endothelium it is not known whether they would have any detrimental effect on other cells expressing this epitope. The ducts are believed to be the source of islet precursor cells (Bonner-Weir *et al,* 1993) and if these are destroyed this may interfere with the generation of islet cells from the foetal pig graft. Since a major reason for using foetal pancreas is to utilise its capacity for growth and development, death or even damage of the islet precursors may negate the use of foetal pancreas but it has yet to be shown that the expression of Gal(α1-3)Gal is detrimental to duct survival. I do not suggest that in this model Gal(α1-3)Gal would play a role as a target antigen since it is also expressed on murine cells although there was little evidence of its presence except in the infiltrate that developed while rejection was occurring. This may be due to upregulation as a result of immune activation. While it is not surprising that HAR did not

occur in pig to mouse model we have seen an histologically similar picture in non-immunosuppressed cynomolgus monkeys that had a xenograft of foetal pig pancreas. Intact non-infiltrated grafts were present at 6 days in a monkey given conventional "triple immunosuppression" (Mandel *et al,* 1995). Cynomolgus monkeys are quite capable of mounting a HAR response against porcine tissue. Species differences may determine the precise nature of an anti-graft response and it is comforting to see that a pathologically similar response developed in a species more closely related to humans. Also results from a Swedish group have shown that HAR rejection was not a problem using foetal pig islets in humans with IDDM (Groth *et al,* 1994).

Finally the aim of this thesis was to determine the best gestational age and culture time for foetal pancreas isografts to reverse diabetes and see if allografts and discordant xenografts can be used with T cell immunosuppressive treatment to reverse chronically diabetic animals. I also wanted to see if xenografts are going to be rejected more vigorous than allografts and if they were going to be susceptible to the recurrence of the autoimmune disease as are the isografts. This study clearly demonstrated that foetal pancreas may be a useful source of islet tissue and can develop into functional grafts. There are still many problems outstanding but it seams that even xenografts of foetal pancreas can function when suitable immunosuppression is used, at least in mice. Notably, however, HAR does not seem to be a problem in this model of xenotransplantation and in NOD mice at least, even recurrence autoimmune disease seem not to be a major problem. Thus, it seems very worth while to explore further the use of islet xenografts in an attempt to solve a major clinical problem, ie. a cure for type I IDDM.

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APPENDIX 1

Preparations for buffers and tissue culture media

1. Mouse tonicity phosphate buffered saline (0.02M PO_4)

2.850 g/l $Na_2HPO_4.2H_2O$

0.625 g/l $NaH_2PO_4.2H_2O$

8.7 g/l NaCl

pH 7.3, iso-osmotic with mouse serum (308 mosmoles) and the autoclaved.

2. Mouse tonicity Hepes-buffered balanced salt solution.

0.168M	NaCl	121 vols
0.168M	KCl	4 vols
0.112M	$CaCl_2$	3 vols
0.168M	$MgSO_4$	1 vols

iso-osmotic with mouse serum (308 mosmoles), pH 7.2 6 vols and autoclaved.

3. Mouse Tonicity Dulbecco's Modified Eagle's Medium.

2 x 10 sachets

68 g $NaHCO_3$

2 vials penicillin (600mg/vial)

2 vials sreptomycin sulphate

dissolved seprately and make up to 20:1 final volume

iso-osmotic with mouse serum (303 mosmles)

gas back to pH 7.0

filter through 0.22 um Millipore Membrane under 10% CO_2

aliquot 90 ml into sterile bottles and store at 4^oC

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