

STUDIES ON THE ISOLATION AND TRANSPLANTATION OF
MAMMALIAN PANCREATIC ISLETS

by

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A handwritten signature in dark ink, appearing to read "S. P. Lake". The signature is fluid and cursive, with the first name "S." and last name "Lake" clearly distinguishable.

Stephen P. Lake

July, 1989.

To Sarah, Emily and Eloise.

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SYNOPSIS

A review is made of the relationship between the disease diabetes mellitus and the insulin-secreting B cells of the islets of Langerhans. In addition, an account is given of the clinical and experimental methods of treatment including insulin therapy and transplantation of the pancreas both as an intact organ and as isolated endocrine tissue (islet transplantation). In particular the methods which have been used to release and isolate islets from the rodent, large animal and human pancreas are discussed. Furthermore, the metabolic effects of transplantation, results of allogeneic transplantation and techniques of islet preservation are described.

In this thesis I have set out to determine whether the standard islet isolation method (using density gradients of Ficoll medium) could be improved by utilising an alternative density gradient medium (Bovine Serum Albumin). Using a rodent model, islet yield, purity and viability (both in vitro & in vivo) were shown to be improved with the BSA method over the standard Ficoll technique. A second stage was subsequently performed, using a novel test gradient system, to optimise the BSA gradient method further by definition of the precise density of rat islets and exocrine tissue.

The BSA gradient method was then adapted to the isolation of canine islets. To overcome the difficulties in processing large volumes of digest produced from the collagenase digested pancreas, an IBM 2991 cell separator was adapted to produce large volume density gradients. Although high yields of highly purified and viable islets were obtained, these were insufficient to prevent diabetes ensuing in autotransplanted pancreatectomised dogs.

An animal model was developed which would potentially be applicable for the in vivo assessment of human islet viability. The nude (athymic) rat, allogeneic and xenogeneic (mouse) islet transplants and a short diabetic induction period with streptozotocin were all used.

Finally, the BSA gradient methodology and IBM 2991 cell processor were used for the large scale purification of intact human islets which were shown to be viable both in vitro, and in vivo following transplantation into diabetic nude rats.

CHAPTER 1

**REVIEW OF DIABETES MELLITUS AND ITS TREATMENT BY
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I. DIABETES MELLITUS: THE DISEASE AND CURRENT METHODS OF TREATMENT

1.1 HISTORICAL ASPECTS

a. Ancient Period

The term diabetes, which is Ionic Greek and means 'to run through a siphon', was coined by Aretaeus of Cappadocia (AD 81 to 138) who noted the large amount of urine that 'runs through the kidneys' in this disease. Although Aretaeus was one of the first to give us a clinical description, the phenomenon of polyuria may have been observed at a much earlier time since it is mentioned in several of the ancient papyri (Papaspysros, 1964).

The sweet taste and large quantities of urine were noted by both Japanese and Chinese physicians of the 2nd and 3rd centuries (Wong, 1932) and a reference to 'urine of honey' appears in old Sanskrit texts from India. During the 9th, 10th and 11th centuries the disease was studied by several eminent Arabian physicians including Avicenna (960-1037) who observed such symptoms as abnormal appetite, sweetness of the urine, gangrene and loss of sexual function. There are few European references to diabetes during the Middle Ages until Theophrastus Bombastus Von Hohenheim (1493-1541) evaporated the urine of a diabetic patient and obtained a white powdery residue which he mistook for salt,

b. Diagnostic Period

During the 17th century Thomas Willis (1621-1675) rediscovered the sweetness of the diabetic urine and was also the first to separate diabetes mellitus from diabetes insipidus, the latter lacking glycosuria (Willis, 1676). He stated that, in diabetes, sugar appears first in the blood, then in the urine and he suggested 'mal-nourishment and lime

water' as therapeutic measures. The fact that glycosuria is a constant and characteristic feature of the disease became generally accepted and it was ultimately William Cullen (1710-1790) who added the qualifying adjective 'mellitus'.

c. Empirical Treatment

John Rollo in 1797 initiated a period of empirical treatment of diabetes by prescribing an exclusive diet of meat. The therapeutic effect of such a low carbohydrate, high protein diet probably resulted from partial starvation. Rollo also recorded diabetic cataract and compared the acetone induced odour of diabetic patients to that of decaying apples. A second major step in the pre-insulin treatment of diabetes was taken by Apollinar Bouchardat (1806-1886) who observed that during the siege of Paris in the Franco-Prussian War of 1870/71 all of his patients with diabetes improved. He devised specific diets in which carbohydrate was replaced with fat and emphasised the importance of caloric restriction. Later in 1885 he confirmed that the sugar in the urine of diabetes was grape sugar (i.e. glucose) and that it could be detected with the help of a polaroscope and copper solutions.

d. Relationship with the Pancreas

It was not until the 19th century that diabetes and the pancreas began to be linked in the developing concept of this disease. Earlier than this, Gallen for instance, believed that diabetes resulted from injury to the kidneys while Willis implicated the blood and Rollo, the stomach (Papaspoulos, 1964).

The first reference to the pancreas as a separate organ has been attributed to Herophilus of Chalkidon (300 BC) (Lazarus, 1962) although it was Rufus of Ephesus, 200 years later, who coined the term 'pancreas'

(pan = all, kreas = flesh) (Papaspnyros, 1964). The first thorough descriptions of the pancreas stem from the 16th century and include Andreas Vesalius (1514-1564) and Gabrille Fallopio (1523-1562) (Schwarz, 1909). Georg Wirsung in 1642 recorded the major pancreatic duct in detail and Giovanni Santorini followed with his description of the accessory duct of the gland 100 years later.

e. Experimental Period

Theoretically the association between diabetes and the pancreas could have been made as early as 1683 when John Conrad Brunner (1653-1727) succeeded in removing the pancreas of several dogs and keeping the animals alive (Brunner, 1683). He noted that the animals displayed polydipsia and polyuria but failed to recognise the symptoms as being caused by diabetes. His observations were soon forgotten until von Mering and Minkowski repeated his experiments in 1889 and established beyond doubt that total pancreatectomy in dogs caused diabetes (von Mering, 1890). From that date diabetes was linked closely to the pancreas and all research centred upon this organ.

Initially, it was believed that the external pancreatic secretion played a role in maintaining normal blood sugar levels. Hedon in 1893, however, observed that diabetes was absent in partially pancreatectomised dogs as long as the pancreas remained vascularised and despite the discharge of the 'external secretions' onto the skin. Attention was thus drawn to the possibility that an 'internal' secretory function was instrumental in blood sugar homeostasis. During the same year Laguesse suggested that the islets of Langerhans (a name he proposed and which had been described by Paul Langerhans (1849-1888) in his doctoral thesis of 1869) were the source of the postulated internal secretion. The existence of a relationship between the islets of Langerhans and an

internal secretory function was corroborated by reports indicating a relationship between the islets and carbohydrate metabolism. For example, Dieckhoff (1895) and Ssobolew (1902) both recorded the complete absence of islets in some cases of diabetes. Furthermore, MacCallum (1909) showed that pancreatic duct ligation in animals resulted in atrophy of the exocrine parenchyma whereas the islets survived. Diabetes did not ensue until subsequent complete removal of the organ.

Soon after the beginning of the 20th century several authors recorded a number of histological alterations in the islets of Langerhans of diabetic individuals. Opie (1901) described hyalinisation and sclerosis of the islets, and during the same year Weichselbaum and Stangl found a reduction in islet number and size with vacuolization of islet cells, the latter being termed 'hydropic degeneration'. In the meantime, histology of normal pancreatic islets became a field for intensive study, with Diamare (1899) and Schulze (1899) being the first to realise that the islets of Langerhans contained more than one cell type. Further progress in staining technology was achieved by Lane (1907) who introduced the designation A cell and β -cell, and by his teacher, Bensley, who subsequently changed the term, β -cell to B cell. A third cell type, the D cell, was identified in 1931 by Bloom.

f. Initial Hormone Therapy

The successful isolation of the 'blood sugar lowering hormone' of the islets of Langerhans by Banting and Best in 1921 represents an important landmark in the history of diabetes. Their extract from canine and beef pancreas was given to pancreatectomised dogs and resulted in rapid amelioration of the diabetic state in many of these animals. When given to human diabetics the effect was erratic at first, but the extracts were purified by their co-worker Collip (1923) who succeeded in producing an extract that was less toxic and more

efficient. Banting and Best, at first, named the isolated hormone 'Isletin' but at the insistence of McCleod, in whose laboratory the two young researchers worked, it came to be known as insulin, a term already proposed by De Mayer in 1909. Abel in 1926 obtained insulin in crystalline form and Scott (1934) established zinc as indispensable for the crystallisation of this hormone. The production of long acting protamine zinc insulin by Hagedorn and co-workers in 1936 initiated a new phase with the successful treatment of diabetes with insulin.

g. Insulin Release from the B Cell

The first direct demonstration of the presence of insulin actually within the pancreatic B cells was accomplished with a fluorescent antibody technique by Lacy and Davies in 1957. More recently the steps involved in the biosynthesis, storage and release of insulin from normal islets of Langerhans have been clarified. Briefly, insulin is synthesised by the rough endoplasmic reticulum of the B cell as a precursor molecule called pre-proinsulin. Following enzymatic removal of the 'pre' sequence of amino acids, proinsulin is packaged into secretory granules within the Golgi apparatus of the cell. A small peptide (C-peptide) is cleaved from the molecule during transit of the granule to the cell membrane. Fusion of the granules with the B cell membrane, a process called exocytosis, allows release of insulin (now 2 peptide chains joined by disulphide bonds) into the space surrounding the cells (Orci, 1974).

1.2 CURRENT CONCEPTS

a. Classification

In recent years it has become accepted that diabetes is not a single disease but rather a group of disorders characterised by hyperglycaemia due to a deficiency of insulin production. In some cases, diabetes follows surgical removal of the pancreas or destruction of the islets resulting from pancreatic diseases such as haemochromatosis, pancreatitis and pancreatic tumours. In the majority of individuals, however, the cause is unclear or 'idiopathic'. In such cases, the most generally accepted classification of diabetes is that produced by an International Workgroup sponsored by the National Diabetes Data Group of the NIH (1979). Accordingly, the insulin-dependent, ketosis-prone diabetic is considered a separate subclass of diabetes, Type 1. The non insulin-dependent, non ketosis-prone type of diabetic that is not secondary to other disease or condition is also a distinct subclass, Type 2.

b. Epidemiology

The number of people affected by diabetes in the United Kingdom is between 1% and 2% of the population, which is equivalent to 0.5 to 1 million people. Of these approximately 250,000 are insulin-dependent. Thirty thousand are children under the age of 16, with 3,000 joining their ranks annually (British Diabetic Association Figures, 1988). Although the incidence varies round the world, with 'developed' countries having a higher prevalence than 'developing' countries, close to 100 million people are affected (BDA, 1988). Furthermore, the overall incidence of childhood diabetes increased by 80% during the period 1966 to 1976 and continues to rise (Patterson, 1983).

Reports from America indicate that the mortality amongst Type 1 diabetics is 3/1,000/year for ages below 25 years and 20/1,000/year for age 25 years and over. These mortality rates are between 5 and 10 times greater than for age matched non-diabetic individuals (NIH publication 1468, 1985). Approximately 12% of Type 1 diabetics die within 20 years of the onset of diabetes. The primary cause of death below the age of 20 years is acute diabetic complications. At older ages chronic complications (see below) account for substantial numbers of deaths. Furthermore, diabetics have less years of schooling, are less likely to be employed and have lower family incomes than the general population (NIH, 1985).

c. Aetiology and Pathogenesis

i) Type 1 diabetes

Complete understanding of the aetiology and pathogenesis of Type 1 diabetes continues to elude researchers. The accumulated evidence suggests that at some stage, in a genetically susceptible individual, an injury of some sort (probably viral induced) occurs which is directed specifically at the B cell. This injury leads to an immune mediated destructive process of the B cells within the islets.

Diabetes is to some extent a genetically determined disease, although we do not inherit the disease per se. However, the combination of 'environmental factors' with a genetic predisposition may lead to the condition. The striking relationship between Type 1 diabetes and the human leukocyte antigens (HLA) B8, B15, DR3 and DR4 suggests that the genes in the HLA region of chromosome 6 have a role in conferring the susceptibility. In contrast, other genes at the DR locus (e.g. DR2) appear to confer resistance.

The suggestion that Type 1 diabetes is viral induced has been proposed for more than a century, with several characteristics including

age distribution, seasonality, family clustering and the temporal relationship between viral illness and the onset of diabetes supporting the concept that an infectious agent initiates this disorder:-

Typically, Type 1 diabetes occurs in children and adolescents with epidemiological studies indicating that the incidence steadily increases after 9 months of age to reach a peak at 10-12 years. The incidence then decreases sharply to form a plateau in late adolescence (Bloom, 1975).

The seasonal distribution of diabetes was first noted in 1926 (Adams). Subsequent investigations, in at least 6 countries, have confirmed that more than 85% of cases occur during a 6 month period commencing in August of any 1 year (Gamble, 1980a).

A number of studies have commented on the temporal relationship between infectious diseases (e.g. mumps, chicken-pox and influenza) and the onset of symptoms of diabetes (John, 1949). Furthermore, other reports have noted outbreaks of diabetes, with clustering of cases occurring during a short time span, in a geographically confined area (Huff, 1974; Gamble, 1980b).

The concept that diabetes results from an autoimmune destruction of the cells has been supported by the demonstration of islet cell autoantibodies (ICAs) in the sera of diabetic subjects. This was first reported in 1974 (Bottazzo) and has subsequently been documented in many individuals with Type 1 diabetes (Bright, 1982). These autoantibodies recognise islet cell antigens and can be detected before the onset of clinical diabetes (Irvine, 1976). The frequency is, however, highest near the time of diagnosis and falls thereafter (Neufeld, 1980).

ii) Type 2 diabetes

The absolute or near absolute deficiency of insulin appears to be the prime factor in Type 1 diabetes. In contrast, the metabolic derangement of Type 2 diabetes appears not only to be due to impaired

B cell function but also the tissues of patients fail to respond normally to insulin, i.e. they are 'insulin resistant'.

The strongest evidence in favour of an underlying genetic influence in Type 2 diabetes comes from twin studies (Barnett, 1981). A 90% concordance rate was noted for Type 2 diabetes in monozygotic twins, as opposed to only around 45% concordance rate in monozygotic ketosis-prone Type 1 diabetic twins. Furthermore, 40% of the Type 2 diabetic twins had first degree relatives with diabetes and, importantly, at the time of diagnosis many of the Type 2 twin-pairs had lived apart for many years in quite different environments. Additional support for a genetic component comes from population studies. For example, in Nauran families glucose intolerance was shown to develop in 40% of offspring of 2 diabetic parents, in 6% of children of a 'normal' parent and a parent with impaired glucose tolerance and in no child of 2 'normal' parents (Zimmet, 1982).

The major environmental factors frequently associated with the generation of Type 2 diabetes are:- age, obesity, diet, exercise and psychosocial stress (see Zimmet, 1982 - for review).

A detailed review of the relationship between insulin secretion and insulin resistance in the pathogenesis of Type 2 diabetes is beyond the scope of this thesis. Both, however, appear to be essential characteristics of patients with Type 2 diabetes (see Alford, 1986 - for review).

d. Pancreatic Pathology

i) Macroscopic

The pancreas of a patient with diabetes shows few gross abnormalities except that it is often small, firm and reduced in weight (Gepts, 1965).

ii) Microscopic

Exocrine tissue

Small foci of acute pancreatitis are often seen in the pancreas of acute Type 1 diabetics, probably representing terminal events induced by dehydration and circulatory failure. In chronic Type 1 diabetics the acinar tissue may appear atrophic with interlobular and intralobular fibrosis. These findings may explain the reduction in pancreatic weight.

Islets

Three distinct histological types of islets are seen:-

psuedoatrophic, hyperactive and PP islets. Psuedoatrophic islets predominate and are composed of cords of small cells with dense nuclei and scanty cytoplasm. Histologically these cells are active endocrine cells comprising two thirds glucagon cells and one third somatostatin cells with virtual absence of B cells. Hyperactive islets are rarer and are found in Type 1 diabetics with a short clinical duration. The constituent cells tend to be large with a dense nucleus and have been shown to be degranulated B cells containing large amounts of RNA granules (Gepts, 1965). PP islets are so-called because of their composition of cells secreting pancreatic polypeptide and represent the prevalent type of islet in the juxtaduodenal segment of the pancreas.

A number of other changes have been observed in the islets of Type 1 diabetics and include hydropic change (accumulation of glycogen), hyalinosis (deposition of a cellular material between the islet cells and the capillaries), islet hypertrophy (probably secondary to excessive stimulation/compensation for atrophy of the majority of other islets) and of most recent interest, inflammatory infiltration of the islets. This process, termed 'insulitis' by Von Meyenburg (1948) has been recognised for many years (Opie, 1901). Insulitis characteristically appears as an inflammation, by small lymphocytes with scanty cytoplasm,

confined to some (usually not all) of the islets of a given individual (Fig. 1.1)(Gepts, 1965). Occasionally a few large cells, possibly macrophages, are present. The majority of islets show the pattern characteristic of psuedoatrophic islets, i.e. small endocrine cells with associated collapse of the islet framework and fibrosis. Lymphocytes progressively disappear from the islets in which all the B cells have been destroyed. The infiltrating cells appear in large numbers in foci of B cell regeneration and are never found in islets composed of PP cells. This appearance, of islets in various stages of apparent hyperplasia, infiltration (and presumably injury) by lymphocytes with atrophy and fibrosis suggests stages in an immunological (or autoimmune) reaction.

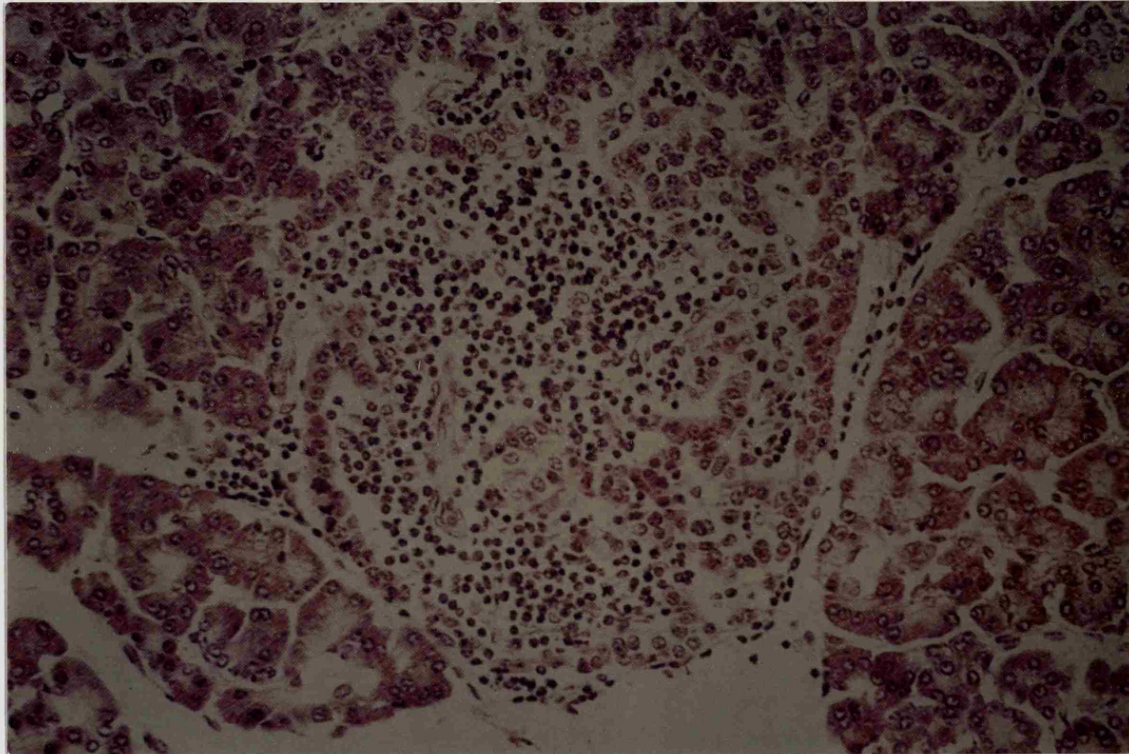


Figure 1.1 Photomicrograph of the pancreas of a recently diagnosed Type 1 diabetic patient showing a diffuse lymphocytic infiltration of an islet (insulitis) with atrophy of islet cell cords. H & E stain.

1.3 EXPERIMENTAL MODELS

Once the basic morphology and function of islet cells and their role in human diabetes had been elucidated, further insight into the aetiology and pathology of diabetes was obtained by the production and use of a number of animal models.

a. Total Pancreatectomy

Complete excision of the pancreas was probably first successfully performed by Brunner in 1683, although he failed to recognise the significance of the polydipsia and polyuria that developed. Two hundred years later Von Mering and Minkowski repeated the experiment, using dogs and cats, and established that diabetes ensued following total pancreatectomy (Von Mering, 1890). More recently Markowitz (1964) fully described the operative technique and a substantial number of subsequent publications have confirmed the value of this model. Dogs become diabetic within 24 hours of total pancreatectomy with a survival (untreated) of less than 7 days (Markowitz, 1964). The metabolic disturbances of hyperglycaemia, low blood alanine, raised plasma lipids and ketosis (all of which are characteristic of insulin dependent diabetes in humans) occur in the pancreatectomised dog (Alderson, 1984).

Pancreatectomy has also been performed in the rat (Scow, 1957), however, the multiple duct system and proximity of the bile duct make this a difficult operation.

b. Chemical Induction

Diabetogenic drugs can be separated into two general classes. Drugs such as alloxan and streptozotocin produce permanent diabetes by destruction of B cells with consequent insulin deficiency. Agents such as cyproheptadine and the benzothiadiazines do not irreversibly destroy

the B cell but cause a temporary decrease in available insulin and/or induce secondary effects which counteract the action of insulin. For the purpose of this thesis, only drugs causing permanent diabetes will be considered.

i) Alloxan

Alloxan (2,4,5,6-tetraoxohexahydropyrimidine) was first reported to be diabetogenic by Dunn in 1943. Since then it has been extensively used to induce diabetes in animal species such as rabbit, dog, mouse, cat, sheep and monkey (Lukens, 1948; Mahler, 1971). Alloxan has a half life in blood of approximately 1 minute (Leech, 1945) and results in a triphasic blood glucose response after injection (Lukens, 1948). Following an initial hyperglycaemic phase, a period of marked hypoglycaemia occurs with hyperglycaemia finally recurring at 24 hours. Although alloxan is an effective drug for inducing diabetes, its use is limited by the hypoglycaemic phase which may be profound and induce convulsions and death.

ii) Streptozotocin

Streptozotocin or 2-deoxy-2-(3 methyl-3-nitrosurea)1-D-glucopyranose) was first reported as an antibacterial agent in 1959 (Herr) and was obtained from the fermentation culture of *Streptomyces achromogenes*. Its diabetogenic property was reported in 1963 (Rakieten) and the drug was subsequently synthesized. Streptozotocin has been shown to be diabetogenic in the rat, dog, mouse, guinea pig, and monkey (Rakieten, 1965; Brodsky, 1968; Schein, 1973). It is now the most widely used chemical agent for inducing diabetes in animals. The effect of streptozotocin is dose dependent and provided sufficient is given by single bolus injection, permanent hyperglycaemia develops after 24 hours (Junod, 1969). The overall response to streptozotocin is

triphasic with initial hyperglycaemia after 1-2 hours, followed by hypoglycaemia for 6-12 hours and by permanent hyperglycaemia at 24 hours. The initial hyperglycaemia is probably secondary to inhibition of insulin release because there is no associated increase in plasma insulin. The hypoglycaemic phase is due to increased plasma insulin, without decreased pancreatic insulin content, suggesting that insulin production can keep pace with release at this time (Schein, 1968; Junod, 1969). The final hyperglycaemic phase results from a deficiency of insulin since both plasma and pancreatic insulin are reduced (Junod, 1969).

c. Viral Induction

A viral aetiology for diabetes mellitus was first proposed over 50 years ago when Gunderson (1927) documented an association between community outbreaks of mumps and the occurrence of new cases of abrupt onset diabetes. Since that time reports have repeatedly implicated the role of mumps virus (Hinden, 1962) and more recently, rubella and group B coxsackie viruses (Gamble, 1969). Several different viruses have been shown to preferentially attack the B cells of the islets in experimental animals. Models that simulate Type I diabetes have been developed in mice, including the M variant of encephalomyocarditis virus (EMC) (Craighead, 1971). Although a pathogenic role for this agent in man is unlikely, EMC provides a model of diabetes that closely resembles the human disease. One major problem with EMC virus, in rodents, has been a high mortality secondary to myocarditis.

d. Spontaneous Development

In 1950 Ingalls described a new mutation in the mouse characterised by profound obesity and diabetes. Since then the pathology and physiology of a number of spontaneous diabetic syndromes in laboratory

rats and mice have been described. The models include two basic groups which manifest either non lethal or lethal syndromes. In the former these animals either experience a full life span with the disease or may eventually return to the normoglycaemic state. In contrast, in the lethal category, the diabetic syndrome is severe and significantly reduces life span. Examples of the strains which suffer from non lethal diabetes are C57B1/6J OB/OB (the obese mouse) (Ingalls, 1950), the Toronto KK rat (Nakamura, 1967) and the sand rat or psammomys (Schmidt-Nielsen, 1964). In these animals a significant B cell proliferation, in response to diabetes, is seen and is usually associated with elevated plasma insulin. On the other hand, the lethal examples (C57B1/KsJ DB/DB, BB (Bio Breeding) rat and NOD (Non-obese diabetic) mouse exhibit decreased B cell numbers, low levels of plasma insulin and severe metabolic abnormalities (Hummel, 1966; Nakhooda, 1977; Makino, 1980). The NOD mouse and the BB rat are particularly interesting because they demonstrate marked lymphocytic infiltration of the islets and are currently considered to represent the animal equivalent of Type I diabetes. The lymphocytic insulinitis is seen prior to and during the acute phase of hyperglycaemia and the use of immunosuppressive agents and neonatal thymectomy protects against the onset of diabetes (Like, 1980). Rodents such as the BB rat require intensive animal husbandry with daily injections for their maintenance. Thus, until recently, their use has been limited to specific research centres.

1.4 TREATMENT WITH INSULIN

The successful isolation of insulin by Banting and Best in 1921 was an important milestone in the treatment of diabetes. Other investigators (Rennie, 1906; Scott, 1911; Gley, 1922) had tried to accomplish the same but had failed, either because of technical difficulties or because their extracts were too toxic for trials in animals or humans. Banting and Best prepared their extracts from normal and duct ligated canine, adult bovine and foetal calf pancreata. When administered to pancreatectomised dogs these extracts resulted in a rapid amelioration of the diabetic state in many of the animals. Initial results in humans were inconsistent, but following the production of long acting protamine zinc insulin (Hagedorn, 1936) a new era of successful treatment of diabetes commenced.

a. Intermittent Subcutaneous Insulin

The standard method of administration of insulin has always been by intermittent subcutaneous injection. A number of advances have been made over the last 50 years in this therapy. The first was modification of the insulin vehicle, to allow variation of the timing of hypoglycaemic action, thereby making preparations described as either 'short', 'intermediate' or 'long-acting' (Krayenbuhl, 1946; Hallas-Moller, 1952). A further advance was the increasing purification of insulin (Mirsky, 1966; Schlichtkrull, 1974) so that tolerance and efficacy of preparation was improved, with a reduction of the side effects of lipodystrophy and insulin antibody production. The third major achievement, which followed the determination of the amino acid sequence of human insulin (Nicol, 1960) was the development of techniques of human insulin production by recombinant DNA technology

(Goeddal, 1979) and by modification of porcine insulin through amino acid substitution (Markussen, 1980).

Insulin therapy radically altered the life expectancy of diabetic patients, however, the chronic complications which affect the eye, kidney, blood vessels and nerves became evident. Although the point is controversial, the degree of glucose control does seem to determine the relative frequency of diabetic complications (Pirart, 1978; Unger, 1985). Maintenance of normal blood glucose levels is difficult to achieve by intermittent injection of even 'slow release' preparations of insulin. Thus, new strategies of insulin delivery have been developed.

b. Artificial Endocrine Pancreas

The most advanced insulin delivery system currently available is the artificial pancreas which contains a glucose sensor and microcomputer which monitors and controls blood glucose levels continuously (Albisser, 1974). Excellent blood glucose control has been achieved, but the machines are bulky and the glucose sensor needs to be replaced every 36-48 hours. Thus, although potentially of great value, these machines are currently for research purposes only (Rizza, 1980).

c. Continuous Infusion

Improved glucose control has also been achieved by use of the simpler method of continuous insulin infusion, using a battery driven pump and subcutaneous injection via a cannula (Pickup, 1978; Tamborlane, 1979). Regular blood glucose measurements are made by the patient and good control can be achieved. Disadvantages are that ketoacidosis may rapidly occur if the cannula does slip out because the insulin used with this type of pump has no repository effect (Mecklenburg, 1982). Furthermore, significant infective complications can occur at the site of injection (Mecklenburg, 1984).

1.5 CHRONIC COMPLICATIONS

The successful treatment of diabetes with insulin has reduced the number of deaths from acute diabetic complications. Substantial numbers of patients now survive for many years following the onset of the disease and many go on to develop long-term complications. Figure 1.2 illustrates the cumulative risks of complications or 'manifestations of diabetes' following the onset of Type 1 diabetics. Complications are not common in the first decade although a progressive increase in nephropathy, retinopathy and arteriopathy occur through the 2nd, 3rd and 4th decades of the illness. Important questions are what causes the complications and can they be prevented or even reversed?

a. Pathological Effects

i) Nephropathy

Kimmelstiel and Wilson in 1936 described spherical hyaline masses in the glomeruli of eight patients who died of renal failure following an illness characterised by albuminuria, oedema and hypertension. The glomerular lesion was attributed to diabetes because 7 of the 8 patients were known to have this disorder. Initially the observation was treated with scepticism, however, subsequent autopsies on diabetic patients confirmed the existence of specific renal histopathological changes in such individuals (Bell, 1942; 1946). The use of percutaneous renal biopsies and the electron microscope, introduced in the 1950s, has led to a greater understanding of the changes in diabetic nephropathy. The earliest changes consist of basement membrane thickening with an accumulation of basement membrane like material in the mesangium. Ultimately, distortion of endothelial cells occurs, as deposits expand, with resulting capillary closure and glomerular destruction (Lannigan, 1964; Osterby, 1984).

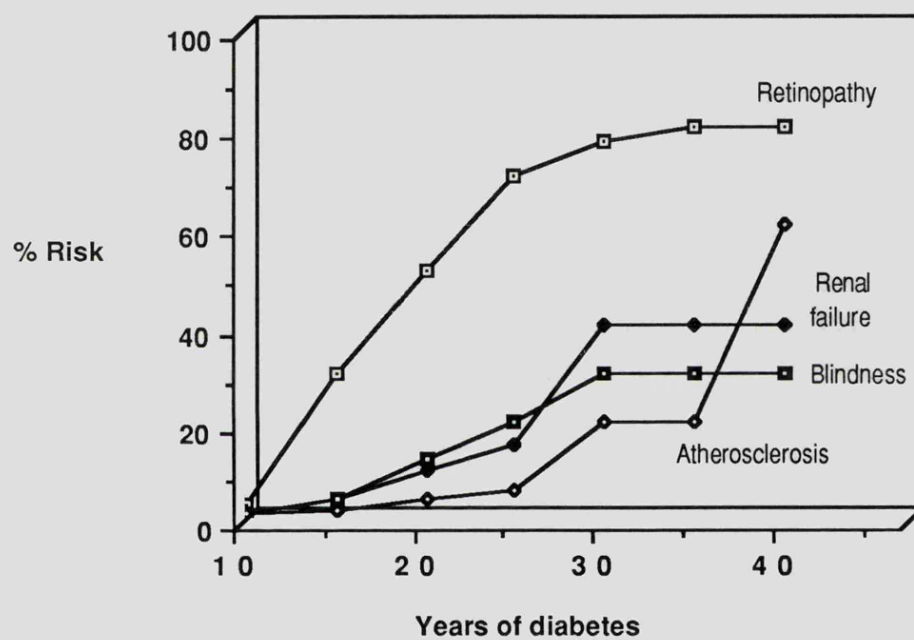


FIGURE 1.2 Risk of end-organ disease following onset of Type 1 diabetes.

(Adapted from Knowles, 1971)

Clinically, nephropathy develops in up to 45% of Type 1 diabetics, of whom approximately two thirds will develop renal failure. Once established, diabetic nephropathy progresses relentlessly to end stage renal failure over a period of about 7 years (range 5-20) (Kussman, 1976). Such individuals, at present, account for approximately 25% of patients on renal replacement programmes.

ii) Retinopathy

Diabetic retinopathy is uncommon during the first few years of diabetes, however, the prevalence subsequently steadily increases (Fig. 1.2). While retinopathy may remain mild, a substantial proportion of patients with early onset diabetes carry a significant (50% in 25 years) risk of vision loss (Knowles, 1971). The first recognisable abnormality in diabetic retinopathy is dilatation of the retinal capillaries (microaneurysm). Progression of the disease results in new blood vessel formation (neovascularisation) or proliferative retinopathy which may affect the vessels on the optic nerve head or more peripheral regions of the retina. Such vessel proliferation results in not only deterioration in vision on its own but also is associated with severe adverse sequelae, such as haemorrhage, retinal detachment or neovascular glaucoma.

iii) Arteriopathy

Widespread coronary artery disease is common in patients with diabetes, particularly in those with advanced nephropathy. Abnormal electro-cardiographs are found in 50-70% of patients (Libertino, 1980). Coronary angiography has shown the presence of significant coronary occlusive disease in 20-100% of patients (depending on the definition of coronary occlusion (Bennett, 1978)). Left ventricular hypertrophy is common and usually severe in patients with advanced nephropathy

Diabetic individuals receiving renal replacement therapy by dialysis or transplantation have a high mortality, with ischaemic heart disease as the most common cause of death even many years after renal transplantation. Amongst those diabetic patients surviving longer than ten years after renal transplantation, 20% suffer myocardial infarction and 15% cerebrovascular accident.

Peripheral vascular disease affecting the feet and hands is an important cause of morbidity in diabetics with nephropathy. Atheroma affecting the large vessels is common, particularly in elderly patients. Although the exact prevalence of peripheral vascular disease is not known, amongst those considered for renal transplantation 10-20% have evidence of peripheral vascular disease (absent foot pulses, claudication and gangrene) and about 1-2% have had amputations (Grenfell, 1985).

b. Cause of Complications

Current evidence suggests that there appear to be many different factors involved in the genesis of arteriopathy, nephropathy and retinopathy including not only genetic and nutritional factors but also biochemical abnormalities in the tissues (Keen, 1982).

i) Genetic Factors

The existence of genetic heterogeneity in diabetes (Nelson, 1975) supports the concept that the complications are not caused by a single genetic factor. Thus, whilst it has been shown that microangiopathy is present in some individuals with secondary, non-genetic types of human diabetes (West, 1978), it was absent in non-diabetic children whose parents were both diabetic (Williamson, 1977) and in non-diabetic twins whose siblings had diabetic complications (Karam, 1976). Hereditary factors may have an important influence in determining risk and rate of development of complications; with hyperglycaemia and its attendant

metabolic disturbance having at least a permissive role in bringing these complications to clinical expression (Cahill, 1976).

ii) Metabolic

The evidence suggesting that the late complications of diabetes may be secondary to metabolic derangement is based on both animal and clinical studies.

In animal studies, microangiopathy occurs in spontaneous and induced diabetes in mice, apes, monkeys, baboons, dogs, carp and the Chinese hamster (Camerini-Davalos, 1973). Although the nature of the lesions found in animals is different from those in diabetic humans (Siperstein, 1977), the short duration of diabetes in animals before death could explain the observed differences. Retinal and glomerular capillary changes are seen in dogs with chemically induced diabetes and these have been ameliorated by good diabetic control over a prolonged (5 years) period (Engerman, 1977). A correlation between the degree of metabolic derangement and capillary basement membrane thickening has also been reported in the monkey (Howard, 1975). In addition, the Minnesota group (Lee, 1974) have reported that normal rat kidneys develop mesangial thickening when transplanted to diabetic individuals. Also, in the rat the glomerular changes were reversed by transplantation of the affected kidneys into a non-diabetic host (Lee, 1974) suggesting that the diabetic micro-environment had influenced the development of the renal lesions.

Studies have attempted to establish a relationship between diabetic control and complications in humans (Cahill, 1976, Siperstein, 1977). These studies were largely retrospective, non-randomised and performed before the means to achieve normoglycaemia and to monitor control objectively were developed. There does, however, appear to be a correlation between hyperglycaemia and prevalence, severity and rate of

progression of complications. In a review of 4,400 patients there was a strong association between poor control and incidence and severity of neuropathy, retinopathy and nephropathy (Pirart, 1977). A correlation between the degree of diabetic control and frequency and severity of complications has been reported whilst a greater progression of complications in association with poor control has been shown (Job, 1976; Graf, 1979).

c. Reversibility of Complications

i) Animal Studies

In the rat, transplantation of pancreatic islets (reviewed below) into animals with recently induced diabetes not only normalised carbohydrate metabolism but also resulted in reversal of the renal lesions within weeks of the transplant (Maur, 1975; 1976). However, when transplantation was performed in rats with long-standing diabetes, the transplants failed to reverse the thickening of the mesangium (Steffes, 1980). The finding that intensive insulin therapy alone could prevent the progression of the mesangial thickening in rats during the early stages of glomerulopathy (Rasch, 1979) suggests that there is an early phase when the course of the diabetic glomerulopathy can be reversed. A relatively irreversible phase, is ultimately reached in long standing disease.

ii) Clinical

Eschwege (1979) in a small study, reported that amelioration of glycaemic control resulted in improvement of retinopathy. In addition a correlation has been reported between the rate of progression of proteinuria and poor metabolic control of diabetes (Miki, 1972). Increased urinary albumin excretion and other renal function abnormalities have been normalised by improved insulin therapy (Viberti, 1979).

With improved insulin therapy, self-monitoring and implementation of more physiological regimes of insulin delivery (e.g. multiple injections or continuous insulin infusion) metabolic parameters such as alanine, pyruvate, B-hydroxybutyrate, free fatty acids and amino acids had been shown to be normalised (Pickup, 1979). However, little data is available regarding the effect of this treatment on the long term complications. This is largely because this form of intensive treatment has only been used for a few years and in a relatively small number of patients. Investigators have, however, shown improvement of motor nerve conduction velocity after normalisation of glucose control by these methods (Pietri, 1978).

The data concerning the efficacy of long term normoglycaemia on the evolution of established diabetic retinopathy and nephropathy is controversial. Indeed, some patients show acceleration of proliferative retinopathy within several months of initiating intensive treatment. For example, Tamborlane (1982) examined the effect of long term (1-2 years) pump treatment on microvascular severity and despite improved glucose control, 4 of the 24 eyes with retinopathy progressed, none improved and 6 remained stationary. In addition, no improvement has been observed in patients with marked proteinuria (Viberti, 1982). Although it appears that established diabetic microangiopathy is not reversed by optimal control, the question remains as to whether further progression can be avoided.

The feasibility of achieving near normal glycaemic control has been demonstrated and this has promoted the execution of several randomised trials. Hollman (1983) found stable renal and sensory nerve function in those who maintained good control, as opposed to those with poor control, who developed renal and neuropathic changes. Wiseman (1985) reported normalisation of increased glomerular filtration rate

but not of kidney size in 6 Type I diabetic patients randomly assigned to intensive insulin therapy. The Steno Study Group have shown in 1982 and 1983 reduced albumin excretion rates in intensively treated patients in contrast to those on conventional treatment. In addition, the same group have shown initial worsening of retinopathy, which improved in time, in patients treated with intensive therapy as opposed to progression in the conventionally treated patients.

In conclusion, the question of the effectiveness of improved metabolic control on diabetic complications is still largely speculative. However, there are indications from animal and clinical studies that improved control (particularly if reversed to normality) has an advantageous effect on the early diabetic complications though the point is more tenuous in established complications.

1.6 TRANSPLANTATION OF THE INTACT PANCREAS

The use of exogenous insulin in the treatment of diabetes has fundamentally altered the outlook of patients with this disease. It is however, even at best, an unphysiological and relatively imprecise method of treatment. The injection of insulin, peripherally, even when controlled by a continuous glucose sensor, as in the artificial endocrine pancreas, cannot match the action of endocrine tissue *in vivo*. Replenishment of the lost B cells by transplantation of the pancreas has thus been an attractive concept, as a treatment of this disease, for many years.

Transplantation of the pancreas for diabetes is complicated by the anatomical fact that the necessary endocrine cells constitute less than 2% of the pancreatic cellular population and are scattered throughout the pancreatic substance in the islets of Langerhans. The presence of the exocrine cells and their secretions of digestive enzymes caused the initial difficulty in the isolation of insulin. It is probably of no surprise that these cells have also appreciably increased the problems associated with successful pancreas transplantation.

The first recorded human pancreas transplant was performed in 1893, on a 15 year old boy who was treated by subcutaneous implantation of three 'brazil nut size' pieces of freshly slaughtered sheep's pancreas (Williams, 1894). The boy died comatosed after three days when histological examination of the graft showed only a 'fibrous stroma'. Since that time extensive research on pancreas transplantation has been performed both in animals and humans utilising a range of pancreatic preparations.

a. Non-vascularised Grafts

i) Animal Studies

Direct implantation of pancreatic pieces into the liver and spleen of a dog was first performed by Ottolenghi (1901) but the graft necrosed. Kyrle (1908) autotransplanted vascularised segments of dog and guinea pig pancreas to the spleen and then ligated their pedicles. The grafts survived but their function was not studied. Martina (1908) described the intra-splenic autotransplantation of a free pancreatic segment in a dog with simultaneous excision of the remaining gland. Although the dog became diabetic it survived for three months. Pratt (1913) reported a series of intra-splenic autotransplants of intact and fragmented pancreas in 8 dogs and 2 cats. The majority of grafts necrosed. However in one dog, the vascularised uncinate process was transplanted and subsequently, 22 days later, the remaining pancreas was resected and the graft pedicle ligated. This particular graft appeared to survive 209 days, during which time the urinary glucose fluctuated from 0 to 7%. Subsequent histology showed scattered acini but no islets. A similar technique was successfully developed by Ivy (1926) who autotransplanted a portion of the pancreas by initially transferring it to the abdominal subcutaneous tissue without interruption of its blood supply. Three to four weeks later the vascular pedicle was ligated and the graft survived with a new blood supply from surrounding tissues. Badile (1930) transplanted pieces of cat pancreas into the liver, spleen, omentum, gastric submucosa, retroperitoneal and subcutaneous tissues. Allotransplants were rejected whereas autotransplants survived for up to 105 days within the gastric mucosa.

Pancreatic tissue grafts are potentially at risk from the exocrine

cell secretion. Attempts had been made as early as 1892 (Thirolloix) to suppress pancreatic exocrine secretion by injection of the duct with oil and lamp black. Thirolloix demonstrated that, in the dog, the splenic segment alone, although fibrosed, could alone maintain normoglycaemia. Brancati (1929) used duct ligation to suppress exocrine function and succeeded in autotransplanting free segments of canine pancreas into the greater omentum. Histology of the grafts showed normal islets among atrophied acinar tissue at 28 and 34 days. Selvaggi (1936) subsequently and unsuccessfully allotransplanted normal and duct ligated segments of canine pancreas into the iliac fossa of chickens.

The above detailed experiments confirmed that autotransplanted pancreatic pieces could survive and maintain normoglycaemia provided that they became vascularised from surrounding tissue. Interest in the transplantation of non-vascularised pancreatic pieces in animals then waned although occasional reports can be found. For example, Brooks (1959) autotransplanted vascularised canine pancreas into the rectus muscle and subsequently allotransplanted large fragments, which did not function.

ii) Clinical Studies

There are few reports of transplantation of pancreatic pieces into humans since the original report by Williams (1894). Ssoblew in 1902 and Allan (1903) suggested it as a treatment for diabetes but subsequent correspondents (e.g. Montsarrat, 1903) expressed serious doubts as to the value of such a procedure. Pybus (1924) described two patients who received subcutaneous implants of non-vascularised sliced human pancreas which were rejected, although, a reduction in glycosuria was seen in one patient. Luisada in 1927 transplanted pieces of duct ligated baboon pancreas into the tunica vaginalis of two young diabetics but with no discernible effect.

b. Vascularised Grafts

Transplantation of the pancreas as an organ graft by vascular suture has been performed for a number of years. A legion of techniques have been employed. Pancreatic organ grafts can be whole organ (with or without the duodenum) or segment (tail or body plus tail). The technical variation can be further divided into those that establish exocrine drainage into the bowel or other viscus and those which do not (duct ligated, open or obliterated). Most experimental work has been performed on outbred animals such as the dog.

i) Animal Studies

Transplantation of the canine pancreas with maintenance of exocrine secretion was initially performed utilising the duodenum as a conduit to the skin (Brooks, 1959). Largiader (1967) subsequently modified the technique with exocrine drainage to the recipient's own bowel by use of a Roux en Y loop. Although other investigators (Ruiz, 1972; Toledo-Pereyra, 1975) have utilised this model, vascular torsion and thrombosis were common and the duodenum was prone to necrosis and ulceration. Furthermore, leakage of exocrine secretion was potentially serious because of activation of the exocrine enzymes by the duodenal secretions. Aquino (1973) obviated some of the problems by excision of the duodenum with the exception of the ampulla.

Transplantation of a pancreatic segment is simpler than pancreatico-duodenal grafting and avoids some of the problems related to the duodenum. Gliedman (1973) successfully transplanted one lobe of the pancreas in dogs and anastomosed the pancreatic duct to the ureter. Such grafts have also been performed with anastomosis to the recipient jejunum (Toledo-Pereyra, 1975). However, the problems of anastomotic leakage, enzyme activation and local necrosis still occur.

A number of techniques have been described to allow transplantation of the pancreas without provision for exocrine drainage. Ligation of the pancreatic duct is probably the simplest method of suppressing the exocrine cell secretions (Mitchell, 1967; Brynner, 1975). The exocrine tissue tends to atrophy, leaving the endocrine cells intact. The inflammatory process can, however, involve the islets and may result in impaired function (Rausis, 1970). Furthermore, duct ligation does not completely prevent fluid leakage from the pancreas and this can give rise to local problems (Kyriakides, 1976). A number of additional methods have been used, in an attempt to further reduce exocrine function, for example, steroids (Kyriakides, 1976), drugs which inhibit enzyme synthesis (Castellanos, 1975) and irradiation (Merkel, 1968).

The principle of the technique of duct obliteration is elimination of the exocrine secretions by obliteration of the duct system with a liquid, synthetic polymer injection which sets to form a cast (Dubernand, 1978). Injection of the duct system of one lobe of the canine pancreas, in situ, resulted in a variable course; some pancreata showing severe fibrosis that involved the islets whilst others appeared to atrophy and leave normal islets (Dubernand, 1978). Kyriakides (1979a) and Baumgartner (1981) injected neoprene into canine auto and allografts and demonstrated a low complication rate in autografts although, a higher infection rate was seen in the immunosuppressed allograft recipients. Histological examination at the end of one year showed persistent inflammation (Baumgartner, 1981). Other agents, including acrylate glue (Papachristou, 1979) and polyisoprene (McMaster, 1980) have been injected with higher success rates and fewer complications than duct ligation techniques.

A number of grafts have been performed, initially in the pig (Kyriakides, 1979b), with free drainage of enzyme into the peritoneal

cavity. There were no significant adverse effects except for a transient hyperamylasaemia. Subsequent studies in the dog (Baumgartner, 1980) confirmed that the peritoneum could absorb pancreatic secretions. The duct gradually obliterates after the first week (Idezuki, 1969) and, interestingly, histological examination has shown less inflammation and fibrosis than in the immediately ligated graft (Baumgartner, 1982).

Successful transplantation of immediately vascularised grafts in diabetic animals has been shown to restore blood glucose levels to normal if more than 20% of normal pancreatic mass is transplanted (Sutherland, 1981; Toledo-Pereyra, 1979). Several investigators have reported survival of autograft recipients for months or even years with normoglycaemia and without deterioration of graft function. Blood glucose and insulin levels vary considerably during glucose tolerance testing and may be low, normal or elevated (Lillehei, 1970; Aquino, 1973; Orloff, 1975). The majority of grafts have been performed with a systemic venous drainage. No advantage could be demonstrated in the few experiments performed with drainage to the portal circulation, although plasma insulin levels were lower (Baumgartner, 1981). The combination of systemic venous drainage and denervation of the pancreas appears to be the cause of the elevation of plasma insulin (Bewick, 1981). Whether these minor abnormalities of metabolism are important has not been determined.

ii) Clinical Studies

Pancreas transplantation in man has been carefully documented by the Transplant Registries since the first human vascularised grafts by Kelly (1967). The initial register 'The American College of Surgeons/National Institutes of Health Organ Transplant Registry' recorded 56 cases, in 54 diabetic patients, to its close at the end of June 1977 (Gerrish, 1977). A new register known as the International

Pancreatic Transplant Registry was set up in 1980 and a recent report of this register showed that a total of 1394 pancreas transplants have been performed up to March 1988 (Sutherland, 1989). More than 80% of which have been performed since 1982. As in animal studies, a wide range of techniques have been utilised:- whole or segmental grafts; duct drainage (bowel/other viscus); duct obliterated/ligated/left open.

Overall Results

Transplant Registry figures (Sutherland, 1989) indicate a steady improvement in overall results since 1966 with 12 month functional (insulin independent) rates improving from 3% for the period 1966 to 1977, to 46% (1983-1988). Patient survival rates were 40% and 82% for the corresponding periods. The three most common duct management techniques performed in this latter period (polymer injection, enteric drainage and bladder drainage) were associated with equivalent graft survival rates and together were significantly better (47% vs 16% at one year) than duct ligation, free intraperitoneal drainage or ureteric drainage. There was no significant difference in graft survival for whole or segmental pancreas transplants. Immunosuppressive regimes that included both cyclosporin A (CyA) and azathioprine were associated with higher graft survival rates than those that included only one of the drugs. For technically successful grafts the 1 year survival rates were 67% (azathioprine plus CyA), 54% (CyA but no azathioprine) and 39% (azathioprine but no CyA).

Pancreas graft and recipient survival rates differed according to whether a kidney was transplanted and according to the timing of transplantation. Pancreas graft survival was 53% at 1 year for cases in which a simultaneous kidney was transplanted, 40% for cases in which a kidney had been previously transplanted and 32% for cases in which a kidney had never been transplanted. The difference was significant

($p < 0.001$) for the first category versus the other two. Conversely patient survival rates were highest in recipients of pancreas grafts alone, 90% at 1 year vs. 88% in recipients of a pancreas after a kidney and 77% in recipients of a simultaneous pancreas-kidney transplant. Patient survival was also higher in those diabetics without rather than with end-stage diabetic nephropathy.

Although the graft survival and patient survival figures are well documented in the Registry reports and indicate a continued improvement in pancreas transplantation, there are several fundamental points which must be considered and include overall morbidity, risks from transplantation of the unwanted exocrine tissue, need for continued immunosuppression and effect on chronic complications. Overall more than 25% of the grafts fail for 'technical reasons', including thrombosis and infection. This represents a high morbidity level for the transplanted individuals. Furthermore, transplantation of the entire pancreas for treatment of diabetes is, even when successful, an unsound proposition because 98% of the transplanted tissue (the exocrine component) is not only unwanted but is also one of the principle causes of the morbidity.

II TRANSPLANTATION OF ISLET TISSUE

The possibility of transplantation of islet enriched tissue without the detrimental effects of surrounding exocrine tissue has been approached by the use of preparations of isolated islets from the adult pancreas and also the use of foetal tissue.

1.7 STUDIES IN THE RODENT

For the purposes of this thesis, in which mice have not been used, the following review will focus on rat studies.

a. Preparation of Isolated Islets

The first report of islet isolation was by Bensley (1911) who used microdissection to obtain islets from the guinea-pig pancreas. Although this method was elaborated by both Norberg (1942) and Hellerstrom (1964), the number of islets produced was small (80 islets/mouse) and studies were limited to morphological examination and biochemical analyses.

A crucial advance in the technique of islet isolation was the use of the enzyme collagenase to disrupt the architecture of diced pancreas and liberate morphologically intact islets (Moskalewski, 1965). This initial technique was subsequently improved by Lacy (1967) who described distension of the rat pancreas with balanced salt solution to facilitate resection and mincing prior to incubation in collagenase. A yield of 75-100 isolated islets per pancreas was obtained from the digest, predominantly by the handpicking of individual islets with a wire loop. Alternatively, islets were purified by centrifugation, after layering of the digest on a discontinuous, sucrose density gradient. This method resulted in a higher yield of 300 islets/pancreas.

The first report of isolated islet transplantation in the rat was by Younoszai (1970) who transplanted isologous islets into the

peritoneal cavity of 5 diabetic rats and demonstrated a reduction of hyperglycaemia in 3 rats and normoglycaemia, for 4 days, in one rat. The effect of isolated islet transplantation in the rat was further documented by Ballinger in 1972 who demonstrated survival of grafted islets, prepared from several isologous donors, transplanted either intraperitoneally or intramuscularly into diabetic recipients. Survival of the recipient rats was extended by this treatment, when compared to controls. In addition, diabetes was ameliorated with maintenance or gain of body weight, reduction of glycosuria and, in some animals, complete post-transplantation normoglycaemia.

These initial experiments demonstrated that islets could be effectively isolated from the rodent pancreas and utilised for the treatment of diabetes. These early experiments also indicated one of the major problems of islet transplantation, that of the ability to isolate large numbers of viable islets with minimal contaminating tissue. Islet isolation is a multistage procedure, involving pancreatic resection, collagenase digestion and islet purification. Much of the more recent research, aimed at optimising yields has concentrated on improving these individual stages in the isolation process.

i) Pre-treatment of the Pancreas

Pancreatic exocrine atrophy after duct ligation was first described by Scott (1912). Keen (1965) reported some success with this technique as a measure to aid microdissection of islets in the rat but the presence of multiple ducts was a hindrance. Subsequently, chemical means to induce exocrine cell atrophy were reported utilising pilocarpine (Vrbova, 1979) and DL-Ethionine (Payne, 1979). These agents appear to facilitate the isolation of viable islets in the rodent, but require a period of administration prior to pancreatic resection. Furthermore, the effect of these chemicals on other organs might make

them unsuitable for transplantation. For these reasons, the chemical induction of exocrine atrophy is unlikely, ultimately, to be of value for human islet isolation.

ii) Collagenase Digestion

The method described by Ballinger (1972) of pancreatic distension, mincing and incubation in collagenase solution remained the standard initial technique for islet isolation until relatively recently. The major problems associated with this stage include variability of the individual batches of collagenase enzyme and the determination of the optimal 'end point' of digestion. Moskalewski (1965) indicated, in his report of islet isolation by collagenase digestion, the need to 'find the optimal concentration and isolation time for every new batch of enzyme because of the difference in their activities'. Too short a period of incubation results in entrapment of the islets within the exocrine tissue; too long an incubation period results in digestion and disruption of the islets themselves. Shibata (1976) attempted to overcome the problem by the use of a filtration-digestion method incorporating a digestion chamber with a wire screen. Theoretically, repeated washing and digestion cycles allowed elution of the islets from the chamber, once released from the pancreatic tissue. Resultant overall islet yield was improved (400 islets/rat pancreas) but consisted of a larger number of smaller islets and thus the final islet mass was only slightly increased.

Improvements in the collagenase digestion stage have recently been reported, for the mouse (Gotoh, 1985) and rat (Sutton, 1986), by incorporation of the enzyme into the initial distending salt solution followed by resection of the pancreas and static incubation. This method allows effective distribution of the enzyme throughout the pancreatic substance and eliminates the need for the damaging mincing

stage. Islet yields were improved but islets from two donor rats were still required for prompt reversal of diabetes in a single recipient rat (Sutton, 1986).

iii) Purification of Islets

The second stage of islet isolation involves the separation of the islets from the unwanted exocrine tissue. Although intact islets can be isolated from the pancreatic digest by handpicking with a finely drawn pipette or a wire loop, for the purposes of transplantation this method is inefficient and time consuming.

A number of techniques for the purification of rodent islets have been described with the specific aim of increasing yield and overall purity whilst maintaining viability. Much of this work has centred on basic cell separation techniques which rely on the intrinsic properties of the cellular constituents such as cell size or density to achieve separation (Leif, 1964; Shortman, 1972).

Lacy (1967) described unit gravity sedimentation in a vertical conical test tube to assist islet purification. This method, which utilises cell size as the discriminating factor was, however, inefficient in that only 75 islets per pancreas were obtained. Furthermore, handpicking of islets was still required to produce a pure islet preparation.

The variation of buoyant density amongst different cell types has been appreciated for many years (Leif, 1964) and has led to the development of a number of methods for physical separation. One of the simplest methods is that of neutral density separation in which cells are centrifuged in a dense liquid medium and are segregated into two fractions, one denser and one lighter than the suspending medium. Multiple layered discontinuous density gradients are an extension of this methodology, and allow the separation of different cell types over a wide

range of densities. The conditions used for density gradient cell separation must maintain cell viability and for this reason aqueous solutions are employed and toxic materials and extremes of pH and osmotic pressure are avoided (Shortman, 1972).

The initial density gradient used to purify rodent islets was a discontinuous gradient of 1.4–1.8 M sucrose (Lacy, 1967) and an improved yield of islets (300 islets/rat pancreas) was demonstrated compared to unit gravity sedimentation. The islets so produced were, however, 'damaged' and were not suitable even for in vitro metabolic studies. This damage was probably secondary to the hyperosmolality of the sucrose solutions (Lacy, 1967) and subsequently Ficoll, a polymer of sucrose, was substituted as the gradient medium on the basis that it provided a better osmotic environment for the islets (Lindall, 1969).

For transplantation studies the crucial assessment of islet viability is in vivo, i.e. their ability to reverse diabetes after transplantation. Interestingly, variable success of islet transplantation was reported with Ficoll isolated islets (Scharp, 1973), with variability appearing to correlate with different batches of Ficoll. It was suggested that the Ficoll solutions contained low molecular weight substances which affected the islet cell membrane adversely. Improved in vivo islet viability was reported with dialysed Ficoll (Scharp, 1973) and subsequently with newer Ficoll formulations (Nash, 1976). These solutions were still, however, hyperosmotic at the densities required for islet isolation. Several iso-osmotic density gradient media, including Ficoll-Hypaque (Tze, 1976), Metrizamide (Raydt, 1977), Ficoll-conray (Okeda, 1979) and Percoll (Brunstedt, 1980; Yamamoto, 1981) have all been studied in an attempt to improve results. Islets prepared on these iso-osmotic gradients have been shown to have

normal morphology and insulin release characteristics on in vitro testing although in vivo viability was not studied.

At the time of commencing this study no detailed comparison between different density gradient media had been made and, despite its hyperosmolality, a density gradient of Ficoll was the standard technique for islet purification.

b. Metabolic Effects of Isogeneic Islet Transplantation

In many studies, it has been established that islet transplantation can reverse diabetes in the rodent. The number of islets required for reversal of hyperglycaemia appears to depend on a variety of factors including, severity of the diabetic state and integrity of the islets (Rabinovitch, 1976; Koncz, 1976). Normoglycaemia has been restored in the diabetic rat by intraportal transplantation of as few as 240 islets (Henriksson, 1977). Hyperglycaemia was, however, ameliorated faster if larger numbers of islets were injected (Henriksson, 1977). In general, with transplantation of greater than 1000 islets normoglycaemia usually occurred within 1-2 days of transplantation. If sufficient islets were transplanted, the metabolic abnormalities in the diabetic rat were reversed. Blood glucose levels returned to normal, glycosuria was abolished, polyuria and polydipsia were alleviated, circulating insulin levels became normal or even elevated and glucose tolerance curves were normalised (Kemp, 1973; Lorenz, 1975; Feldman, 1977). Minor abnormalities of glucose tolerance have been attributed to the ectopic location of the islets, reduced islet mass or lack of innervation (Trimble, 1980).

Isolated islets have been transplanted into a number of different sites. Although the majority of studies have utilised the intraportal route, the spleen and the renal subcapsular space have been shown to be effective sites (Reece-Smith, 1981; Mellgren, 1986). Diabetes has

rarely been reversed by transplantation into subcutaneous, intramuscular, intrapancreatic or intrasalivary gland tissue (Kemp, 1973; Georgakakis, 1977). Histological survival of islets at these sites has, however, been observed.

Neovascularisation of the islets has been shown to occur following transplantation (Griffith, 1977). Differing rates of this process may explain the variability of islet survival in different sites (Mellgren, 1986). With specific regard to the liver, quantitative studies have suggested that at least 50% of the islets embolised into the portal vein survive (Pipeleers-Marichal, 1976; Trimble, 1980).

c. Results of Allogeneic Islet Transplantation

A substantial number of reports have investigated the effects of allogeneic islet transplantation in the rodent. Multiple factors including species, heterogeneity of donor:recipient combination pairs; variability of islet source; transplant engraftment site; potency of biological immunosuppressant and effectiveness of immunosuppressants such as steroids and cyclosporin A on the severity of diabetes makes full comparisons difficult. A variety of treatment protocols affecting the donor tissue or recipient animal which will prolong allograft survival have, however, been described.

i) Pre-treatment of Donor Islets

The concept behind the prevention of islet transplant rejection without the use of immunosuppressive drugs has been that lymphoid cells within the islets and not the endocrine cells themselves initiate the rejection process (Lafferty, 1984). This 'passenger leucocyte' concept has been tested by the use of methods which appear to destroy or alter the lymphoid cells but do not affect the islets cells.

Lacy (1979) demonstrated that rejection of rat islet allografts could be prevented by the use of low temperature culture of the donor

islets, if combined with a single injection of anti-lymphocyte serum into the recipient. Subsequently it was shown that islet cells express only Class I histocompatibility antigens, whereas the antigen-presenting or dendritic cells express both Class I and Class II (Faustman, 1980). The prevention of mouse islet allograft rejection was then demonstrated with either incubation of the donor islets and antibody to Class II antigens plus complement (Faustman, 1981) or by treatment of the islets with a monoclonal antibody to dendritic cells plus complement (Faustman, 1984).

Additional protocols which have been shown to prevent rejection of islet grafts in rodents include: culture of mouse islet aggregates in 95% O₂ and ultraviolet irradiation of donor rat islets (Lacy, 1984). In rats with a strong immune response a temporary suppression of the immune system with Cyclosporin A, in conjunction with treatment of the donor islets with U.V. irradiation (Hardy, 1984), low temperature culture (Lacy, 1979) or Ia antibody will prevent rejection (Terasaka, 1986).

ii) Immunosuppression of the Recipient

Early reports of the use of general immunosuppression of the recipient, by chemical or biological agents, were relatively unsuccessful in that only moderate prolongation of graft survival was achieved in relatively small numbers of animals (Sutherland, 1981 - for review). More recently, prolongation of rat allograft survival has been demonstrated with the use of agents such as: 15-Deoxyspergualin (Walters, 1987) and interleukin-2 receptor antibody (Hahn, 1987).

iii) Immuno-isolation of the Islets

An alternative technique for the prevention of rejection of islet grafts has been to protect them from the recipients' immune system by encasement of the tissue within specific chambers. Such techniques should not only protect the islets but also allow free diffusion of

secretagogues and essential nutrients. The initial methods utilised quite large chambers (Millipore or Nucleopore) containing cellulose or polycarbonate membranes. Although successful reversal of diabetes was achieved (Marotas, 1976; Gates, 1977) the major problem has been fibrosis of surrounding tissue which limits graft survival and the diffusion of insulin and glucose. Recently, the use of alginate-polylysine-alginate envelopes to encapsulate individual islets has shown promise because of this materials' bio-compatibility (O'Shea, 1984; Sun, 1987). Effective studies of the use of this material with human islets must, however, await the availability of large numbers of purified islets.

d. Preservation of Islet Tissue

The preservation of isolated rodent islets may facilitate transplantation studies. Several methods, including tissue culture, cold storage and cryopreservation have been investigated.

i) Tissue Culture

A number of reports have shown that rodent islets can be cultured at 37°C for periods of up to several weeks and retain their ability to secrete insulin and reverse diabetes once transplanted (Nakagawara, 1977; Weber, 1977; Andersson, 1978). Several different culture media were successfully used to maintain the islets. Factors such as the presence of serum, the glucose concentration, and pH all contributed to the efficiency of the medium utilised.

Diminution of amylase content of the culture fluid has been demonstrated during culture of pancreatic tissue prior to transplantation (Matas, 1977). This technique may thus provide a means of purification of islet tissue by deletion of exocrine cells and enzymes.

ii) Cold Storage

The first report of this relatively simple technique showed a marked reduction in insulin release by islets after 3 days storage in Hanks' solution at 4°C (Knight, 1973). Subsequently, maintenance of the ability of isolated islets to release insulin was demonstrated by Frankel (1976). For the optimal technique, a high glucose (18 mmol/l) containing, amino acid supplemented medium and a temperature of 8°C with short weekly incubations at 37°C was used. With these improved conditions insulin release was maintained for at least a week, but ultimately became impaired, with insulin release falling to 30% of the original value by 5 weeks. Diminution of islet viability has also been shown by in vivo studies, in which, reliable reversal of diabetes by transplantation of 24 hour but not 72 hour cultured islets was demonstrated (Morrow, 1982). It would thus appear that hypothermic storage can only be utilised for short periods of time.

iii) Cryopreservation

A variety of cells and tissue fragments can be stored at low temperatures (in liquid nitrogen) for prolonged periods. A number of studies have investigated the use of different freezing protocols and cryoprotectants for the preservation of islet or islet containing tissue (Bank, 1979; Rajotte, 1981; Taylor, 1983; Sandler, 1986). Although islet tissue was 'preserved', diminution in viability both in vitro (e.g. insulin release) and in vivo (transplantation) was demonstrated.

Cryopreservation has also been used to aid islet purification on the basis that acinar and islet cells have optimal conditions for cryogenic preservation. Selective destruction of acinar cells, under conditions which were innocuous for islet cells, has been demonstrated (Bank, 1983).

e. Transplantation of Foetal Tissue

The use of foetal rodent pancreas to reverse diabetes attracted attention because such tissues have a capacity for growth and differentiation after transplantation (Brown, 1978). In addition, although the overall B cell mass is relatively small, there is a favourably high ratio between endocrine and exocrine tissue content (Brown, 1978). The low islet mass and required period of time for differentiation of the endocrine component, however, results in the need for multiple donor transplantation if diabetes is to be ameliorated completely and with a short latent period. In general, relatively simple techniques have been used to transplant foetal tissue, either free as an intact, whole organ graft (McEvoy, 1978) or as dispersed tissue produced by collagenase digestion (Hegre, 1976). Purified islets can be obtained by tissue culture of the fragments and selective loss of the exocrine cells (Hellerstrom, 1979).

It was initially hoped that rodent foetal pancreas would be less immunogenic than adult islet tissue. Studies have, however, shown that rodent foetal pancreas is highly immunogenic and attempts to allotransplant foetal pancreas have met with limited success (Simeonovic, 1988).

1.8 STUDIES IN LARGE ANIMALS

Following the successful reversal of diabetes in the rodent by isolated islet transplantation, attempts were made to develop similar models in larger animals (pig, monkey and dog). The methods of islet isolation initially used were based on those described for the rodent, i.e. pancreatic distension, mincing and, in some studies, islet isolation. A significant problem in the large animal has been the lack of an inbred strain and thus, for isogeneic experiments single donor:single recipient engraftment must be used. Although multiple donors can be utilised and allogeneic tissue transplanted, the major problem in the assessment of success has been dissociating failure of graft function from transplant rejection.

a. Preparation of Islet Tissue

Sutherland (1974) had limited success with intramuscular and intraperitoneal auto- and allotransplantation of isolated porcine islets, prepared by collagenase digestion of the resected pancreas with separation on Ficoll density gradients. Prolongation of life was demonstrated (13 days v 6 days, in non-transplanted controls) with evidence of insulin secretion by the graft, although all animals became and remained hyperglycaemic post-transplantation. Subsequently, Scharp (1975) transplanted Ficoll isolated islets from the Rhesus monkey (prepared in a digestion/filtration chamber to maximise yield) into allogeneic recipients in which diabetes had been induced by partial pancreatectomy and streptozotocin injection. Although improvements in post-transplantation glucose tolerance tests were documented, results from this type of animal model have been difficult to interpret because of the inability to determine the contribution made to the overall post-transplantation glucose control by the remaining pancreas

(Sutherland, 1981).

A major contribution to the development of a large animal model was made by Mirkovitch (1976; 1977) who reported the successful autotransplantation of pancreatic tissue in the dog following total pancreatectomy with prevention of diabetes. The resected pancreas was injected with collagenase, minced and incubated to produce micro-fragments which were then dispersed, without any attempt at purification, throughout the splenic substance by direct infusion. The suggested key elements of success were the production of tiny pieces of tissue (all less than 2 mm in diameter), the omission of islet purification and the widespread dissemination of the tissue throughout a well vascularised organ.

A number of parameters have been shown to be important for the preparation of islet microfragment grafts in the dog. Kretschmer (1977a) demonstrated that the duration of the collagenase digestion was critical. Furthermore, Mehigan (1981) showed that not only was the process dependent on the batch of collagenase (as in the rat) but also that the outcome was markedly affected by the method and degree of pancreatic disruption. A mechanical mincing technique, with production of a 'moderate' particle size resulted in a higher degree of graft success than hand mincing or mincing to a small particle size. Subsequent improvements were made by elimination of the mincing stage by intraductal delivery of collagenase (Horaguchi, 1981). The efficacy of this technique has been confirmed by a number of reports including comparative studies by Griffin (1986) and Hesse (1986). Preliminary preparation of the pancreas, by duct ligation, to reduce the amount of exocrine tissue was shown to be detrimental to micrograft transplantation (Mehigan, 1980; Yamauchi, 1985) unlike similar studies in the rat.

Repeated attempts to improve the purity of the tissue transplanted have been made since the first successful microfragment transplants in 1976. Noel (1982) utilised overnight culture and then Ficoll density gradients to isolate canine islets which were transplanted directly into the spleen. Hyperglycaemia was reversed by such transplants in three dogs, however this study was marred by the use of the incomplete model of diabetes of partial pancreatectomy and streptozotocin injection. Serial sieving has been shown to slightly improve the fragment purity, as measured by insulin/amylase ratios, when compared to whole pancreas (Griffin, 1986; Hesse, 1986). Irradiation (Nason, 1986) and cryopreservation (Evans, 1987) have been used to attempt purification of tissue prior to engraftment, with limited effect.

Although the majority of work in large animals has been performed in the dog, Gray (1986a) reported the successful autotransplantation of isolated islets in the pancreatectomised Cynomologous monkey. Islets were prepared by techniques developed for human islet isolation (see below) using intraductal collagenase digestion and purification with serial sieving and Ficoll density gradients. Islet purity in the final preparation was approximately 10%.

b. Metabolic Effects of Isogeneic Transplantation

Successful islet transplantation in the pancreatectomised dog has been shown to maintain normoglycaemia, although many animals become moderately hyperglycaemic for 2-10 days post-transplantation (Mirkovitch, 1977; Hesse, 1986) presumably because of a period of neovascularisation following implantation. Basal serum insulin levels were similar to normal (Mirkovitch, 1977; Hesse, 1986) although glucose clearance and insulin release during glucose tolerance testing were generally significantly reduced (Mirkovitch, 1977; Warnock, 1983;

Hesse, 1986). All the principal hormonal and metabolic abnormalities that exist in diabetic dogs were reversed in the short term by islet transplantation (Alderson, 1984). In some studies an improvement in graft function has been documented between 1 and 3 months post-transplantation (Alderson, 1984; Alejandro 1986). A significant (70%) long term graft failure rate has, however, been demonstrated in both dog and monkey at 15 months and 18 months respectively (Alejandro, 1986; Sutton, 1987). Chronic stimulation of a low beta-cell mass has been suggested as a cause of such failure (Alejandro, 1986; Sutton, 1987) particularly as successful long term graft function can be predicted from metabolic assessment shortly after (6 weeks) initial grafting (Sutton, 1987).

The spleen has been utilised as the site of engraftment in the majority of canine microfragment transplants because of its vascularity and the large volume of tissue which must be injected. The method of transplantation has generally been by direct puncture (Mirkovitch, 1977) or reflux into the terminal splenic veins (Warnock, 1983). Insulin containing tissue has been demonstrated by histological methods within the splenic substance post-transplantation, with in some cases, a degree of surrounding fibrosis (Hesse, 1986; Griffin, 1986). Although microfragment transplantation has been performed by injection into the liver via the portal vein (Kolb, 1977; Kretschmer, 1977b), portal hypertension and disseminated intravascular coagulopathy develop with potentially lethal effects (Mehigan, 1980; Miller, 1983). Such features were not demonstrated with purer islet tissue transplantation as performed in the dog (Alejandro, 1985) and the monkey (Gray, 1986a). The renal subcapsular site has also been used as the site of microfragment engraftment in the dog (Toledo-Pereyra, 1984). The value of this site has, however, not been confirmed by all workers (Hesse, 1986).

c. Allogeneic Transplantation

Early studies indicated that chemical immunosuppressive agents were ineffective at prolonging allogeneic graft survival (Kretschmer, 1979; Kolb, 1980a). More recently, prolonged survival of allogeneic islet transplants (both microfragment and purified islets) has been demonstrated with use of cyclosporin A (Alejandro, 1985; Williams, 1986). Relatively simple measures such as short term tissue culture or treatment of fresh islets with anti-Ia monoclonal antibodies and complement are inadequate to prevent rejection in outbred dogs unless combined with low dosage cyclosporin A (Alejandro, 1987a). Of interest, however, Toledo-Pereyra (1984b) has demonstrated prolonged survival of allogeneic canine microfragments transplanted under the renal capsule without immunosuppression.

d. Preservation of Islet Tissue

Cold storage (4°C for 24 hours) and cryopreservation (-196°C) have both been shown to preserve canine tissue viability sufficiently for successful engraftment (Schulak, 1978; Walsh, 1984) with long term function (Kneteman, 1986a).

1.9 STUDIES WITH HUMAN ISLETS

a. Adult/Infant Donor Tissue

i) Early Experience

The first report of the isolation of human islets of Langerhans was by Ashcroft in 1971. He obtained a portion of pancreas from a four year old child, at surgery, and isolated a small number of islets by distension, mincing and collagenase digestion of the tissue. Insulin release from and glucose utilisation by the islets were demonstrated. The ability to isolate islets in this manner was confirmed by Ballinger (1972) who used the same technique but with Ficoll gradient separation to isolate islets from a segment of pancreas resected from a twelve year old boy. Similarly, Sutherland (1974) isolated histologically intact islets from a series of 30 cadaveric adult pancreata. Biochemical criteria were used not only to assess the purity of the preparation but also to confirm the ability of the isolated islets to synthesise protein. This early paper indicated some of the problems of human islet isolation, in particular the poor yield and difficulties experienced in technical terms (amount of glassware, reagents required) of processing large amounts of human tissue.

In an attempt to reduce the amount of contaminating tissue Sutherland (1976) then determined the ratio of endocrine to exocrine tissue in a series of twelve human infant (<1 year) pancreata. The results of this study indicated that human infant pancreas contained a significantly higher ratio of insulin to amylase than the adult pancreas. Purification was thus potentially not required. Furthermore, viability of the tissue was confirmed by measurement of insulin release by in vitro glucose stimulation. A series of ten human transplants (4 adult and 6 infant) were then performed in 7 patients by the Minnesota group utilising the methods described above (Najarian, 1977).

Minimal success was, however achieved, with no cases of complete insulin withdrawal (Fig. 1.3).

A number of additional groups were attempting transplantation of allogeneic islet tissue at this time (Kolb, 1980b; Valente, 1980; Sutherland, 1980)(Fig. 1.3). There were few indications of success but, fortunately, no patient appeared to be harmed by these procedures. During the same period islet transplants were also performed in several series of patients undergoing near total pancreatectomy for chronic pancreatitis (Cameron, 1980; Najarian, 1980; Traverso, 1981; Hinshaw, 1981). In these studies the pancreas was resected and processed in a similar manner to the allografts, in that collagenase digestion, mincing and injection into the portal vein were performed. Several of these autograft reports indicated success, some patients having a lower insulin requirement than would normally have been expected, with increased levels of C-peptide release. A major drawback of these studies was, however, the inability to determine the contribution to metabolic control of the remaining non-resected pancreas. Furthermore, this technique began to fall into disrepute, due to reports of portal hypertension, hepatic infarction and liver failure which complicated the injection of unpurified pancreatic digest into the portal vein (Walsh, 1982; Memsic, 1984). More recently, Toledo-Pereyra (1986) has transplanted pancreatic fragments into the renal subcapsular space which has resulted in the development of abscesses in the perirenal and subhepatic regions.

ii) Improved Methods of Islet Isolation

Although the clinical trials of islet transplantation in diabetic patients faltered during the early 1980's following the lack of success and development of complications, the quest to improve human islet isolation techniques has continued. A major impulse to the development

of more efficient human islet isolation was achieved by Gray (1984) who reported an improved technique using intraductal injection of collagenase solution and incubation at 39°C, followed by disruption of the digested tissue by shaking and aspiration through differing sized needles. Finally, purification was performed by sieving and centrifugation on Ficoll gradients. An average yield of 1100 islets per gram of pancreas was achieved, although purity was less than 30%.

Subsequent reports have confirmed the value of intraductal delivery of collagenase. Kneteman (1986b) perfused the pancreas with collagenase via the duct and the tissue was then teased apart and purified by filtration. Approximately 85,000 islets per pancreas were obtained with a purity of 20-40%. Kuhn (1986) perfused the pancreas with collagenase and used Velcro strips to retain the partially digested tissue. An average yield of 80,000 islets was obtained. Kneteman (1987) utilised the distension method and then liberated the islets by the passage of the tissue through a tissue macerator. The final washing of the islets was performed in a cell elutriator. The yield of islets per pancreas was increased to 150,000 to 250,000 islets/pancreas. Such improvements persuaded one group (St. Louis) to perform a series of unpurified islet transplants in six diabetic patients with functioning kidney grafts (Scharp, 1987)(Fig. 1.3). Function of the islet grafts was indicated by an increase in C peptide level and decrease in insulin requirement of 50-90% in three patients for 2-3 months. In addition, 5 islet cell transplants were performed in 4 patients by the Miami group (Alejandro, 1987b). Ductal instillation of collagenase and hypertonic sucrose-EGTA solution was used to disrupt the pancreas and minimal islet purification was performed by unit gravity sedimentation and culture with anti-Ia monoclonal antibody. In all 5 patients tissue was safely

engrafted into the liver but function could only be demonstrated in one individual by a reduction in insulin requirement.

The above reports showed that large numbers of islets could be liberated from the pancreas by intraductal delivery of collagenase enzyme. However, the significant problem of how to efficiently isolate large numbers of pure islets from this crude digest remained.

iii) Human Islet Viability

The isolation of large numbers of pure human islets is not the only consideration for successful transplantation studies. The isolation process itself may be potentially damaging to the islets. It is thus crucial that the isolated islets are viable and able to respond appropriately to glucose stimulation, with reversal of the diabetic state once transplanted. In vitro biochemical techniques have predominantly been used to assess islet viability. Measurement of insulin release from islets after stimulation with differing concentrations of glucose has commonly been used either 'statically' by incubation (Ashcroft 1971; Ballinger, 1972; Andersson, 1976; Gray, 1984; Warnock, 1987) or in a continuous system by 'perifusion' (Sutherland, 1976; Kneteman, 1986 & 1987; Scharp, 1987; Warnock, 1987). Glucose utilisation (Ashcroft, 1971), protein (insulin) synthesis (Sutherland, 1974; Lundgren, 1977) and oxygen utilisation (Andersson, 1977; Lundgren, 1977) have also been documented. More recently the use of vital stains to confirm overall viability has been described (Gray, 1984; Warnock, 1987). None of these techniques can, however, indicate whether the islet tissue will function appropriately in vivo.

Attempts have been made to confirm in vivo human islet viability by transplantation into a specific animal model, the nude (athymic) rodent. Such animals have a deficient immune system due to congenital thymic

aplasia and are unable to reject transplanted xenogeneic tissue (Festing, 1981).

The first report of transplantation of human pancreatic tissue into the nude rodent was by Povlsen (1974), when several portions of human foetal pancreas were transplanted subcutaneously into a non-diabetic nude mouse. Histological examination of the excised tissue at 64 days post-transplantation revealed a relatively normal lobular appearance with no sign of rejection. A number of groups subsequently reported further success with transplantation of human foetal pancreas into the non-diabetic nude mouse with observation of histological differentiation and the maturation of endocrine tissue (Usadel, 1980; Mandel, 1982; Schwedes, 1983; Tuch, 1984).

Viable insulin containing tissue with no sign of rejection was also demonstrated histologically, at two weeks post-implantation, in four survivors out of fifteen non-diabetic nude mice transplanted with freshly isolated human adult islets by Gray (1984) and recently, in the nude rat, with cryopreserved human islets (Warnock, 1987). Although these reports indicated the potential value of this animal model for xenotransplant studies, the assessment of the functional ability of transplanted tissue is only possible in diabetic recipients.

Buschard (1976) was the first to report reversal of streptozotocin induced diabetes in 3 out of 6 nude mice transplanted subcutaneously with 1-3 neonatal rat whole pancreas grafts. Blood glucose returned to normal 2 weeks after transplantation and the animals survived and gained weight for 27-93 days. Intraperitoneal transplantation of isolated human islets (2 cases) and allogeneic mouse islets (4 successes out of 8 transplants) into diabetic nude mouse was subsequently reported (Andersson, 1976; Lundgren, 1977). As indicated above the criteria for success was a reduction in blood glucose over a period of time. Glucose

levels did not, however, return to normal and there was no confirmation of graft function by glucose tolerance testing, reversal to the diabetic state following graft removal or histological confirmation of graft survival. More recently, the maturation of human foetal pancreas in diabetic nude mouse recipients, including one case of reversal of diabetes has been demonstrated by Tuch (1985). Graft function at 52 weeks post-transplantation in the normoglycaemic animal was confirmed by recurrence of diabetes on graft removal. The relative paucity of reports on pancreas transplantation in the diabetic nude rodent, probably reflects the poor survival of such animals not only in their normal immunologically incompetent state but also after induction of diabetes.

iv) Preservation of Human Islets

For human islet transplantation studies, the ability to store isolated islets for periods of time by tissue culture and/or cryopreservation would be advantageous.

Tissue culture

The capacity of isolated human islets to survive in tissue culture without loss of function for 1-3 weeks has been demonstrated (Andersson, 1976; Lundgren, 1977). Furthermore an attempt was made to demonstrate the ability of these islets to reverse diabetes by transplantation into diabetic athymic mice (Lundgren, 1977). Prolongation of survival, associated with a reduction in hyperglycaemia, was obtained in one of the transplanted animals.

Tissue culture has also been used to purify pancreatic digest (Reemstma, 1980). In this study, the tissue culture technique while resulting in islet purification, also caused a loss of islets, with only 1-5% survival after one week of culture. However, more recently, Scharp (1987) has demonstrated the ability of human islets to survive and

function after culture at 24°C for one week. The success of this technique has potential importance for immunological studies because of the demonstrated selective loss of antigen presenting cells in rodent islets when cultured in analogous conditions.

Cryopreservation

Kneteman (1986) and Warnock (1987) have demonstrated the cryopreservation of small numbers of human islets utilising standard techniques. Evidence of damage was however apparent from secretion and electron micrographic studies.

b. Foetal Tissue

An alternative source of human pancreatic tissue for transplantation is the human foetus. The presence of insulin containing islets of Langerhans can be demonstrated by the 10th week of gestation (Villem, 1969). The first report of the isolation of foetal tissue was in 1970 (Espinosa, 1970) and the methodology used to produce 'foetal islets' (pancreatic resection, chopping, collagenase digestion and subsequent culture) has remained largely unchanged to date. Potential advantages of the use of such tissue are the high endocrine to exocrine cell ratio which obviates the need for purification, and the potential for growth and maturation in vivo (Tuch, 1985).

The first report of a diabetic individual being transplanted with human foetal pancreas was by the Stockholm group in 1980 (Groth). A 45 year old diabetic was transplanted with fragments from 6 foetal pancreata into the portal vein. Insulin requirement post-transplantation was unchanged but function of tissue was suspected by the presence of C-peptide in the urine.

A number of groups have subsequently reported the transplantation of human diabetics with this type of graft and up to November 1988 just under 400 individuals have been transplanted with foetal tissue

(Hering, 1988). Most studies have used tissue from multiple donors, of gestational age 9-22 weeks, with a culture period of 1-70 days, and an intraportal or intramuscular transplant site in an immunosuppressed recipient (Hering, 1988). Unfortunately documentation on donor tissue preparation, diabetic status of the recipient, effect of islet transplantation on metabolic and chronic complications is lacking. Thus assessment of the reported findings, which in some studies indicate a 100% success, is difficult. Many reports indicate a relative change in insulin requirement, but this finding is not considered to be a reliable determinant of success. Thus it seems that, at present, confirmation of the success of foetal pancreas transplantation in humans is unproven.

Furthermore, the considerable ethical implications of utilising human foetal tissue have recently become most prominent, particularly as most of the tissue used is harvested from induced abortions (Marwick, 1988). In the United States all clinical studies on foetal tissue have currently been halted pending a report from the National Institute of Health (Marwick, 1988).

Figure 1.3 : Summary of clinical experience of allotransplantation of adult islet tissue

Reference	Date	Institut ⁿ	No. of cases	Donor Tissue	Preparation	Digestion	Disruption	Purification	Tx Site	Immunosuppression	Insulin Dose Reduction
Najarian	1977	Minnesota	10 in 7 pt	Infant (6) Adult (4)	Distension + Mincing	Collagenase	Tituration	Nil Ficoll grad	I. Perit (5) I.M. (1) I.P. (4)	Pred+Aza (3) Pred+Aza+ALG (4)	26-75% N=7, 2-78w
Kolb	1980	Zurich	7	Adult (6) Infant (1)	Distension + Mincing	Collagenase	Nil	Nil	I.P. (4) Spleen (3)	Pred+Aza+Cyclop	100%, N=1, 10-20 m
Valente	1980	Genova	13	Adult	Mincing	Collagenase	Nil	Nil	S.C. (10) I.P. (1)	Diffusn chamb	100%, N=2 12-77%, N=10
Sutherland	1980	Minnesota	8 in 7 pt	Adult	Mincing	Collagenase	Nil	Nil	I.P. (8)	Pred+Aza+ALG	100%, N=2, 2-3d 0%, N=6.
Bandlien	1987	Detroit	7 in 6 pt	?	Mincing	Nil	Nil	Nil	Spleen (2) Renal (6)	Pred+Aza+ALG	50%, N=2.
Alejandro	1987	Miami	5 in 4 pt	Adult		Ductal coll } EGTA	Tituration	Unit gravity	I.P. (5)	Pred+Aza+CSA	50%, N=?
Scharp	1987	St Louis	6	Adult		Ductal coll	Macerator	Elutriator	Spleen (6)	Pred+Aza	50-90%, N=5, 1-3m
Key	I.M. - I.P. - Pred - Aza - ALG -	Intramuscular Intraportal Prednisolone Azathioprine Antilymphocyte globulin			d - day w - week m - month						

1.10 SUMMARY AND SCOPE OF THE PRESENT STUDY

This chapter has documented many of the fundamental discoveries that have elucidated the relationship between the disease diabetes mellitus and the B cell of the islets of Langerhans. This disease is certainly common and has a multi-factorial aetiology and pathogenesis, with current evidence favouring a selective autoimmune destruction of the B cell population. Although the ideal therapy for diabetes would be prevention, there are currently no techniques available to achieve this.

Treatment of diabetic individuals with insulin during the last 60 years has been lifesaving. However, chronic complications frequently develop and result in significant morbidity and mortality. Current opinion suggests that this is due to imperfect glucose control and that effective maintenance of normoglycaemia may prevent or even reverse early complications.

An attractive alternative therapy to insulin injection is transplantation of the pancreas to replace the destroyed endocrine tissue. The most frequently used method has been to engraft the intact organ, as a vascularised graft. Functional results of pancreatic gland engraftment have steadily improved in recent years and currently, in technically successful transplants, approach those of other organ grafts. This procedure does however still entail an appreciable morbidity and even mortality secondary to the complexity of the surgical procedure. In addition, the concept of transplantation of the intact gland is also flawed by the fact that transplantation of the exocrine component (98% of the cellular constituents) is at the least unnecessary and at worst responsible for most of the complications.

An alternative treatment for diabetes is, therefore, the transplantation of only the endocrine tissue. In recent years methods have become available for the liberation of intact islets from a variety

of mammalian pancreata. Essentially these techniques have been developed in the rodent and then applied to the pancreata of higher animals. Despite refinements, application to the human pancreas with resultant successful islet transplantation has not occurred. Possible reasons are that for successful islet transplantation, large numbers of highly purified and viable islets are required. Previous studies on human islet isolation have concentrated on the initial collagenase digestion phase of the process with the production of large amounts of islet containing digest. A second stage, to purify the islets from this crude digest is necessary. For the isolation of human islets the process needs to be performed in a rapid, large scale manner which optimises yield, purity and viability of the islets.

The purpose of this present study was thus to :-

- 1) Develop and optimise the methodology for rat islet purification
- 2) Adapt the technique for the large scale isolation and autotransplantation of purified islets from a large animal pancreas
- 3) Develop a method of assessing human islet viability, once transplanted, by use of an animal model
- 4) Adapt the purification techniques developed for the animal pancreas for the large scale isolation of viable human islets of Langerhans.

CHAPTER 2

BOVINE SERUM ALBUMIN DENSITY GRADIENT ISOLATION
OF RAT PANCREATIC ISLETS

Part I

Comparison with Ficoll Density Gradient Isolation

INTRODUCTION

Islet purification involves the separation of the islets from the exocrine tissue. Intact islets can be purified by hand-picking from the pancreatic digest, however, for transplantation purposes this method is inefficient, even for the rodent because of the number of islets involved. The most frequently used alternative method of islet purification has been the use of discontinuous density gradients which rely on the intrinsic density of the individual cells to achieve separation. The most commonly utilised density gradient media has been Ficoll, a polymer of sucrose. In some studies this medium seemed to affect islet viability. Although several alternative density gradient media have been studied in an attempt to improve methods of islet purification, detailed objective comparisons of these density gradient media have not been made. Thus the use of Ficoll as a gradient medium has remained the 'standard' technique.

A density gradient of bovine serum albumin (BSA) was introduced as an erythrocyte fractionation medium in 1964 (Leif) and was subsequently used for separating subpopulations of splenocytes (Sunshine, 1978). Albumin has particular advantages for density gradient separation of cells because solutions have a low viscosity, a general protective effect on cells and, in addition, confer reduced cellular aggregation (Shortman, 1972). Furthermore, high density BSA solutions can be produced which have no effect on the osmotic activity of salt solutions (Shortman, 1972). BSA has not previously been used to isolate pancreatic islets. In this study, in order to test the feasibility of using BSA in such a manner, a number of criteria including islet yield, purity of preparation, insulin release characteristics and isologous islet transplantation were used to compare BSA density gradient centrifugation with the standard Ficoll technique.

MATERIALS AND METHODS

Experimental Animals

Inbred WAG/Ola rats of either sex, weighing 180-220 g, were obtained from colonies maintained by the Biomedical Services Unit of the University of Leicester and were utilised both as pancreatic donors and islet-transplant recipients. These rats were maintained both pre- and post-operatively in appropriate animal unit conditions.

Chemicals and Reagents

Hanks Balanced Salt Solution (HBSS)

Sterile bottles containing 500 ml of Hanks balanced salt solution were obtained from Flow Laboratories, Rickmansworth, UK. Ten ml of Hepes (N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid) was added as a buffering agent, prior to use. Powdered bovine serum albumin (5 g) was subsequently added where appropriate (HBSS/BSA).

Streptozotocin

Streptozotocin was purchased from the Sigma Chemical Company Ltd, Poole, UK and was dissolved, immediately prior to use, in 0.9% saline buffered with 0.05 M citric acid to pH 4.2.

Collagenase

The enzyme collagenase was obtained, as a powder, from both the Boehringer Corporation, Lewes, UK and the Sigma Chemical Company (Sigma Type II) and aliquotted, under sterile conditions, into 50 ml glass conical flasks (7.5 mg and 2.5 mg per rat pancreas respectively) with 0.1 mg of the enzyme, DNA-ase (Sigma).

Ficoll

Ficoll 400 powder was obtained from Pharmacia, Milton Keynes, UK and dissolved in saline to a 25% solution. Subsequent dilutions were made with HBSS.

Bovine Serum Albumin

A 35% sterile stock solution was obtained from Sigma and stored at 4°C. Subsequent dilutions of the stock were made with HBSS.

Isolation of Islets

A mid-line incision was made in each of the donor rats, under ether anaesthesia, and the pancreas, duodenum and common bile duct were exposed. The distal common bile duct was occluded close to the duodenum and following cannulation of the proximal portion of the duct with a size 23 or 25 gauge needle, the pancreas was distended with 10 ml ice-cold HBSS (Fig. 2.1). The pancreas was then rapidly resected (Fig. 2.2), placed in a 50 ml sterile glass beaker, chopped into approximately 1 mm cubes with scissors and washed twice with cold HBSS (Fig. 2.3). The diced tissue was subsequently transferred to a 50 ml conical glass flask into which the 10 mg of collagenase/pancreas had been aliquotted (Fig. 2.4). To this was added 5 ml of warmed (37°C) HBSS and the tissue was digested for 12 minutes, at 37°C, in a shaking water bath. Mechanical disruption was performed by shaking the conical flask, by hand, for 1 minute. The endpoint of digestion was confirmed by assessing the degree of separation of the islets from the exocrine tissue, under a dissecting microscope. The digest was washed with HBSS/BSA through a 500 µm nylon mesh (Fig. 2.5). The pooled filtrate was carefully divided equally between twelve 50 ml glass centrifuge tubes (to give the equivalent of one rat pancreas per tube) and was washed twice more with HBSS/BSA to remove any remaining collagenase (Fig. 2.6). Six tubes containing digest (Fig. 2.7) were taken for each of the gradient methods described below.

Ficoll Density Gradient

Islets were separated on Ficoll by suspending the digest (one pancreas equivalent) in 4 ml of a stock solution of 25% Ficoll overlaid with a discontinuous gradient of 23, 20 and 11% dilutions in HBSS (3 ml of each) in a 14 ml Corex tube (Dupont UK, Stevenage, Herts) (Fig. 2.8) and centrifuged at 2000g for 20 minutes. Islets were aspirated from the 23-20% (B) interface of the Ficoll gradient, and were washed with HBSS/BSA.

BSA Density Gradient

Islet separation on the BSA gradient was performed, as adapted from the method of Sunshine (1978), in a similar fashion to that used for Ficoll. The digest equivalent of one rat pancreas was suspended in 3 ml of 35% stock solution and overlaid with 29, 26, 23 and 10% dilutions of BSA in HBSS (2.5 ml each) (Fig. 2.9). The gradient was centrifuged at 18,000g for 30 minutes at 4°C. Islets were aspirated from the 26-23% (B') interface of the BSA gradient and were washed with HBSS/BSA.

Measurement of Osmolality

Osmolality of the gradient solutions used was measured by freezing point depression (Advanced Instruments Model 3L Laboratory Osmometer supplied by Analytical Supplies Ltd, Little Eaton, UK). BSA and Ficoll solutions were first diluted 1:2 in HBSS and 2 ml aliquots were placed in the freezing vials. The measured osmolality was multiplied by 2 to give a final figure.

Assessment of Islet Yield

a. Islet Count

Islets were aspirated from the appropriate interface of the density gradients and after washing were resuspended in 1 ml of HBSS/BSA. The islets were kept in suspension by continuous agitation of the tube and

triplicate 50 μ l aliquots were taken from the middle of the suspension by use of a 100 μ l pipette (Gilson, Anachem, Luton, UK). These aliquots were then further diluted by the addition of 200 μ l HBSS/BSA and triplicate 50 μ l aliquots were again taken. The number of islets in each sample was counted by an experienced colleague, who was unaware of the sample source, using an inverted phase contrast microscope (Leitz Instruments Ltd, Luton, UK).

b. Insulin Extraction

Tissue was aspirated from the appropriate interfaces and pellets of the gradients, washed twice in HBSS/BSA and made up to a final volume of 1 ml. This was then rapidly macerated in a ground glass tissue homogeniser at 4°C. Insulin was extracted with 9 ml acid/alcohol ($\text{H}_2\text{SO}_4/\text{C}_2\text{H}_5\text{OH}$, 1:40 v:v) at 4°C for 24 hours. Following dilution with phosphate buffer the insulin content was measured using radioimmunoassay (Appendix 1A).

Determination of Islet Purity

Islet purity was assessed by measurement of the amylase content of the tissue after disruption, which was performed as described for insulin, except that acid/alcohol extraction was not used and dilution was with HBSS. Determination of amylase content was performed using the hydrolysis, by the amylase, of a water-insoluble starch polymer carrying a blue dye to form water-soluble blue fragments (Appendix 1B).

Measurement of Insulin Release of Isolated Islets

Insulin release characteristics of the isolated islets were assessed by the use of a perfusion system as described by Lacy (1972). Multiple lots of 100 islets were hand-picked from either the pancreatic digest (non-density gradient isolated islets) or from the islet preparation after isolation on the BSA or Ficoll gradients. Each 100

islet aliquot was placed in an individual chamber (Swinnex 13, Millipore Ltd., Watford, UK) and perfused with HBSS/BSA with a low (5 mmol/l) glucose concentration. After an initial 30 minute stabilisation period, samples were taken at 1 minute intervals for a period of 5 minutes. Perfusion was then continued with HBSS/BSA of high (20 mmol/l) glucose concentration and samples were taken at 1 minute intervals for 15 minutes and then 5 minute intervals for 45 minutes. All samples were frozen at -20°C for later insulin assay.

Induction of Diabetes in Isologous Recipient Rats

Diabetes was induced by intravenous (femoral vein) injection of 65 mg/kg of freshly prepared streptozotocin. Diabetes was confirmed by regular measurement of blood glucose levels and only animals with consistent blood glucose levels of greater than 20 mmol/l, for more than 2 weeks, were used as islet transplant recipients.

Measurement of Blood Glucose

Animals were bled from the tail vein under light anaesthesia and blood glucose estimation was performed by the glucose oxidase method (Appendix 1C).

Transplantation of Isolated Islets

Isolated islets for transplantation were suspended in 1 ml of HBSS/BSA and drawn up into a 1 ml syringe. The islets were then embolised into the liver by cannulation, with a 23 gauge needle, of the portal vein which was exposed at laparotomy under ether anaesthesia (Fig. 2.10). A small amount of blood was drawn back into the syringe, at least twice, to wash out the syringe. Haemostasis was secured following removal of the needle, by pressure and use of a small plug of Sterispon (Allen & Hanbury's Ltd., Greenford, UK) over the puncture site. Blood glucose estimations were performed daily for fourteen days

post-transplantation, thrice weekly for a further two weeks and thereafter weekly. A graft was termed successful if the animal became normoglycaemic within 7 days of the transplant and maintained a blood glucose level below 9 mmol/l for at least 100 days.

Experimental Groups

In each of 15 separate experiments, 12 WAG/Ola rats were used as donors and equal aliquots of digest were allocated to 6 BSA and 6 Ficoll gradient tubes.

Statistical Analysis

Non-parametric statistical analysis was used to compare the results of the two gradient methods. A p value of less than 0.05 was deemed to be significant.

RESULTS

Osmolality Measurements

WAG/Ola rat serum was found to have an osmolality of 315 ± 2 mosm/kg (n=6) (Fig. 2.11, arrow). For separation on the standard Ficoll gradient the pancreatic digest was initially suspended in a 25% solution and this was found to be hyperosmotic (450 mosm/kg, Fig. 2.11). The entire Ficoll gradient was, in fact, hyperosmotic throughout including the point at which the islets were recovered (B interface, osmolality 425 - 390 mosm/kg). The BSA solution was, however, iso-osmotic throughout the gradient system (Fig. 2.11).

Islet Yield

a. Islet Count

The number of islets obtained for the BSA gradient was 714 ± 23 (SEM) per pancreas significantly greater than that obtained for the Ficoll (364 ± 39) (n=8 experiments) ($p < 0.001$; Mann Whitney) even though the starting digest was identical (Fig. 2.12).

b. Insulin Extraction

As expected, there was no significant difference between the total amount of insulin extracted from the material in the pellet and islet containing interfaces of the Ficoll and BSA gradients (Fig. 2.13) thus confirming equal distribution of digest between the gradient tubes. The proportion of insulin present in the BSA islet containing interface (B') was 62.2% (Fig. 2.13) significantly higher than the Ficoll islet containing interface (B, 36.6%) ($p < 0.001$, Mann Whitney test) indicating a higher yield of islet tissue. Conversely the proportion of insulin present in the pelleted tissue was significantly greater for the Ficoll gradient than for the BSA gradient.

Purity of Preparation

The majority of exocrine tissue was present in the pellet of both gradients. However, the amylase activity of the tissue obtained from the gradient interfaces was consistently higher throughout the gradient for Ficoll compared with BSA (Table 1). In particular, the amylase activity of the islet containing interface was significantly higher for Ficoll (B) than for BSA (B') ($p < 0.001$, Mann Whitney test) indicating that the islet tissue from the BSA gradient contained a lower level of contaminating exocrine tissue.

Insulin Release Characteristics of Isolated Islets

The insulin release characteristics, in response to glucose stimulation, of BSA isolated islets was similar to those seen with handpicked (non-density gradient isolated) islets (Fig. 2.14). The Ficoll separated islets, on the other hand, showed sub-optimal insulin release.

Transplantation of Isologous Islets

When Ficoll separated islets were used, at least three donors were required per recipient for consistently successful transplantation of isologous islets (Fig. 2.15). With lower ratios transplantation success rapidly declined. However, from the same initial digest, the yield of islets from the BSA gradient was sufficient for single donor:single recipient transplantation success (Fig. 2.15). It was only with an even lower ratio of 1 donor to 2 recipients that the success rate declined.

DISCUSSION

Collagenase digestion combined with Ficoll density gradient separation has been the standard method of isolating islets of Langerhans from the adult rodent pancreas since 1972 and, using this technique, approximately 350 intact islets can be obtained from each adult rat pancreas (Ballinger). In this study BSA density gradient was shown to be a more efficient method of islet isolation than Ficoll in that the final yield was approximately twice the number of islets from an equivalent volume of collagenase digested pancreas. This improved yield was confirmed by determination that the proportion of insulin containing tissue in the islet containing interface (B') of the BSA gradient was significantly greater than that found in the equivalent interface of the Ficoll gradient. In other words, 63.4% of the insulin containing tissue remained in the pellet in the Ficoll gradient and was

thus effectively lost, whereas this was reduced to a 37.8% loss in the BSA gradient.

Contamination of the islet preparation with non-endocrine tissue has been shown to reduce the effectiveness of an islet transplant (Lacy, 1979; Gray, 1986b). The degree of exocrine contamination, as measured by amylase levels, was much reduced at all interfaces of the BSA gradient compared with the Ficoll gradient (Table 1). In particular the amylase contamination of the islet preparation obtained from the BSA gradient was significantly lower than that obtained for the Ficoll gradient. Thus the BSA isolated islets were less contaminated than those isolated with Ficoll.

Islet number and degree of contamination are not the only criteria for successful transplantation; a further fundamental consideration is the viability of the islets obtained. In this study the *in vitro* viability of the isolated islets was assessed by measurement of insulin release in a perfusion system. BSA isolated islets showed a normal insulin release response following glucose stimulation, similar to that seen with non-density gradient isolated islets, while Ficoll isolated islets gave a sub-optimal insulin release (Fig. 2.14).

The final assessment of islet function is the ability to correct diabetes by transplantation. Finch in 1977 showed that approximately 800 islets were just sufficient to reverse streptozotocin induced diabetes in the rat. Using the same Ficoll separation technique in this study, a mean of 364 islets were obtained from a donor pancreas. Thus with Ficoll separation, three donors (ie. 364×3) were required for 100% (6/6) graft success. With only two donors (364×2) or lower, the success rate rapidly declined (for example, 1 donor : 1 recipient ratio, 0/6 success). The BSA gradient separation method yielded 714 islets per pancreas and single donor to single recipient transplantation was

achieved (ie. 1 donor : 1 recipient ratio, 6/6 success) (Fig. 2.15). A smaller number of BSA isolated islets than Ficoll isolated islets were thus required to reverse diabetes. Therefore, the BSA gradient method not only gave a better yield of islets than the Ficoll gradient but also the islets obtained from this gradient functioned better both in vitro and in vivo.

SUMMARY

BSA density gradient separation of adult rat pancreatic islets gave significantly improved results in terms of islet yield, purity of islet preparation, insulin release characteristics and transplantation results.

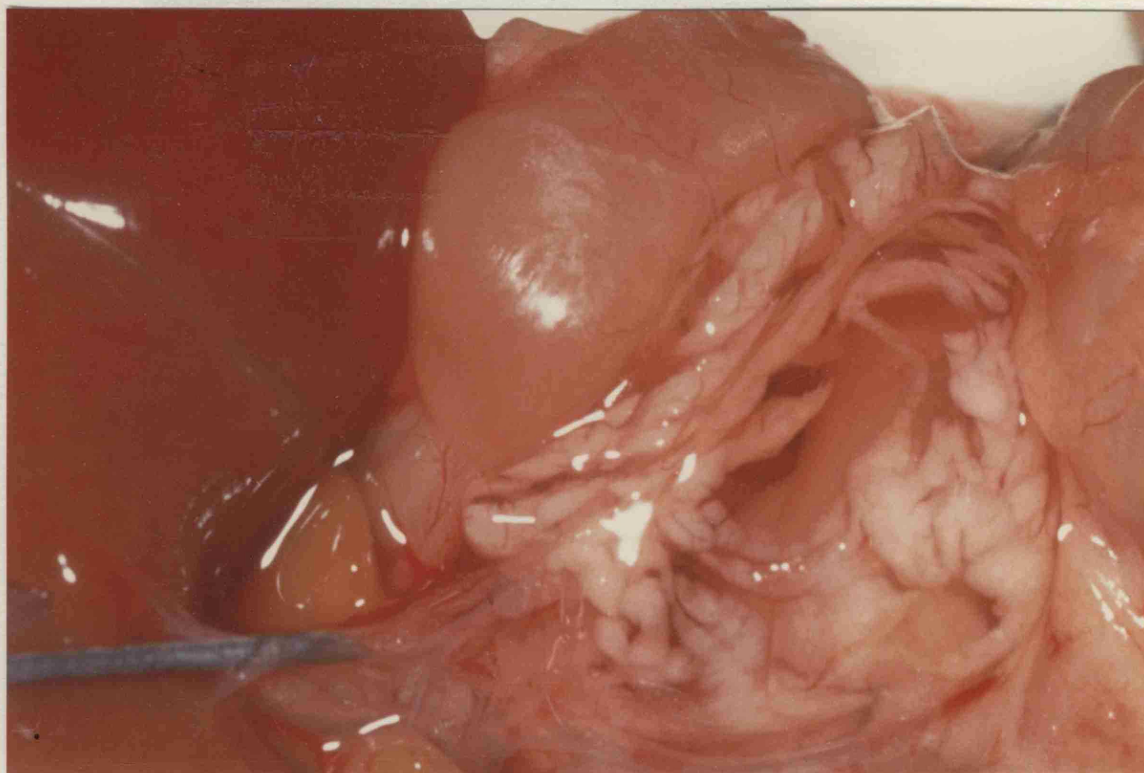


Figure 2.1 Distension of rat pancreas by injection of cold HBSS into the cannulated common bile duct.



Figure 2.2 Distended rat pancreas after resection.

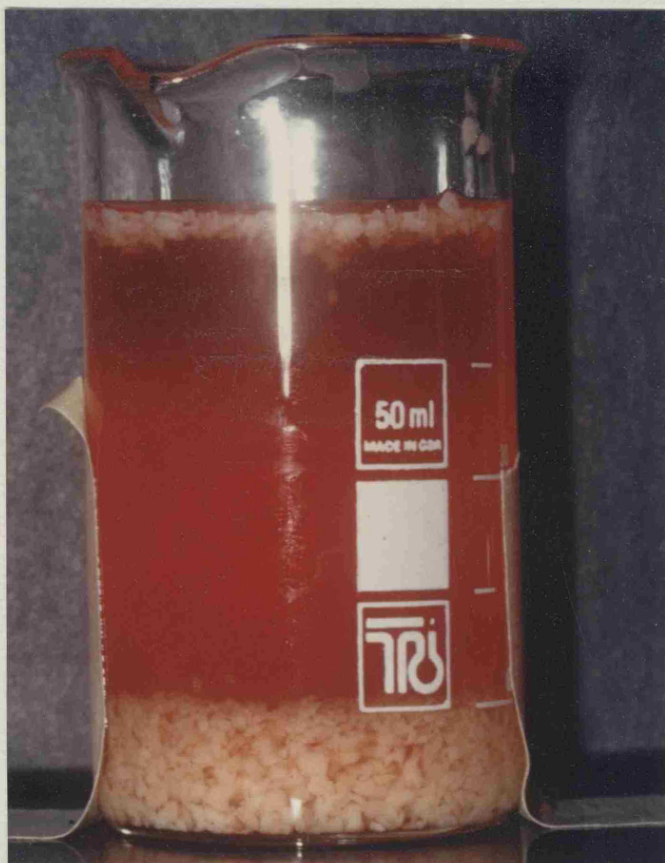


Figure 2.3 Rat pancreatic tissue after dicing with scissors. Small pieces of fat have accumulated on the surface of the medium.



Figure 2.4 Diced pancreas placed in glass conicals with collagenase powder and HBSS prior to incubation at 37°C.



Figure 2.5 Pancreatic digest after washing through a 500 μm nylon mesh to remove undigested pieces of tissue.

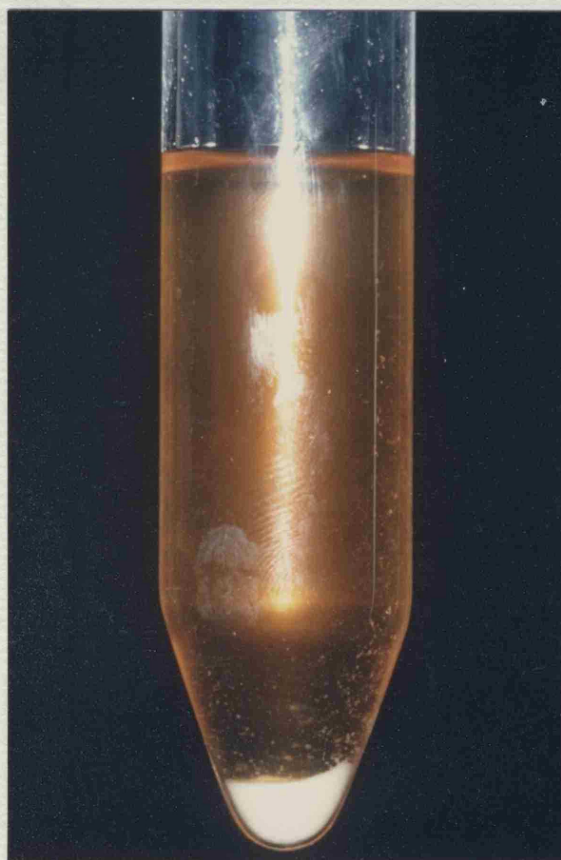


Figure 2.6 Pancreatic digest in centrifuge tube during washing phase.

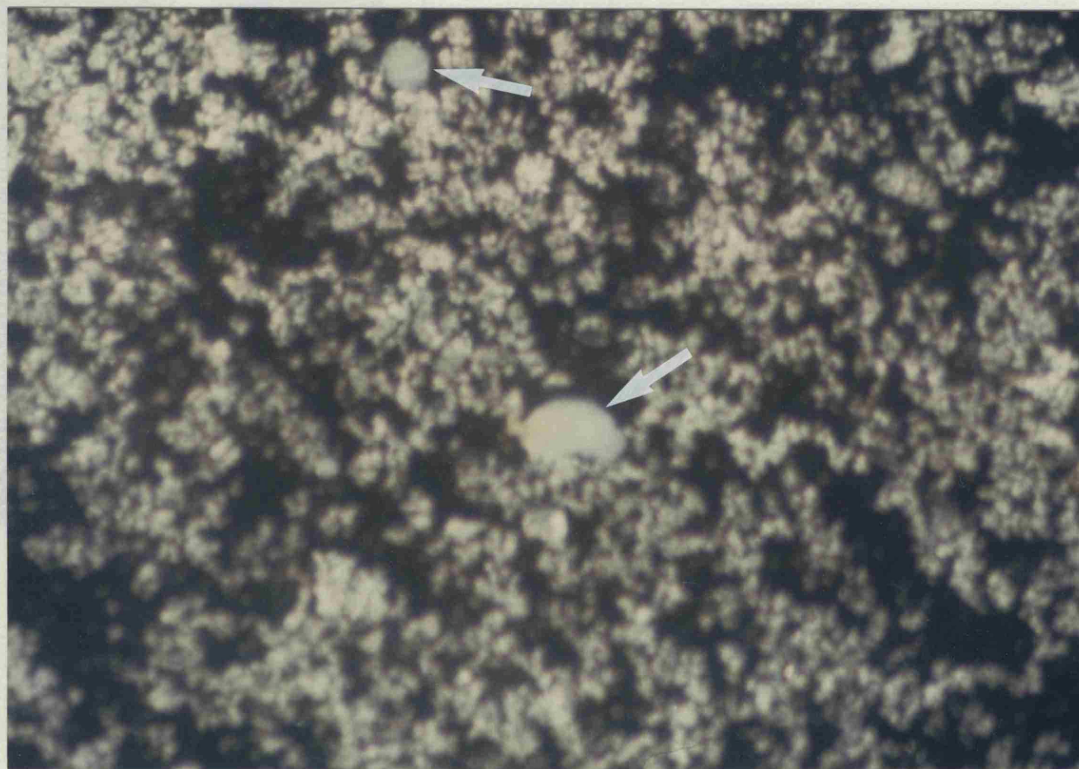


Figure 2.7 Rat pancreatic digest showing intact islets (→) and exocrine cells.
(x 50)

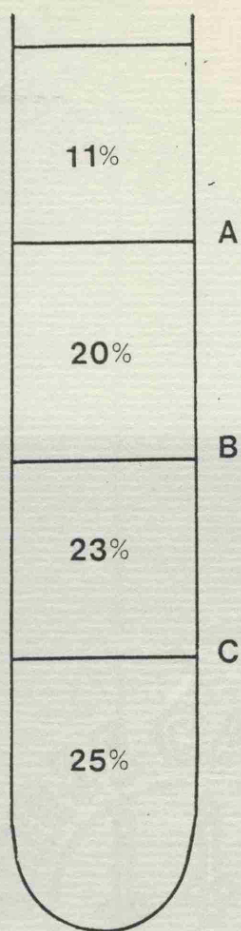
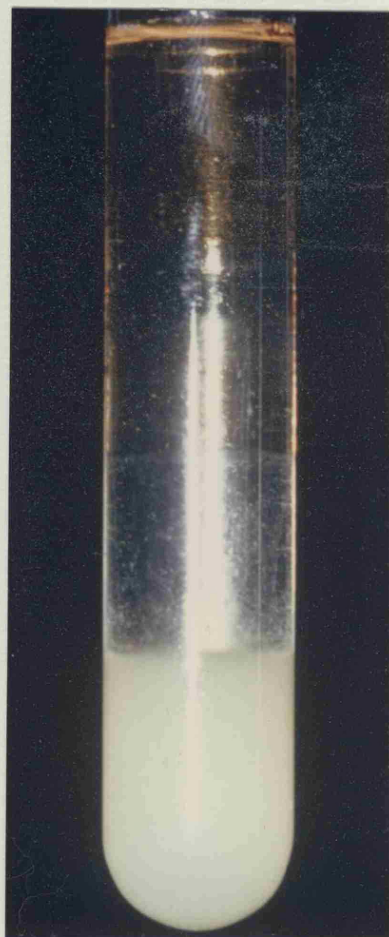
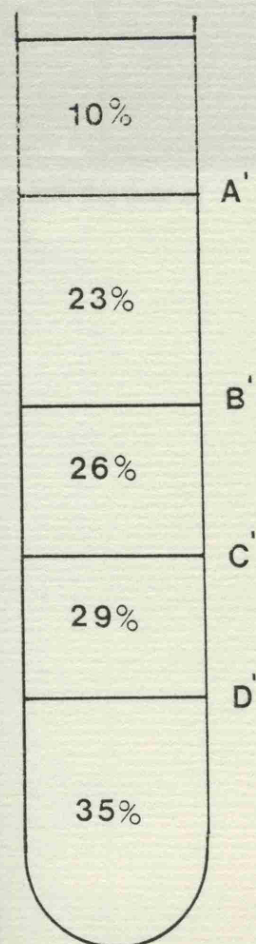


Figure 2.8 Photograph and diagram of **Ficoll** density gradient tube showing concentrations of the constituents and the interfaces.

Figure 2.9 Photograph and diagram of **BSA** density gradient tube showing concentrations of the constituents and the interfaces.



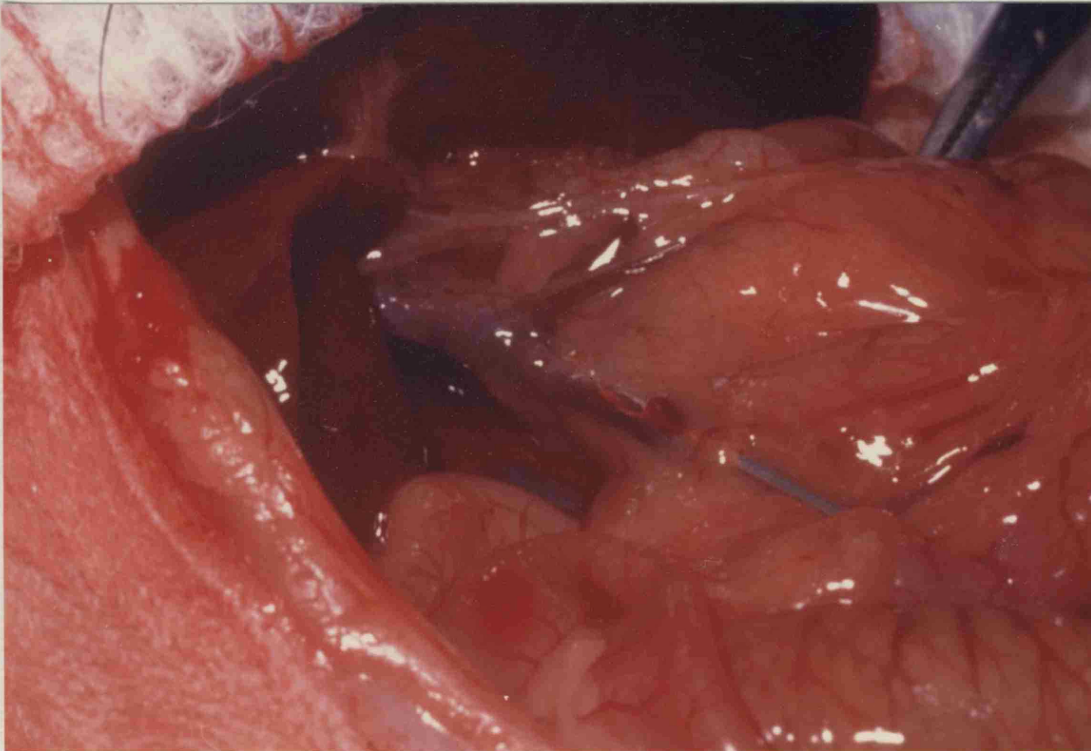


Figure 2.10 Transplantation of purified islets, by embolisation into the liver of diabetic recipients via the portal vein.

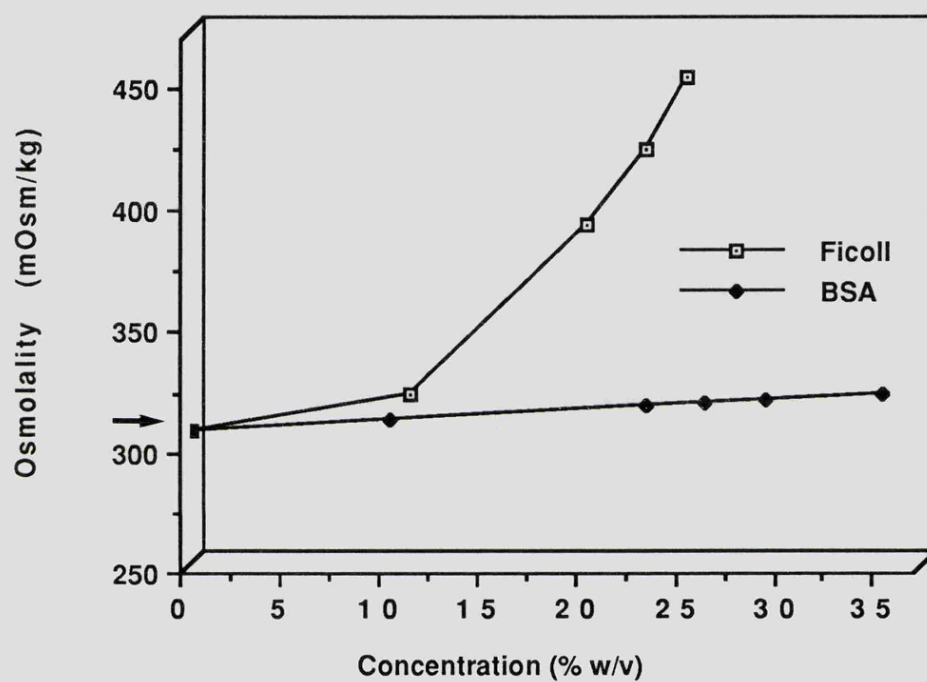


FIGURE 2.11 Comparison of Ficoll and BSA density gradients for effect on osmolality with change in concentration. Arrow indicates rat serum osmolality.

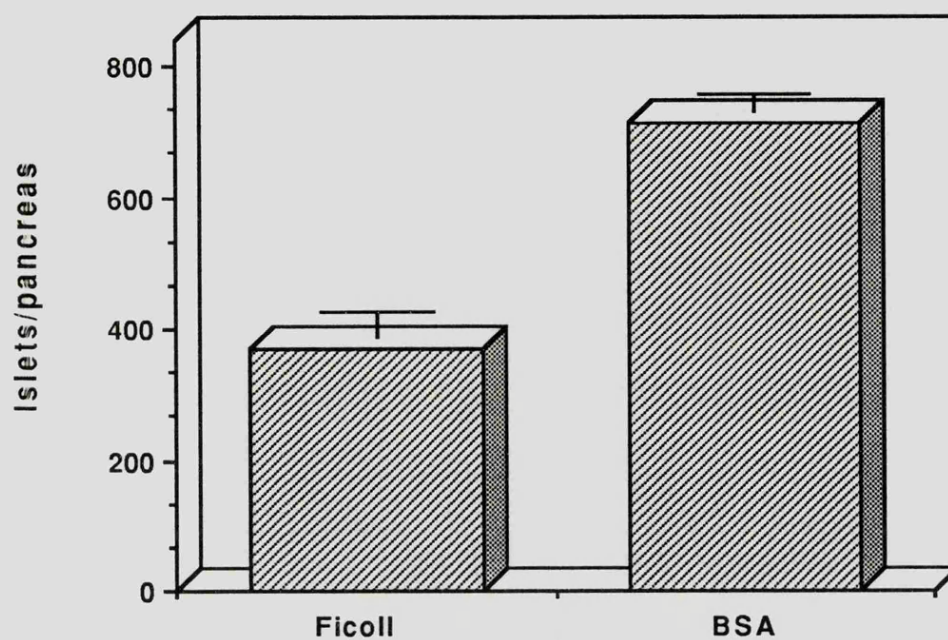


FIGURE 2.12 Comparison between Ficoll and BSA density gradient separation for islet yield.

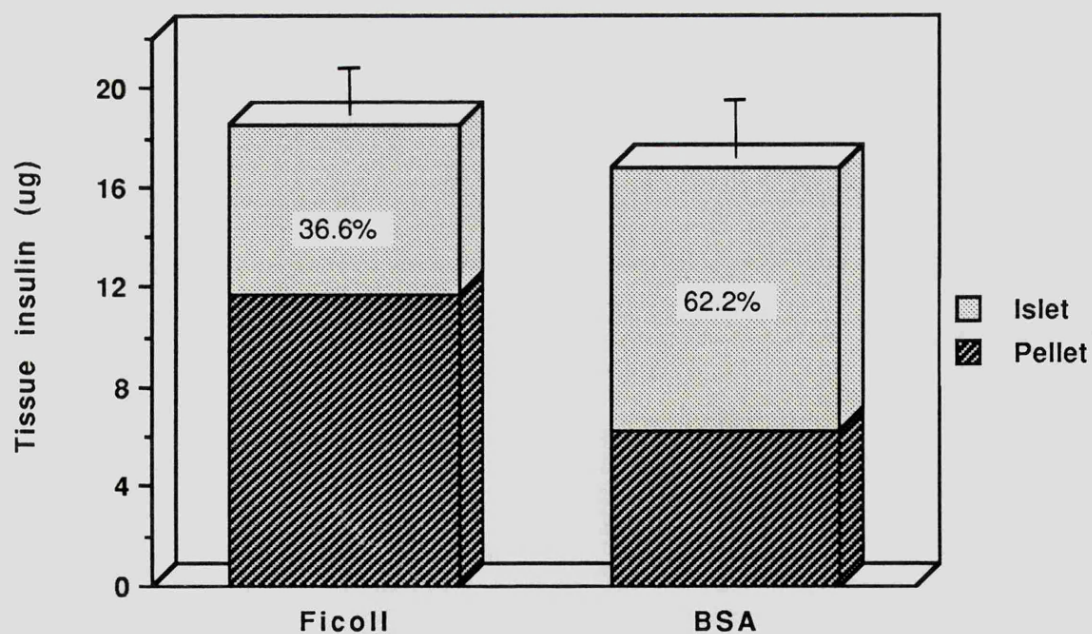


FIGURE 2.13 Comparison of insulin extraction from the material in the pellets and islet-containing interfaces of the Ficoll and BSA density gradients (mean of 8 experiments). The proportion of insulin in the islet interfaces is illustrated.

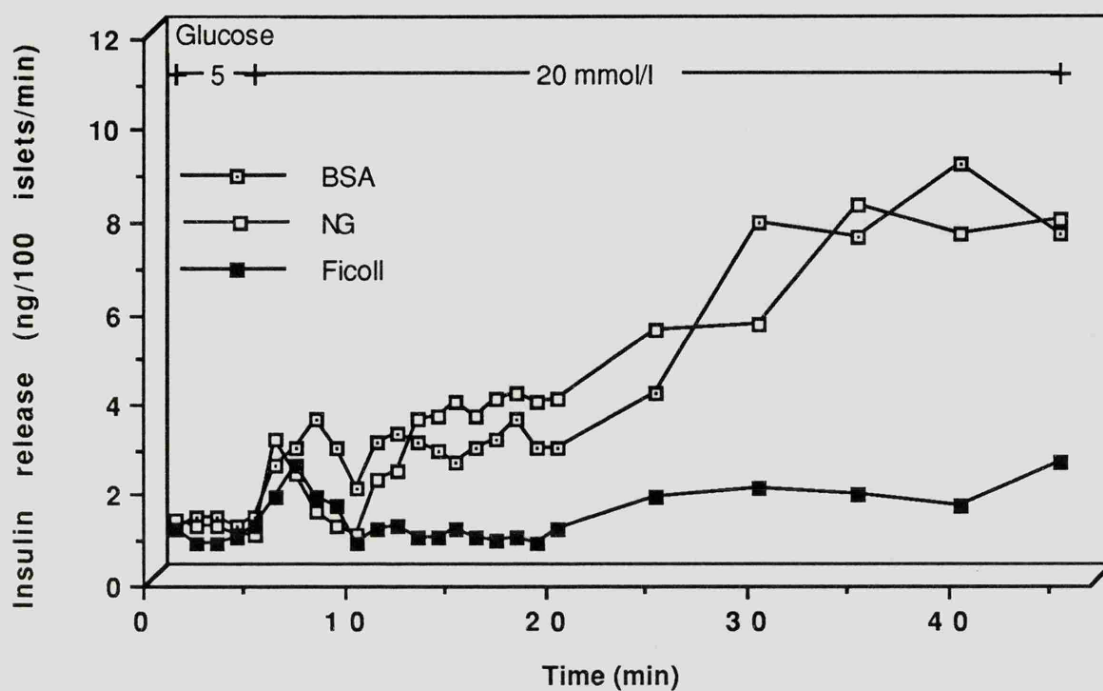


FIGURE 2.14 Insulin release characteristics of non-density gradient, Ficoll and BSA density gradient isolated islets. (Mean of 3 experiments for each isolation method).

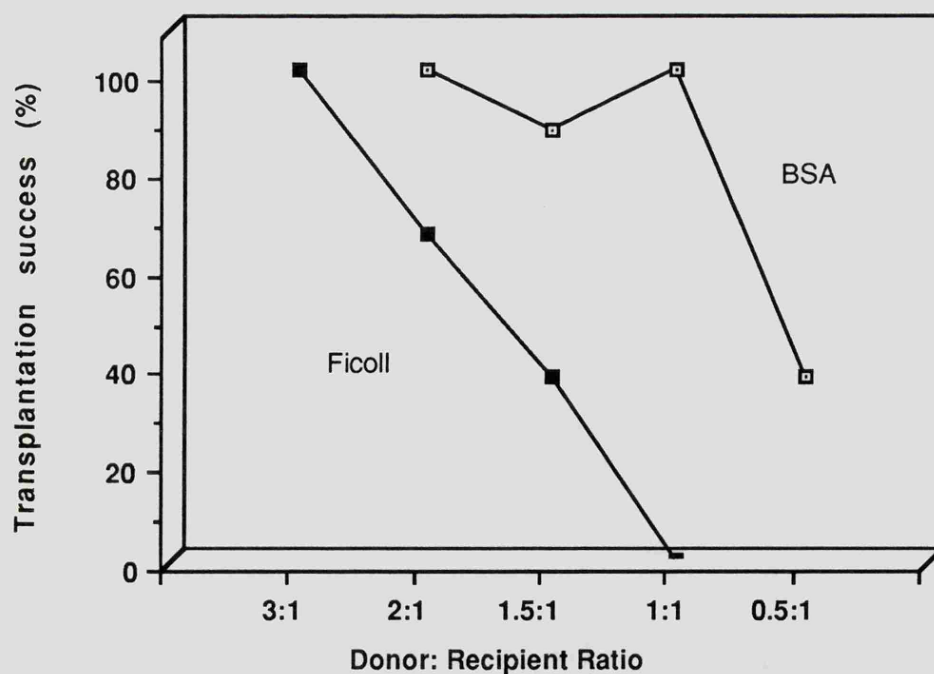


FIGURE 2.15 Comparison between Ficoll and BSA density gradients for success of syngeneic transplantation with a progression of donor:recipient ratios. (For each ratio, recipient number in each group $n=6$, except for the 1.5:1 ratio, $n=8$).

TABLE 1.

Amylase activity of the tissue obtained from the Ficoll and BSA gradient interfaces (one pancreas per gradient) (mean of 8 experiments).

FICOLL				BSA			
	Interface	Amylase (U/l)			Interface	Amylase (U/l)	
A	(20-11%)	1600	± 155	A'	(23-10%)	480	± 32
B	(23-20%)	6133	± 320	B'	(26-23%)	1230	± 164
C	(25-23%)	12840	± 1890	C'	(29-26%)	5660	± 1430
				D'	(35-29%)	9120	± 1850

CHAPTER 2

BOVINE SERUM ALBUMIN DENSITY GRADIENT ISOLATION OF RAT PANCREATIC ISLETS

Part II

A Test Gradient System for Optimising Islet Isolation

INTRODUCTION

In the previous study a purification method for rat pancreatic islets was described, using a density gradient of Bovine Serum Albumin. Improved islet yield, purity and viability were demonstrated when compared with the standard Ficoll technique. Despite the improvements described, the overall yield of islets from rat pancreatic digest was only 62% as judged by insulin extraction. Thus it would appear that a proportion of the islets were retained within the unused (pelleted) material.

Although the approximate density of isolated rodent islets had been indicated by previous authors as 1.06-1.08 for the Lewis rat (Tze, 1976) and 1.065-1.070 for the OB mouse (Buitrago, 1977), the density profile of exocrine tissue has not been accurately documented. By precisely defining the density range of the islet and exocrine cells, a discontinuous gradient could potentially be formulated which maximises the yield of islets and minimises the exocrine contamination.

In this study a test gradient system was devised and used to optimise the density gradient isolation technique for rat pancreatic islets.

MATERIALS AND METHODS

Experimental Animals

Inbred WAG/Ola rats were used as pancreatic donors and islet transplant recipients as described in Part I.

Chemicals and Reagents

Minimal Essential Medium (MEM)

Sterile bottles of Minimal Essential Medium (Flow Labs. Ltd, Herts., UK) were used throughout the remainder of the study because improved viability (judged by Trypan blue exclusion) was observed in islets maintained in this medium over a period of time when compared with HBSS. The medium was prepared by dilution of concentrated MEM (10x) with sterile water and was then supplemented with Streptomycin (100 U/ml), Penicillin (100 U/ml) and Amphotericin (2.5 µg/ml).

Streptozotocin

Diabetes was induced in recipient rats as described in Part I.

Collagenase

High activity Type X1 collagenase (Batch no. 46F-6838, Sigma), which has been specifically formulated for islet isolation, was used for this series of experiments and was dissolved in MEM to a concentration of 0.7 mg/ml with 0.02 mg/ml DNA-ase (Sigma).

Isolation of Islets

Islet isolation was performed as described in Part I except that a number of modifications were made to improve the digestion phase, essentially as described by Gotoh (1985) and Sutton (1986). In particular collagenase was delivered throughout the pancreatic substance by intraductal injection using 5 ml of ice cold MEM, containing collagenase and DNA-ase. Following resection, the pancreas was incubated in 10 ml MEM for 17 minutes at 37°C without prior mincing of

the gland. The incubation vial was then shaken by hand for one minute, the digest was poured through a 500 μ m mesh and was washed with medium to remove the collagenase.

BSA Test Gradient Isolation Method

A series of BSA solutions were prepared by dilution of MEM of a stock 35% BSA solution (density 1.10 g/ml). The density of each of the individual solutions was measured and adjusted by use of a digital densitometer (DMA 35, Parr Scientific Ltd., London, UK) to produce a range of densities 1.077–1.087 g/ml. Pancreatic digest was suspended in stock BSA and was aliquotted (1 ml) into a series of 5 ml test tubes. These individual aliquots were then each overlaid with 1 ml of BSA of defined density and finally overlaid with 1 ml MEM (Fig. 2.16). In each of the tubes the three layers generated two interfaces which were designated as the upper (X) interface (MEM:Test BSA) and lower (Y) interface (Test BSA:1.10 BSA). The tubes were centrifuged (800 g for 20 minutes at 4°C) and the tissue at the X and Y interfaces was extracted for measurement of insulin and amylase content.

Discontinuous Gradient Isolation Method

Following determination of the optimum density of BSA for islet purification, islet isolation from individual rat pancreata was performed on a 5 step discontinuous gradient, essentially as previously described (Part I) except that defined densities of BSA (MEM, 1.070, 1.081, 1.090 and 1.10) and a lower centrifugation speed (800 g) were used. To assess the degree of efficiency of islet isolation, tissue was aspirated from the individual interfaces (designated A-D from the top of the test tube) and from the pellet. The tissue was washed with MEM/BSA and assayed for insulin and amylase content.

Assessment of Islet Yield

Islets were aspirated from the B (1.081-1.070) interface of the discontinuous gradient and were washed with MEM/BSA. Islet counts were performed as described in Part I.

Islet Transplantation

Isolated islets were transplanted intraportally in 1 ml MEM, as described in Part I.

RESULTS

Use of the Test Gradient

The test gradient system was used to define the optimum density of BSA for islet isolation. The three layer test gradient gave two interfaces designated as X (upper) and Y (lower). At densities higher than 1.079 g/ml more than 95% of the insulin containing tissue was found in the X interface (Fig. 2.17); at densities below 1.079 g/ml the yield of insulin containing tissue was reduced. At densities of 1.081 g/ml and below, the percentage of amylase content in the X interface was always less than 15% of the total in the X and Y interfaces; at densities higher than 1.081 g/ml the exocrine contamination in the X interface increased in a linear fashion with regard to density. The density of 1.081 g/ml was thus found to represent the optimum density of BSA required to achieve maximum islet/insulin yield with minimal exocrine/amylase contamination.

Use of the Discontinuous Gradient

The previously described 5 step BSA gradient was then modified using defined densities of BSA. Collagenase digest from 8 rat pancreata was centrifuged separately on individual discontinuous gradients and the tissue obtained from the 4 interfaces (A-D) and the pellet were removed

and extracted for insulin and amylase (Fig. 2.18). The B interface (1.081–1.070) contained greater than 85% of the total insulin on the gradient with minimal exocrine contamination. In an additional series of experiments, islets from individual rat pancreata (n=12) were purified by this method (Fig. 2.19). The mean islet count obtained was 1450 ± 250 islets per rat pancreas.

A final assessment of yield and viability was made by syngeneic islet transplantation into streptozotocin induced diabetic recipients. Sufficient islets were obtained to allow 100% (n=8) successful single donor:single recipient transplantation with prompt (median = 2 days) reversal of diabetic state. An 87.5% success rate was obtained even when islets from a single donor were transplanted into two recipients (n=8), although the median time of reversion to normoglycaemia was 7 days.

DISCUSSION

In this study the defined density of BSA for maximal rat islet purification was found to be 1.081 g/ml (Fig. 2.17). Densities higher than this resulted in increasing contamination of the islet preparation with migration of the exocrine tissue into the islet containing (X) interface. Furthermore, densities lower than 1.079 g/ml resulted in loss of insulin containing tissue.

In the second part of the study the test gradient data obtained above were used to optimise the 5 step BSA gradient and the overall success of islet isolation was confirmed by demonstration that over 85% of the total insulin content of the gradient tubes was now present within the islet containing (B) interface with only minimal exocrine contamination (Fig. 2.18). The overall high yield of islets (1450 islets/pancreas) may be due to the use of the intraductal collagenase method combined with the optimised gradient system.

Purified islet yield/viability was validated by syngeneic transplantation into diabetic recipients. In previous reports, using Ficoll or Dextran gradient purification (Sutton, 1986; Van der Vliet, 1988), the yield/viability of the purified islets was only sufficient to reverse diabetes promptly, in the rat, if two donors:one recipient were used. Delayed reversal of diabetes (up to 14 days) was demonstrated in these previous studies with single donor:single recipient transplantation. In this study using the optimised gradient system, the yield/viability of the isolated islets was sufficient to successfully and promptly (48 hours) reverse diabetes by single donor:single recipient transplantation. It was even possible (7/8 rats) to successfully transplant two recipients from one donor, although, the time of reversal to normoglycaemia was delayed in these rats.

SUMMARY

A novel system for optimising islet isolation was demonstrated. The success of the test gradient system was clearly shown by improved rat islet yield and purity with maintenance of viability.

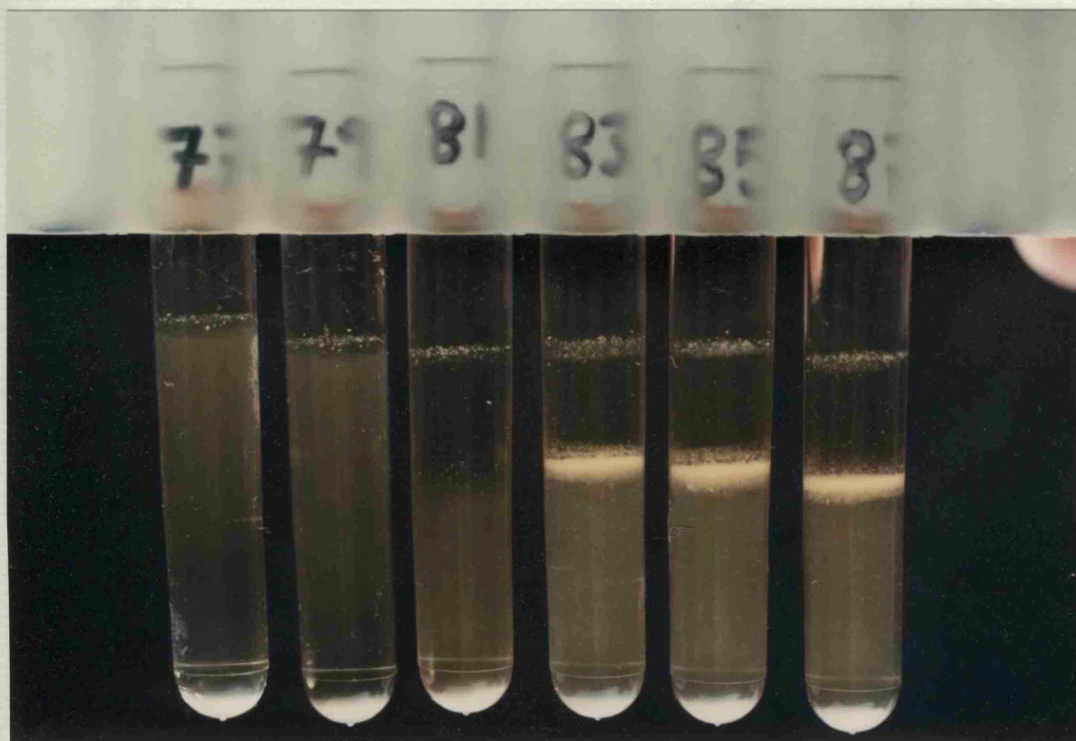


Figure 2.16 BSA test gradient system.

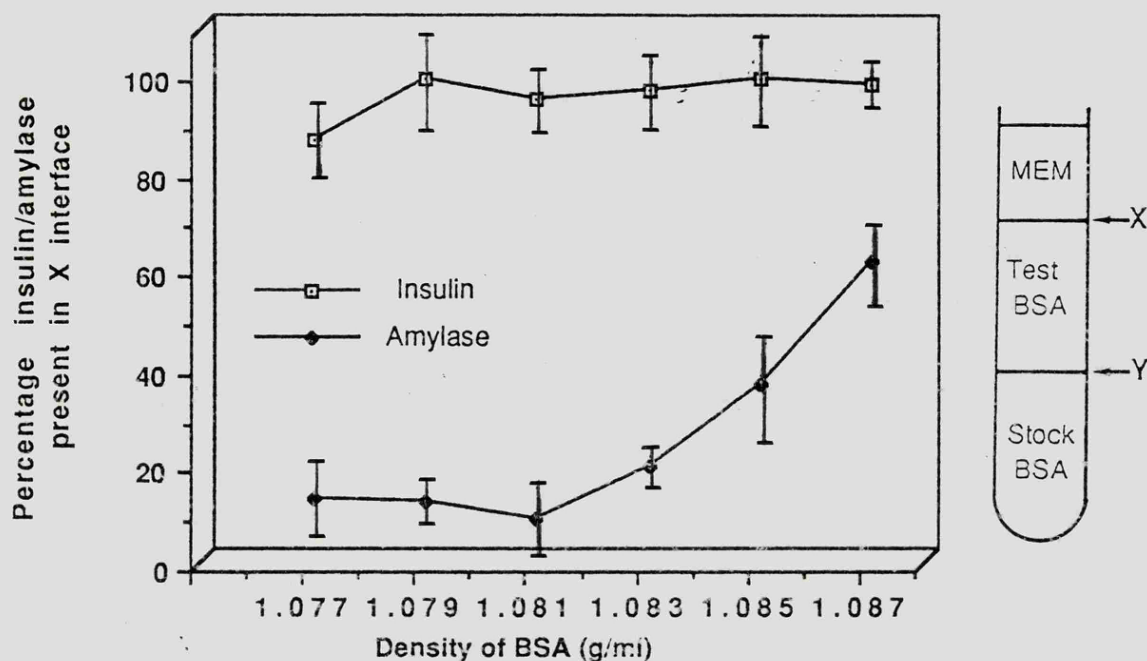


FIGURE 2.17 Analysis of insulin and amylase containing tissue present in the X interface of the BSA test gradient. Results are expressed as the percentage of insulin or amylase present in the X interface compared to the total present (n=3)

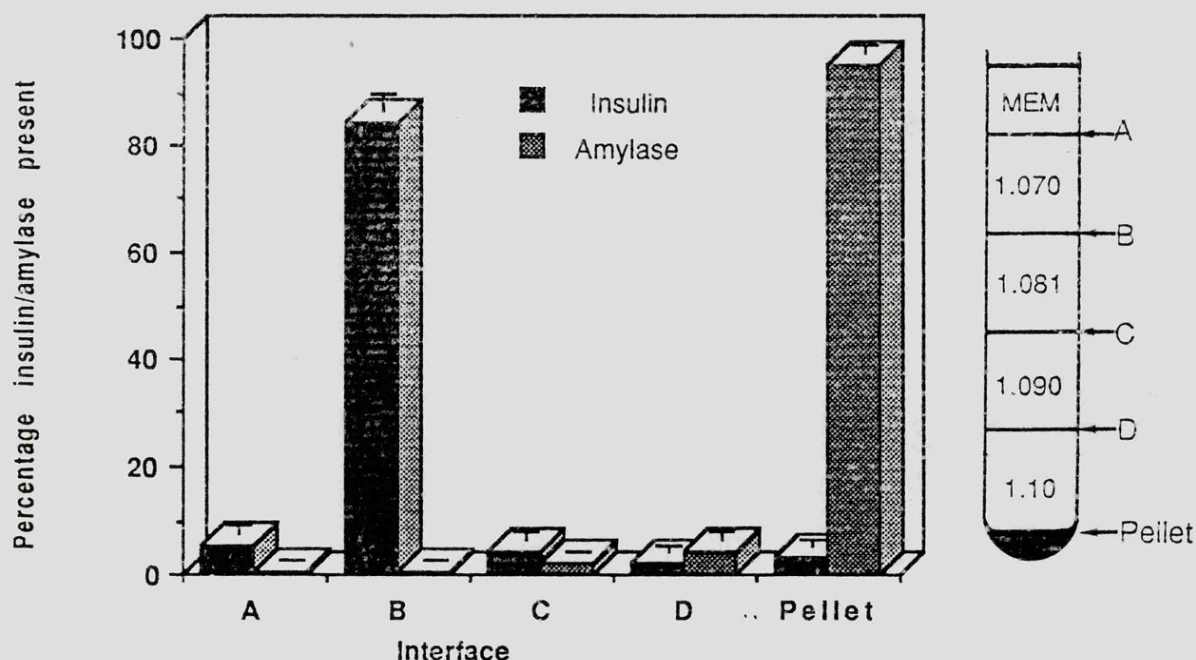


FIGURE 2.18 Percentage amylase and insulin content of individual gradient interfaces (A-D) and pellet obtained from the 5 step BSA discontinuous gradients. Diagram indicates the composition of the individual gradient tubes and the nomenclature used. (n=3)

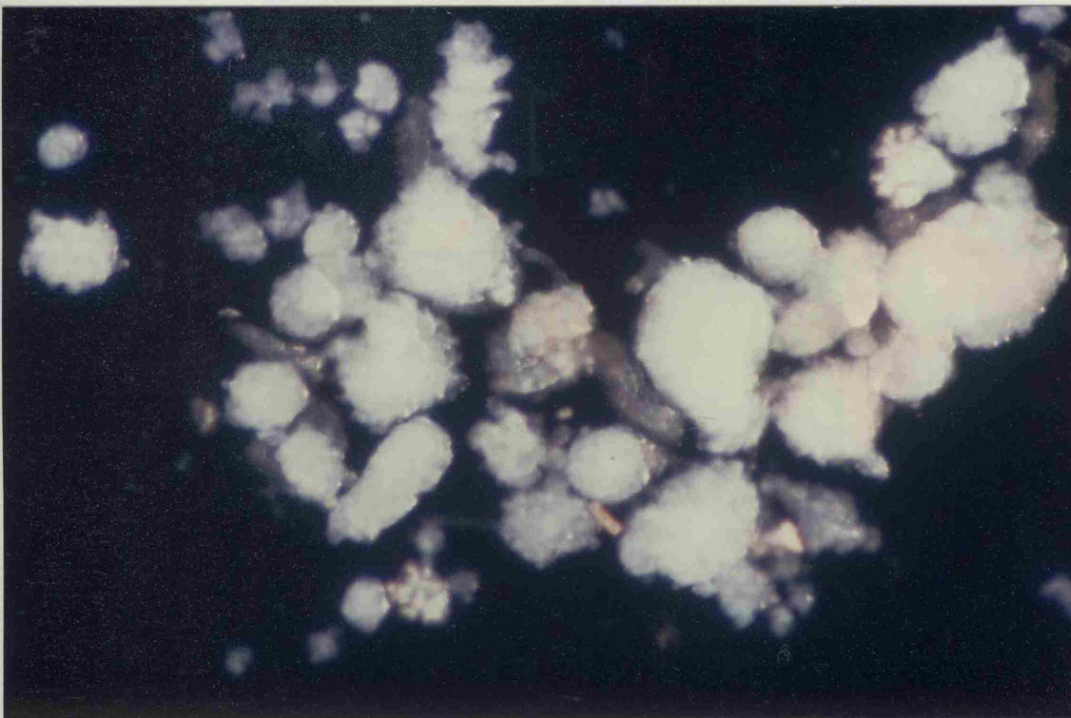


Figure 2.19 BSA purified rat Islets of Langerhans. (x 100)

CHAPTER 3

**THE LARGE SCALE ISOLATION OF PURIFIED CANINE ISLETS OF LANGERHANS
FOR AUTOTRANSPLANTATION**

INTRODUCTION

The initial technique used for 'islet transplantation' in the dog was total pancreatic resection followed by mincing, collagenase digestion and direct injection of the microfragments produced into the spleen (Mirkovitch, 1976). A large number of reports have confirmed the value of this technique, although a major disadvantage has been that large volumes of non-purified islet tissue were transplanted. This puts into question the clinical relevance of such studies in the development of a method for the treatment of diabetic patients. In particular, the human spleen is smaller and more friable than its canine equivalent and is thus unlikely to support engraftment of large volumes of crude pancreatic digest. Furthermore, although an alternative transplant site is into the liver, portal hypertension and disseminated intravascular coagulation have occurred following intrahepatic microfragment grafting both in the dog and in man (Mehigan, 1981; Memsic, 1984).

Transplantation of highly purified islets may overcome these problems. There has been limited previous success with isolated islet transplantation, in the dog, probably because the combination of the digestion and purification stages resulted in excessive loss of islet tissue yield and viability. One of the major problems of purifying islets from crude pancreatic digest of a large animal is the volume of digest which must be processed to obtain adequate numbers of purified, viable islets. Although rodent islet isolation can be performed on a test tube basis, the number of gradient tubes required to process the digest from an entire canine pancreas would be substantial.

An alternative approach may be to use a large volume density gradient. The IBM (now COBE) 2991 Cell Separator (Lakewood, Colorado, USA) was developed as a centrifuge to wash units of red blood cells and to remove the preservative agent from cryopreserved blood

(Jones, 1988). More recently, it has been used to purify bone marrow in preparation for transplantation (Gilmore, 1982). The machine consists of a centrifuge bowl, with a flexible membrane in the bottom, into which fits a sealed (sterile) plastic processing bag. This bag has an arrangement of tubing and a special rotating valve which allows fluids to be pumped into the bag during centrifugation. Beneath the flexible membrane is a hydraulic system which, using an operating system, can displace liquid from the centre of the processing bag through the valve, during centrifugation. The arrangement allows rapid production of large volume density gradients.

Following the development of an improved isolation method for rat pancreatic islets, the aim of this study was twofold:-

- 1) To determine whether viable canine islets could be purified from crude digest by the BSA density gradient method;
- 2) To ascertain whether a large scale discontinuous density gradient technique could be utilised to purify sufficient islets from a single canine pancreas to prevent the onset of diabetes following total pancreatectomy.

MATERIALS AND METHODS

Experimental Animals

For the initial experiments pancreatic lobes were obtained from 10 Beagle dogs undergoing terminal experimentation for vascular and renal transplantation research. The animals were obtained from a colony specifically bred and maintained at the Biomedical Services Unit of the University of Leicester.

For the autotransplantation experiments 9 dogs (4 male, 5 female) weighing 10–15 kg were obtained. Pre- and postoperative care was under the direct supervision of a veterinary surgeon. Animals were given water ad libitum and were fed twice daily with Pedigree chum/mixer (70 g/kg/day). Post-pancreatectomy, the diet was supplemented with Pancrex granules (Paines & Byrne Ltd., Greenford, UK) to overcome exocrine deficiency.

Chemicals and Reagents

Minimal Essential Medium (MEM)

This medium was prepared as detailed in Chapter 2, Part II.

Collagenase

For the initial experiments several different batches of this enzyme were obtained, as a powder, from the Sigma Chemical Company. The enzyme was dissolved in MEM in a variety of concentrations (0.25–1 mg/ml) and DNA-ase (0.02 mg/ml) (Sigma) and calcium chloride (0.015 M) were added. After filtration, 200 ml aliquots were placed in sterile bottles and frozen at -20°C for subsequent use.

For the series of autotransplant experiments a specific batch of Type V enzyme (Batch No. 36F-6819) was used.

Bovine Serum Albumin

BSA was utilised as stated in Chapter 2, Part II.

Anaesthesia of Dogs

All dogs were fasted overnight prior to surgery and were pre-medicated with acepromazine (0.1 mg/kg) and atropine (0.3 mg) 30 minutes prior to induction of anaesthesia with sodium thiopentone (0.5-1 mg/kg). Following intubation with a cuffed endotracheal tube (8-8.5 mm), anaesthesia was maintained by mechanical ventilator with a combination of oxygen, halothane (1.5-2%) and nitrous oxide.

Pancreatectomy

i) Lobectomy

For the initial experiments, the right lobe of the pancreas was delivered, under sterile conditions, through a midline incision. The distal portion of the lobe was excised by division of the feeding vessels and transection of the pancreas itself just proximal to where the body of the pancreas comes in close contact with the duodenum. The pancreas was immediately cooled by immersion in 300 ml ice-cold MEM and was transported back to the Department of Surgery for processing. The duct was cannulated with a single (2 FG) cannula (Portex Ltd., Hythe, Kent) using aseptic techniques under a dissecting microscope.

ii) Total Pancreatectomy

This was performed, under sterile conditions, through a mid-line incision, as originally described by Markowitz (1964) with preservation of the duodenum and its vasculature. The right lobe of the pancreas was first delivered into the wound with the duodenum and the fine mesenteric attachments were divided with preservation of the major vessels. The body of the pancreas was then freed from the pancreaticoduodenal vessels, with careful ligation of the pancreatic branches and thus preservation of the main vessels and branches supplying the duodenum (Fig. 3.1).

The main pancreatic duct, which enters the duodenum approximately 2.5 cm proximal to where the right pancreatic lobe and duodenum become closely applied was identified, ligated close to the duodenum and was marked with a fine (5/0) prolene suture for future cannulation. The splenic portion of the pancreas was freed by division of the mesentery, with preservation of the major vessels. The central, or pyloric, region of the pancreas, was then dissected with ligation of fine vessels and preservation of the bile duct until the two lobes of the pancreas remained attached only by the major vessels. These vessels were clamped (subsequently ligated) and the pancreas was excised (Fig. 3.2) and immediately placed in 300 ml ice-cold MEM. Following pancreatectomy and after haemostasis was ensured, the wound was temporarily closed.

Collagenase Digestion

A variety of collagenase types, batches, concentrations and methods of delivery were used in the series of initial experiments. Liberation of intact islets from the pancreas was found to be optimal with the Sigma V enzyme batch no. 36F-6819, used at a concentration of 0.5 mg/ml and delivered as below.

The resected pancreas (Fig. 3.2) was transported to the laboratory in ice-cold MEM and was weighed. Under aseptic conditions, the major pancreatic ducts were cannulated (using a dissecting microscope) with 2 cannulae (2 or 3 FG) (Portex) which were ligated in place with 4/0 silk sutures (Fig. 3.3). The pancreas was then distended with 1 ml collagenase per gram of tissue with careful observation for leakage. The pancreas was placed in a beaker in a waterbath with 200 ml MEM which had been previously warmed to 37°C, and the gland was perfused (6 ml/minute) with collagenase using a peristaltic pump (Infusomat S31, Braun Melsengen, Germany), sterile tubing and a Y connector (Fig. 3.4).

After 12 minutes the gland was carefully and repeatedly inspected until digestion was considered to be complete, when the gland had a mucoid appearance and fragmented easily on gentle teasing with forceps. The gland was then placed in ice-cold MEM (Fig. 3.5) and rapidly teased apart with forceps so that the tissue was removed from the supporting ductal stroma. The fragments produced were poured into a 500 ml bottle with 200 ml cold MEM and this was gently shaken, on ice, by hand for 5 minutes. The digest was subsequently washed through a 500 μ m mesh, aliquotted into 50 ml centrifuge tubes and the filtrate was washed three times with MEM/BSA. The tissue that collected on top of the filter was replaced in a kidney dish, teased apart, again placed in the bottle with MEM and shaken by hand. This process was repeated three times in all and the filtrates were pooled during the washing procedures. The final digest volume was documented.

Identification of Islets

In the preliminary experiments canine pancreatic digest was visualised through a variety of microscopes and with different methods of illumination. Canine islets were differentiated from the clumps of exocrine cells by their compact, dull, pearly white appearance when viewed through an Olympus microscope against a black background with side, white light illumination at a magnification factor of x40 (Fig. 3.6). In contrast, under the same conditions, exocrine tissue had a shiny appearance with easily visible gaps between individual cells.

BSA Density Gradient Isolation

i) Test Gradient

In order to determine the optimum density of BSA for canine islet purification an expanded test gradient with a range of densities (1.077-1.088) of BSA was prepared by dilution of the stock 35% MEM as described

in Chapter 2, Part II (Fig. 3.7). In these experiments centrifugation was at 800g for 20 minutes at room temperature. The individual interfaces were then aspirated and the tissue was extracted for measurement of insulin and amylase content.

ii) Large Scale Density Gradient Isolation

The digest was initially thoroughly dispersed in 200ml of 35% BSA stock solution with a 20ml sterile pipette and was placed in a sterile 500ml glass bottle which was sealed with a rubber bung after aliquots (15 µl) had been taken for insulin determinations. The digest was then transported to the Department of Haematology, Leicester Royal Infirmary, with 2 sterile bags containing BSA (density 1.082) and MEM (75ml of each) (Fig. 3.8).

A large volume discontinuous BSA density gradient was formed on the IBM 2991 (Fig. 3.9) by firstly running the digest/35% BSA mixture into a processing bag (Fig. 3.10) which had been placed in the centrifuge bowl (Fig. 3.11) of the IBM 2991. The infusion was under gravity and through tube A (Fig. 3.12). Centrifugation was then commenced and a second layer of BSA (75ml, density 1.082) was loaded onto the gradient, by use of a peristaltic pump (Model 503S, Watson-Marlow, Falmouth, UK) through tube B followed by 75ml MEM through tube C (Fig. 3.12). Centrifugation was continued for 20 minutes at 2000 rpm (800g). At this stage the supernatant (MEM) was pumped off to waste (tube D) and the islet containing interface X was collected in a sterile bag through tube E. (Fig. 3.11) The remaining interface Y could then be removed through re-use of tube B.

The bag containing the islets (Fig. 3.13) was placed on ice and transported back to the Department of Surgery where the islets were washed with MEM/BSA in 50 ml centrifuge tubes and aliquots were taken

for the appropriate assays (see later). For autotransplantation the islets were transported back to the Biomedical Services Unit, on ice.

Measurement of Insulin Release of Isolated Islets

Aliquots of 10 islets were aspirated, washed in MEM/BSA and randomly allocated to incubation at 37°C for one hour, in MEM containing either low glucose (5.5 mmol/l) or high glucose (20 mmol/l). At the end of this period, the MEM was carefully aspirated and frozen for later insulin assay by radioimmunoassay (Appendix 1A).

Transplantation of Islets

Following transportation of the isolated islets back to the Biomedical Services Unit the abdominal wound of the anaesthetised dog was reopened and the pancreatic bed and duodenum were inspected to ensure continued haemostasis. A radicle of the splenic vein, in close proximity to the spleen itself, was dissected. Fine ligatures were placed around the vein and after incision a 7FG Portex (Liver Perfusion Cannula) was inserted and passed until it was palpably present in the portal vein just proximal to the liver. A three-way venous manometer set (Travenol, Thetford, Norfolk, UK) was primed with 0.9% saline and connected to the cannula and to a pressure measuring recorder (Model 128A, Kontron Instruments Ltd., Watford, UK). The islets were mixed thoroughly in 20ml MEM, drawn up into a 20ml syringe with a filling cannula. The syringe barrel was then connected to the cannula and the syringe plunger was removed. The islets were thus infused into the portal circulation under gravity after initial base line portal pressure measurement (Fig. 3.14). Portal pressure was subsequently measured 5 and 10 minutes post infusion. At this stage the cannula was removed and the splenic vein radicle ligated.

Monitoring of Transplants

Blood samples for glucose determination were taken daily at 8-8.30 a.m. Animals were bled from the cubital vein, without anaesthesia, and 1ml was placed in a vial containing ACP anticoagulant for blood glucose estimation which was performed by the glucose oxidase method (Appendix 1C). Transplants were deemed to have failed once blood glucose levels became greater than 20 mmol/l with consequent commencement on insulin subcutaneously by the veterinary surgeon who supervised the day to day care of these dogs.

Assessment of Islet Yield

i) Islet Count

Islets were counted by dilution, as described in Chapter 2, Part I.

ii) Insulin Extraction

Insulin extraction was performed as described in Chapter 2, except that in view of the large number of samples, tissue disruption was performed by rapid freezing/thawing (3 times; liquid nitrogen [-196°C]/water[20°C] and sonication (3 minutes).

RESULTS

BSA Test Gradient System

The test gradient system was used to determine the optimum density of BSA required for the purification of canine islets. At densities greater than 1.082 g/ml a large proportion (>75%) of the insulin containing tissue was present in the upper (X) interface (Fig. 3.15). At densities less than 1.082 g/ml a steep decline in the amount of insulin containing tissue present in the X interface was observed. At densities less than 1.079 g/ml the percentage amylase containing tissue in the islet containing interface was 10% or less. At a densities above this level the proportion of amylase containing tissue in the X

interface progressively increased (Fig. 3.15). A density of 1.082 g/ml was chosen as the density for use with the large volume density gradient because it gave the maximum islet yield, although as indicated, there would be a degree of contamination.

Insulin Release of Isolated Islets

In vitro viability of aliquots of BSA isolated islets was confirmed by incubation in MEM containing glucose (Fig. 3.16). An increased release of insulin was observed by those islets incubated in high glucose medium (3.5 ± 0.55 ng/5 islets/hour) compared with low glucose containing medium (1.7 ± 0.14 ng/5 islets/hour).

Autotransplants

In this series of experiments the entire pancreas was resected and used to prepare islets in 9 dogs. The large volume density gradients were produced successfully except for dog No. 3 in which the special rotating valve became occluded during the centrifugation cycle and thereby prevented removal of the supernatant and interfaces. Examination of the valve showed the presence of an albumin plug; in later experiments the valve was washed through with MEM to avoid recurrence of this problem. This tissue was effectively lost in this animal and thus 8 dogs received autotransplants and dog No. 3 became a non-transplant control.

The mean digest volume obtained was 12.1 ± 1.5 ml from a mean weight of pancreas of 32.5 ± 2.5 g (Table 3.1). The mean volume of tissue transplanted was 272 ± 70 μ l which represents 2.2% of the initial digest. The mean number of islets transplanted was $85,000 \pm 23,000$ with a range of 30-198,000 (Table 3.1). Dogs number 2 and 9 had islet counts which were substantially higher than the rest of the group mainly because of the presence of a large number of islet fragments. The mean insulin content

of the islet preparations was $50.2 \pm 8.5\%$ of the total placed in the gradient.

Portal venous pressure rose from a mean of 6.7 ± 0.4 to 10.9 ± 0.8 mmHg, 5 minutes after embolisation of the tissue into the liver. A fall in pressure was then observed to 8.4 ± 0.7 mmHg at 10 minutes post transplantation (Fig. 3.17).

The individual blood glucose and insulin levels for the 8 successfully transplanted dogs are shown in (Fig. 3.18) and are compared in each case with the control (non transplanted) dog. Some of the animals maintained only moderately elevated blood glucose levels for variable periods of time post transplantation. Unfortunately, all ultimately became hyperglycaemic and required the commencement of insulin therapy.

DISCUSSION

Studies in which successful 'islet autotransplantation' has been performed in the dog, have utilised grafts of microfragments of pancreas produced by collagenase digestion of the gland. Such grafts contain not only clumps of exocrine cells but also fragments of islets, intact islets and uncleaved islets, (i.e. islets surrounded by exocrine tissue) (Mirkovitch, 1977; Warnock, 1983). Microfragment grafts transplanted into the spleen have been successful because of omission of the second stage of islet purification (Mirkovitch, 1977). For intrahepatic transplantation, particularly in man, highly purified islet grafts are likely to be required to prevent the complications of portal vein injection, i.e. portal hypertension and disseminated intravascular coagulation. The aim of this study was to develop a method for the mass isolation of islets for autotransplantation. It was thus necessary for large numbers of islets to be cleaved by collagenase digestion and then be purified from the main bulk of the exocrine tissue.

In the first part of the study a number of different collagenase enzyme types, batches and concentrations were tested in order to optimise the digestion process with production of cleaved islets. The most efficient batch was then utilised for the remaining experiments. In these initial stages the test gradient system, as described in Chapter 2, was used to determine the optimum density of BSA for islet purification as measured by insulin/amylase. The optimal concentration of BSA for islet isolation was found to be 1.082 g/ml (Fig. 3.15). Furthermore, viability of the isolated islets was confirmed in vitro by demonstration of insulin release in response to glucose (Fig. 3.16).

In the second part of the study the information obtained with the segmental pancreas studies was utilised in the autotransplant experiments where whole pancreata were digested for islet purification. In the 8 autotransplants a mean of 85,000 islets were produced, contained in a mean volume of 272 μ l. Furthermore, the transplanted tissue volume represented only 2.2% of the origin digest volume however contained more than 50% of the insulin content from the total gradient system. Although an elevation in portal pressure was seen 5 minutes post-engraftment (Fig. 3.17) this was seen to fall back towards normal by 10 minutes. The small tissue volume transplanted did not therefore appear to cause irreversible portal hypertension.

All the animals became markedly hyperglycaemic at varying periods of time following engraftment and thus must be considered as transplant failures. Possible reasons for these failures include insufficient numbers of islets transplanted, poor viability of the tissue, and failure of the islets to become revascularised in the liver vascular bed. Recently, Warnock (1988) has demonstrated a dose related response for successful islet and transplantation in dogs and certainly the 'dosage' of islets given in Warnock's study (120,000 islets/dog) was

higher than in the transplants reported here. In addition, although the in vitro viability of the purified islets was demonstrated, this does not necessarily indicate in vivo viability. Finally, with regard to the site of transplantation, Warnock (1988) compared the liver and spleen as engraftment sites for purified islets in the dog. An increased failure rate was observed with liver engraftment which was suggested to be due to either failure of islet vascularisation or chronic stimulation of the islets by hyperglycaemia in the portal vein.

SUMMARY

Successful large scale purification of isolated islets of Langerhans was demonstrated using discontinuous gradients formed on the IBM 2991 cell separator. Although in vitro viability was demonstrated, islet autotransplants failed to prevent diabetes developing in the longterm in these pancreatectomised dogs.

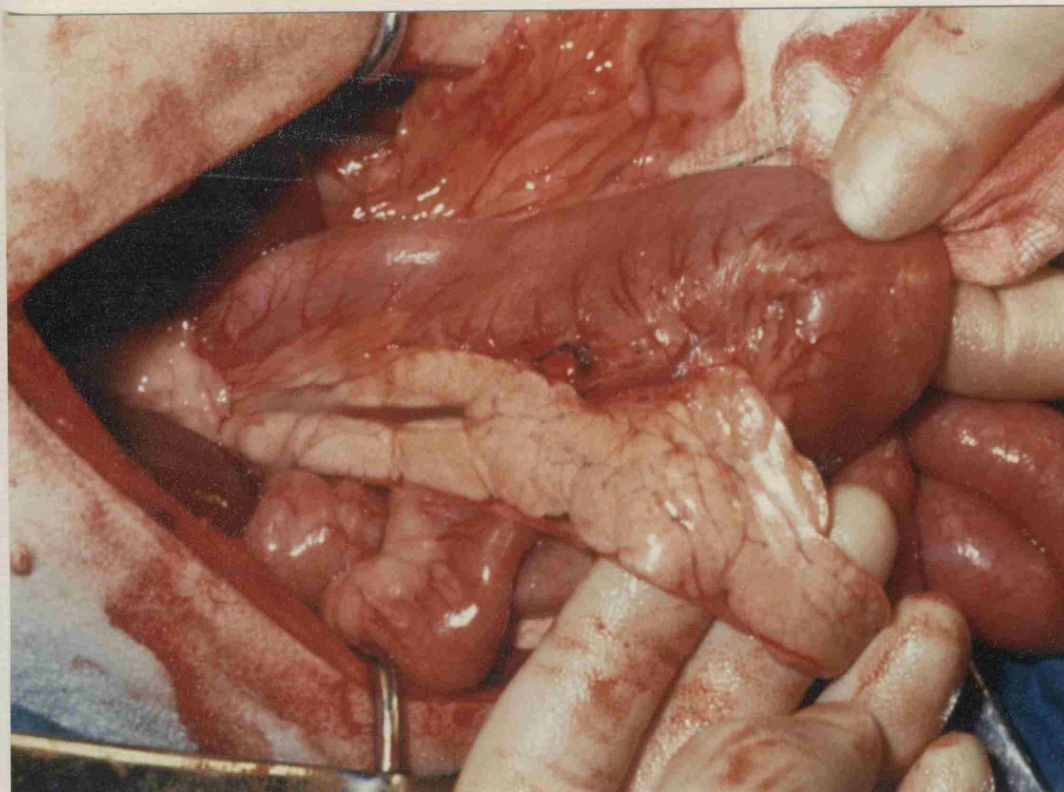


Figure 3.1 Intraoperative photograph showing excision of the pancreas with preservation of the duodenum.

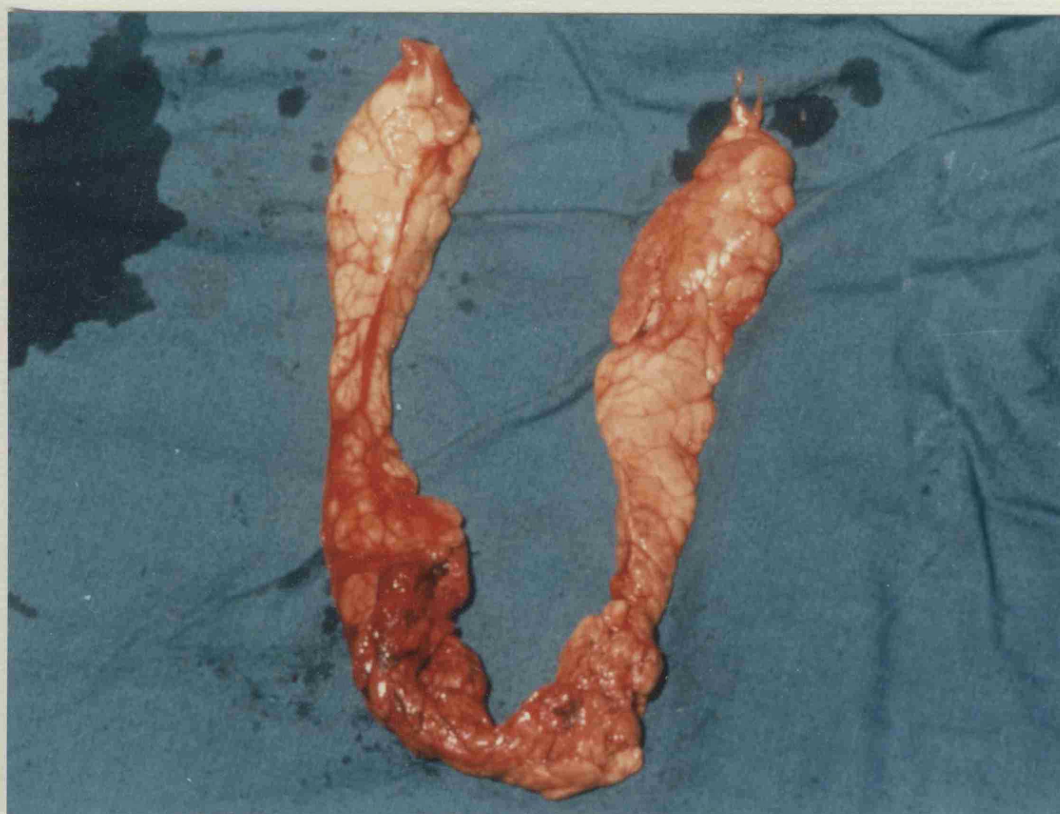


Figure 3.2 Resected canine pancreas.

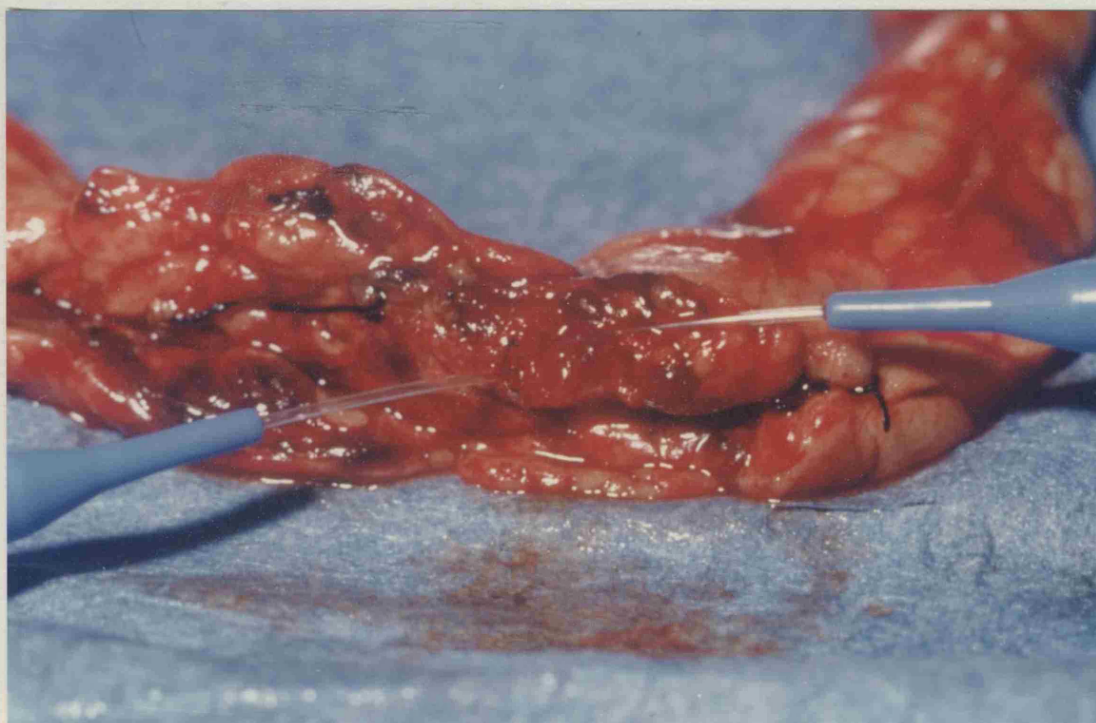


Figure 3.3 Resected pancreas following intraductal cannulation prior to collagenase digestion.



Figure 3.4 Photograph of apparatus used for intraductal collagenase perfusion for digestion.

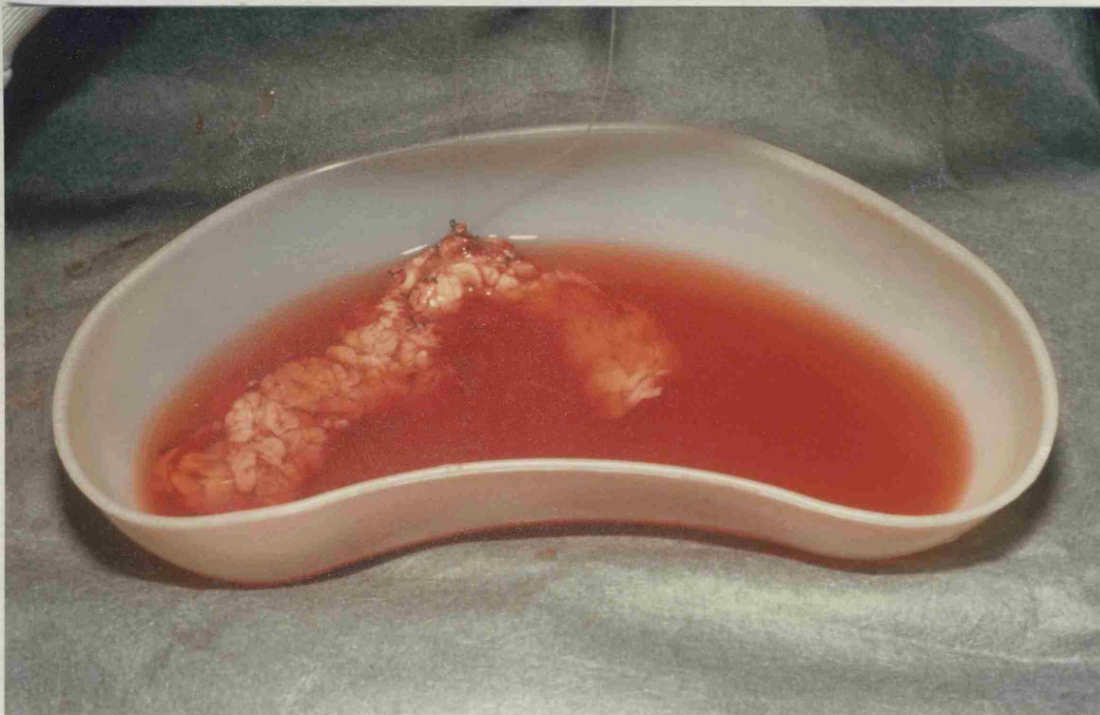


Figure 3.5 Digested canine pancreas prior to teasing apart with forceps.

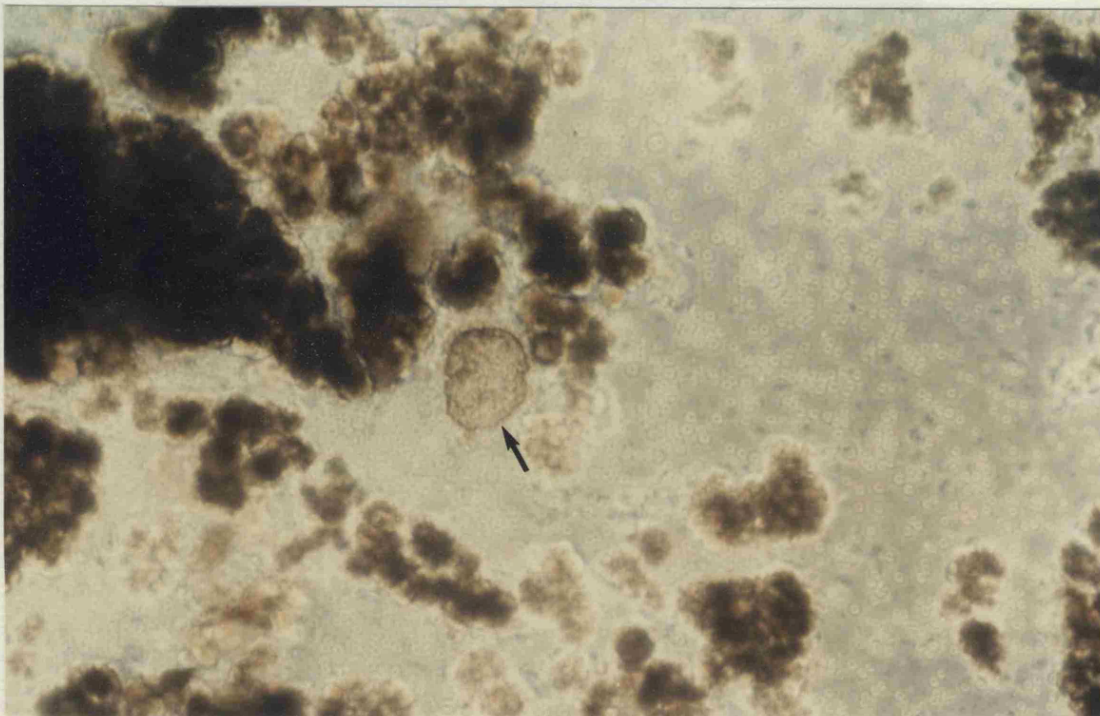


Figure 3.6 Canine pancreatic digest showing intact islet (→) and clumps of exocrine cells.

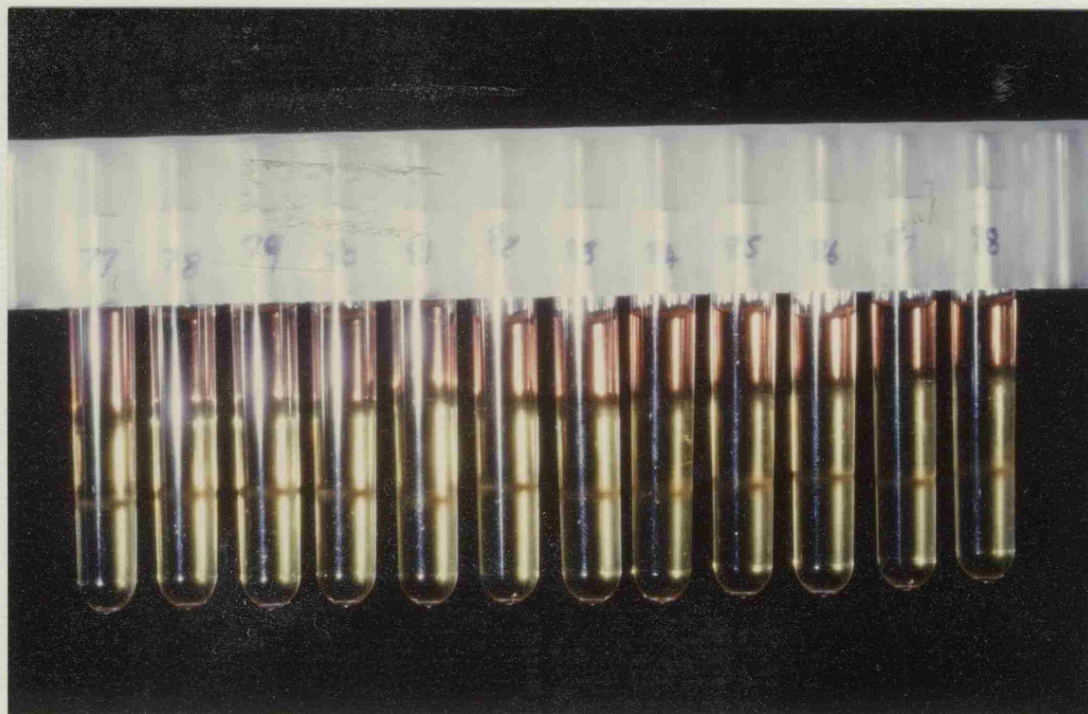


Figure 3.7 BSA test gradient prepared to determine the optimal density of BSA for islet isolation.



Figure 3.8 Dispersed pancreas mixed with 200 ml of stock BSA, with sterile bags containing BSA (density, 1.082) and MEM for large scale density gradient isolation.



Figure 3.9 IBM 2991 cell processor.



Figure 3.10 Sterile enclosed processing bag for use on the IBM 2991 cell processor.

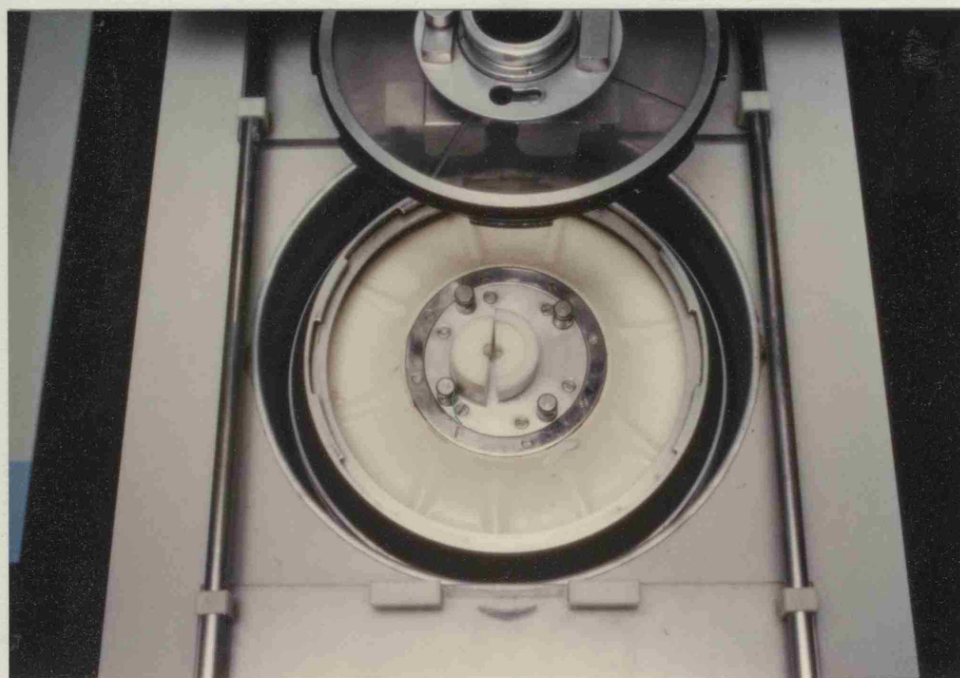


Figure 3.11 Centrifuge bowl of cell processor.

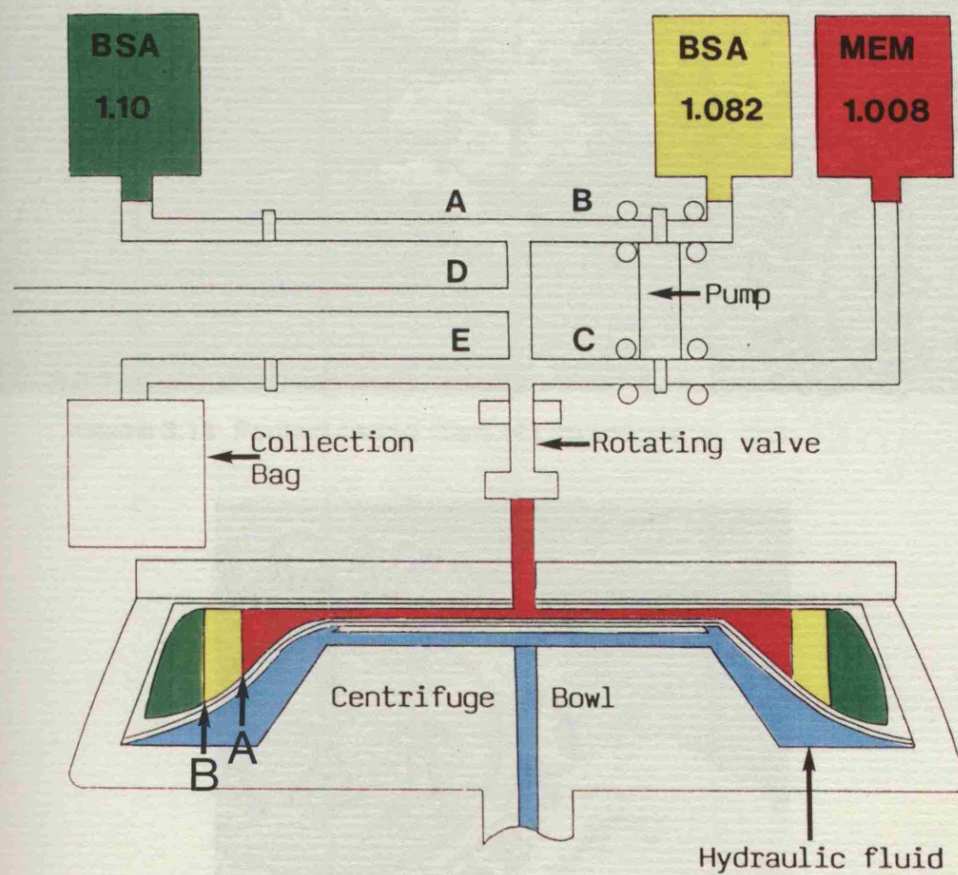


Figure 3.12 Schematic representation of BSA gradient/IBM 2991 separation system for canine islets.

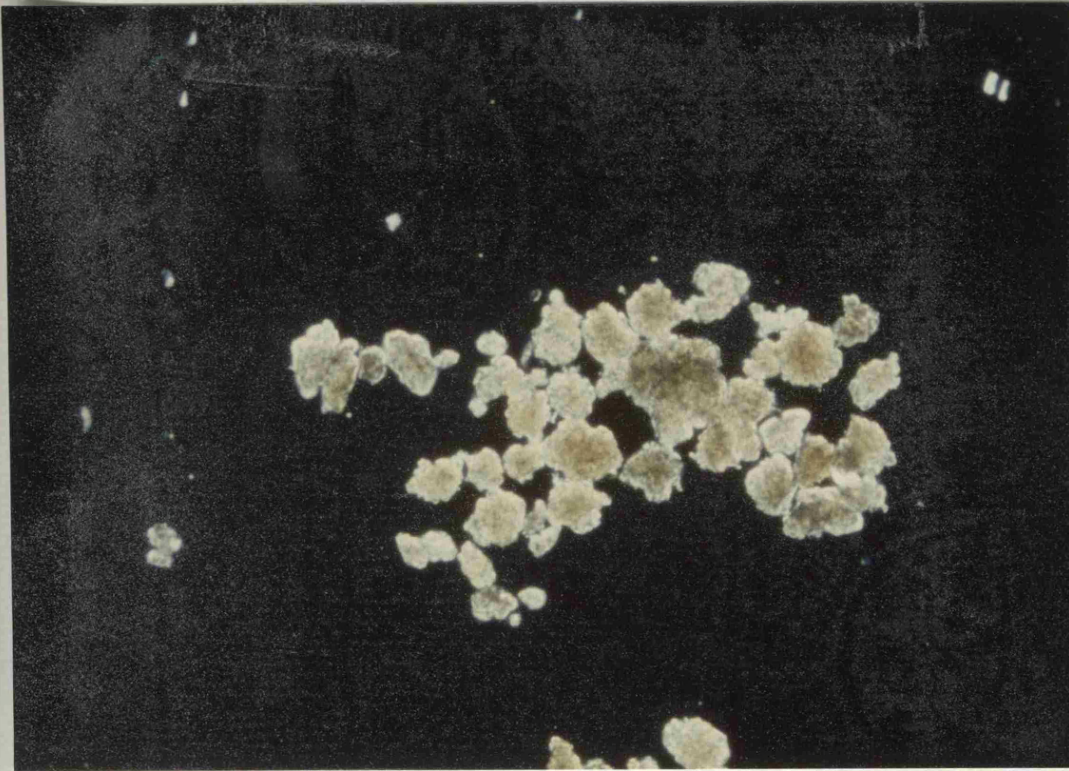


Figure 3.13 Purified canine Islets of Langerhans. (x 100)

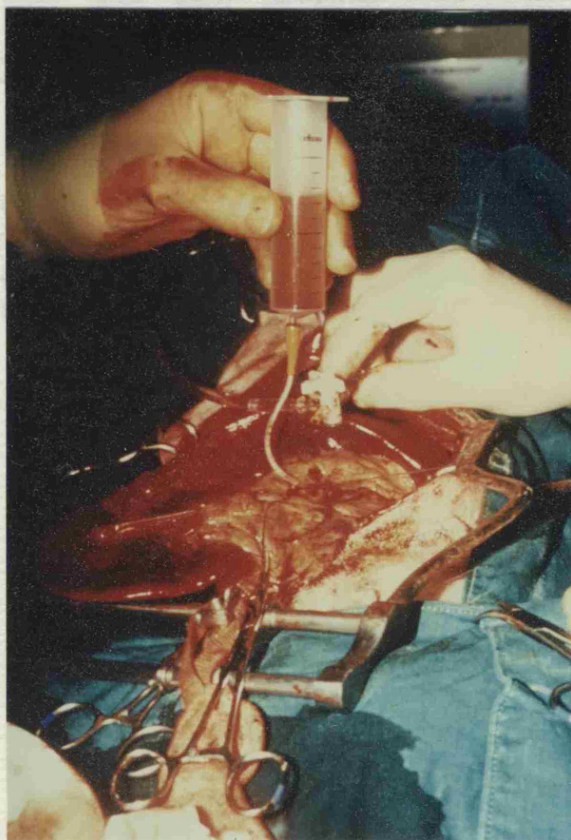


Figure 3.14 Intraportal transplantation of purified islets by gravity infusion.

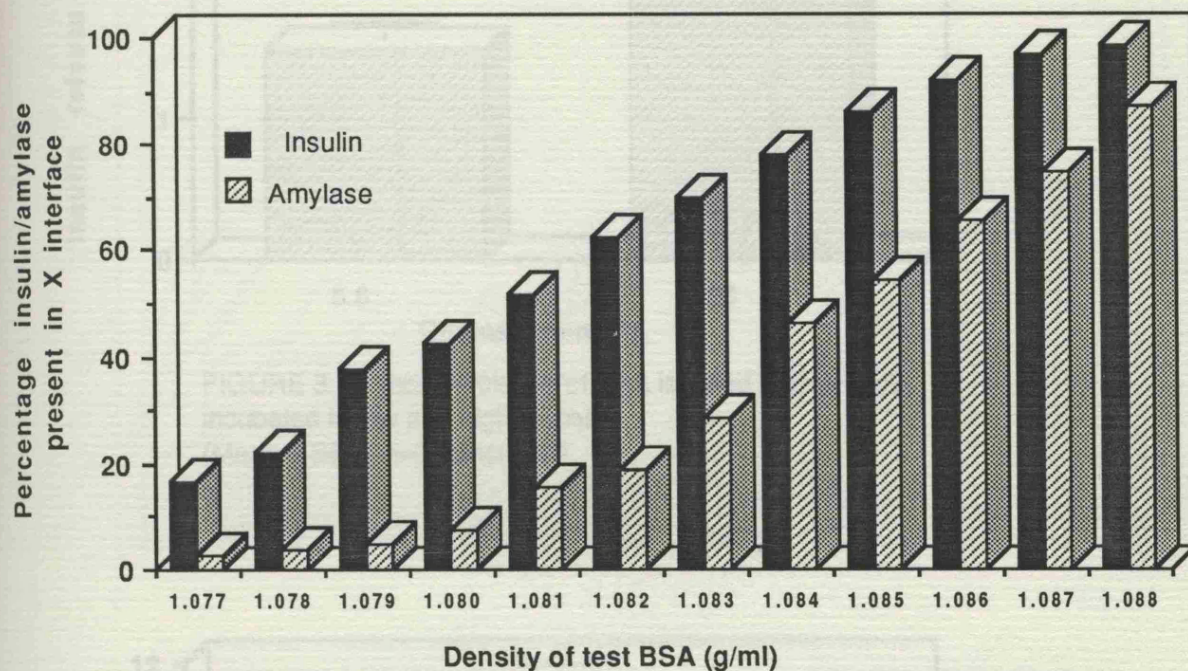


FIGURE 3.15 Analysis of insulin and amylase containing tissue present in the X interface of the canine BSA test gradient system. Results expressed as the mean ($n=5$) percentage of insulin or amylase present compared with the total within the gradient.

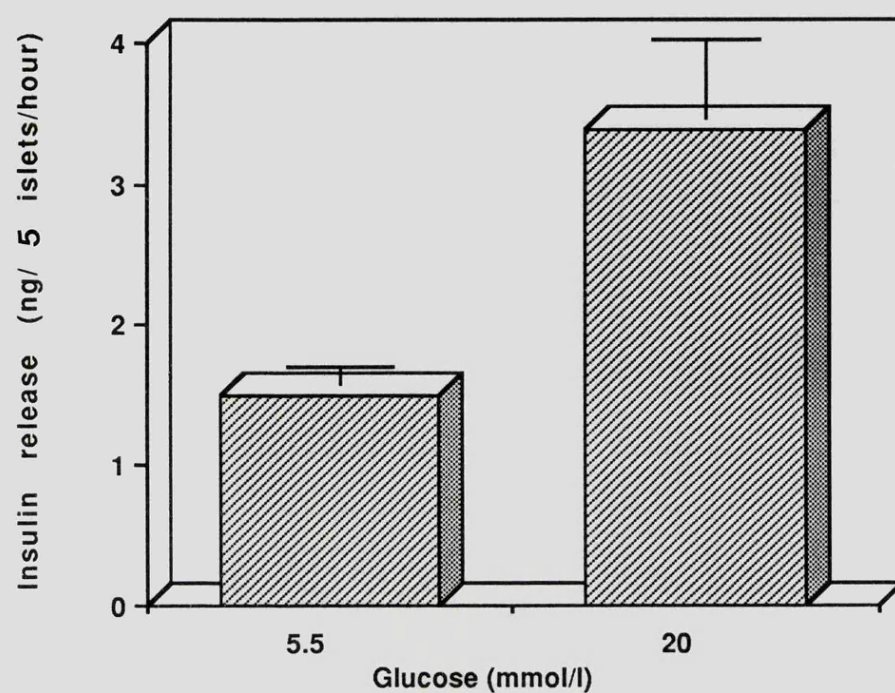


FIGURE 3.16 Insulin release of BSA isolated canine islets incubated in low and high glucose. (Mean $\pm$ SEM, $n=5$ pancreata).

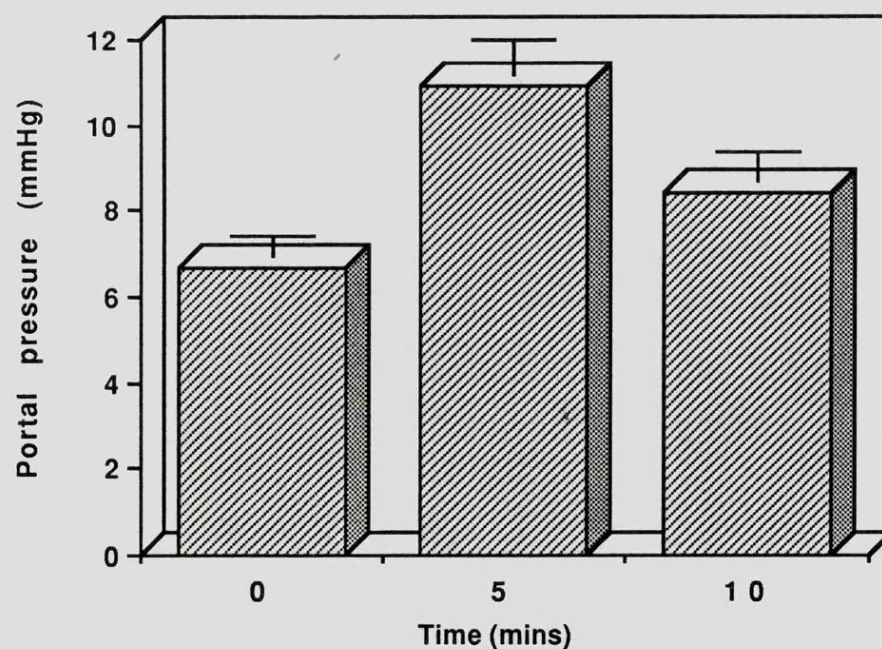


FIGURE 3.17 Mean portal pressure of dogs autotransplanted with purified islets, pre- and post-engraftment. (Mean $\pm$ SEM, $n=8$).

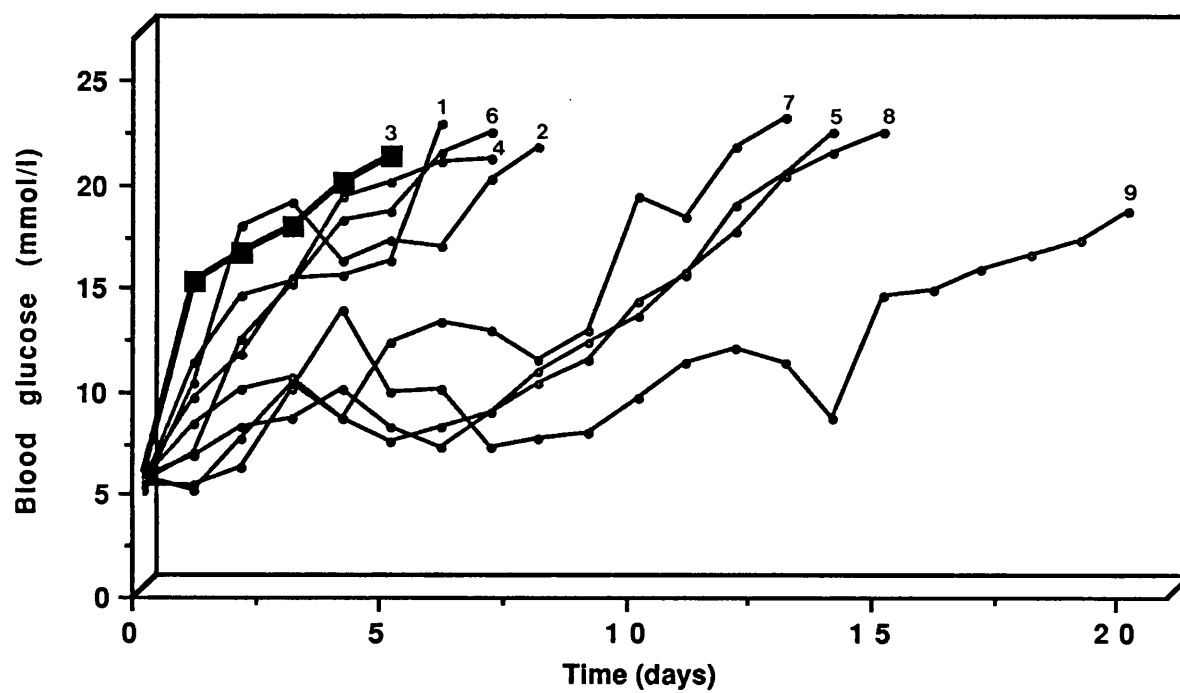


FIGURE 3.18 Serial blood glucose of control (■) and purified islet autotransplanted dogs (n=8).

Table 3.1 Results of BSA Gradient/IBM 2991 Isolation of Canine
Islet Autotransplants

	Mean	SEM	Range
Pancreas weight (g)	32.5	2.5	20-46
Digest volume (ml)	12.1	1.5	7-19.5
Islet transplant volume (ul)	272	70	150-750
Islet count	85,000	23,000	30,000-198,000

See Appendix 2A for details.

CHAPTER 4

ESTABLISHMENT OF AN IN VIVO METHOD OF ASSESSING ISLET VIABILITY UTILISING THE DIABETIC NUDE RAT

INTRODUCTION

Previous methods described for assessing human islet viability have utilised 'in vitro' techniques such as the use of vital stains (Gray, 1984; Warnock, 1987) and measurement of insulin release following glucose stimulation (Ballinger, 1972; Gray, 1984; Warnock, 1987). A method of assessing 'in vivo' viability of isolated islets, by their ability to reverse diabetes, would be a useful adjunct in the development of islet isolation techniques.

The nude (athymic) rodent has been shown to accept human tissue or tumour grafts for prolonged periods of time, without rejection, due to a genetically determined athymic aplasia (Povlsen, 1974; Festing, 1981). Although islet tissue has previously been transplanted into the nude mouse (Usadel, 1980; Mandel, 1984; Schwedes, 1983; Gray, 1984) this has been predominantly into the non-diabetic recipient so that no functional assessment of the ability of the graft to reverse diabetes could be performed. Those reports using streptozotocin-induced diabetic nude mice for islet transplantation studies have had limited success (Lundgren, 1977) probably because of susceptibility to dehydration and infection, particularly if using the conventional 10-14 day period post-induction of diabetes prior to transplantation.

The purpose of this study was to develop a method of successfully transplanting islet tissue into diabetic nude rats which would then allow assessment of function in vivo. In order to achieve this aim allogeneic and xenogeneic (mouse) islet transplantation was performed in the nude rat shortly after induction of diabetes with streptozotocin. Furthermore, transplantation of human thyroid tissue was also performed in order to ensure that the nude rat would accept human endocrine tissue grafts.

MATERIALS AND METHODS

Donor Rats and Islet Isolation

Inbred WAG/Ola rats were used as donors for islet isolation, which was performed as described in Chapter 2, Part II.

Donor Mice and Islet Isolation

Hybrid MF1 x BALB/c F1 mice, of either sex, weighing 20-25 g, were used as islet donors. Islet isolation was performed essentially as described for the rat except that batch testing of collagenase and adjustment of the time needed for digestion were both required. The final method used 2 ml of MEM containing 0.5 mg/ml Type XI collagenase (Batch no. 46F-6839) and a digestion time of 15 minutes. A BSA test gradient (similar to that described in Chapter 2, Part II) was then used to determine the density of mouse islets and exocrine tissue. Islets were then finally purified from the 1.068-1.010 interface of a 4 step BSA gradient with densities of 1.105, 1.090, 1.068 and 1.010 (MEM).

Islet Culture

Culture of isolated islets was performed at 37°C, in an atmosphere of humidified air plus 5% CO₂, in 90 mm petri dishes containing 10 ml of RPMI medium, supplemented with:- foetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml), L-glutamine (2 mmol/l), sodium pyruvate (1 mmol/l), 2-mercaptoethanol (0.1 mmol/l) and hydrocortisone (0.01 µmol/l).

Source of Human Thyroid

Small pieces of thyroid tissue were obtained at operation from 5 patients undergoing thyroidectomy for thyrotoxicosis (n=4) and multinodular goitre (n=1). The individual thyroid tissue was placed in ice-cold MEM immediately after resection and was then chopped into small

fragments (0.5 mm cubes) in the laboratory under sterile conditions. Three pieces from each sample were picked to be transplanted into 1 nude rat and the remainder was retained for histological examination.

Renal Subcapsular Transplants

The renal subcapsular space was used as the engraftment site to facilitate subsequent graft removal.

i) Islets

Freshly isolated or cultured islets were centrifuged into a small pellet for transplantation (Fig. 4.1) and were mixed with 10 μ l of the recipient's tail vein blood. The blood clot was then placed into the left renal subcapsular space after exposure of the kidney through a flank incision under halothane anaesthesia (Fig. 4.2).

ii) Thyroid

Three fragments of tissue were placed into the left renal subcapsular space of each of 5 nude rats by flank incision. Graft removal was performed by nephrectomy at 2,4,6,8 and 12 weeks after transplantation.

Recipient Animals

i) Heterozygous Rats

Heterozygous Rowett hooded rats (+/rnu), weighing 80-100g, were used as islet transplant recipient controls.

ii) Nude Rats

Inbred Rowett homozygous nude rats (rnu/rnu)(Fig. 4.3), of either sex weighing 80-100g were used as islet or thyroid recipients or non transplanted control animals. The colony was kept in conventional conditions, in isolation from other rodent colonies and the rats were subjected to minimal handling. To maintain the colony, homozygous males in which a foetal thymus transplant has been performed at weaning,

(Hetherington, 1975) and heterozygous females were used in breeding units of 1:4.

Induction of Diabetes

Diabetes was induced by femoral vein injection of 70 or 80 mg/kg streptozotocin (Sigma) as described in Chapter 2.

Monitoring of Transplants

Body weight measurement and blood glucose estimations (glucose oxidase method) were performed at regular intervals in all animals throughout the study period. Graft removal was performed by left nephrectomy through a flank incision under halothane anaesthesia.

Glucose Tolerance Tests

These were performed by intravenous injection of 0.5 g/kg glucose and pre- and post-injection (5, 10, 15, 20, 30, 40 minutes) tail vein blood samples were taken for glucose measurements.

Histological Examination of Tissue

Histology of the resected kidneys and islet or thyroid grafts was performed following fixation in 10% formal-saline and embedding in paraffin. Sections (5 μ m) were stained either with haematoxylin and eosin or were specifically stained for insulin by immunohistology (Dako Pap Kit, Dako Ltd., High Wycombe, Bucks.).

RESULTS

Allogeneic Islet Transplants

i) Heterozygous Recipients

Eight heterozygous hooded rats were given 70 mg/kg streptozotocin and all became diabetic (blood glucose >20 mmol/l) within 48 hours at which time the islet transplants were performed. Diabetes was reversed

in all within 24-48 hours of islet transplantation. Rejection (reversion of blood glucose back to >20 mmol/l) occurred in 6/8 at a mean of 9 days post-transplantation and histology of the islet grafts of these 6 animals confirmed a dense lymphocytic inflammatory cell infiltrate (Fig. 4.4). Two animals remained normoglycaemic until graft removal by nephrectomy (at 21 days) when diabetes immediately recurred. Histology of both these grafts however showed a moderate lymphocytic cell infiltrate.

ii) Homozygous (Nude) Recipients

Twenty-four nude rats were given 70 mg/kg streptozotocin and 23 of the 24 became diabetic within 48 hours of injection (mean blood glucose pre-induction of diabetes 5.4 ± 0.4 mmol/l, 24 hours 19.8 ± 1.1 mmol/l and 48 hours 23.8 ± 1.2 mmol/l). One animal remained normoglycaemic and was excluded from further study. Seven animals acted as non-transplanted controls and remained diabetic throughout the study period with a mean survival of 23 ± 1.8 days and a mean weight loss of 0.5 ± 0.2 g/day (Fig. 4.5).

Eight animals were transplanted with 1000-1500 freshly isolated allogeneic islets. Diabetes was effectively reversed in all 8 rats within 48 hours of transplantation and the animals remained normoglycaemic until the graft was removed by nephrectomy when all animals became diabetic (Fig. 4.6).

Diabetes was similarly reversed and normoglycaemia maintained in the 8 diabetic rats receiving two-day cultured islets (Fig. 4.7). Following graft removal 7/8 animals became hyperglycaemic within 72 hours; the remaining animal, however, maintained a normal blood glucose level for 5 days until culled.

An initial mean weight loss of 0.5 ± 0.2 g per day was observed in all the nude rats following induction of diabetes (Fig. 4.5). Weight gain

following transplantation was similar in both freshly isolated and cultured islet groups at 2.0 ± 0.25 g/day, which was significantly greater ($p < 0.001$; Mann Whitney) than control diabetic animals and was identical to the weight gain of normal nude rats (Fig. 4.5).

Macroscopic examination of all the kidneys at the time of resection showed the presence of well vascularised areas of tissue beneath the renal capsule (Fig. 4.8). Histological examination showed a band of endocrine tissue which stained strongly for insulin (Fig. 4.9). There was no evidence of an inflammatory cell infiltrate in any of the grafts.

Xenogeneic Transplants

i) Islet

Seven nude rats were given 80 mg/kg streptozotocin. All became diabetic within 48 hours of injection (Fig. 4.10) and were transplanted with 900-1000 mouse islets which had been cultured for 2 days. Diabetes was reversed in all 7 recipients and these animals remained normoglycaemic (Fig. 4.10) and gained weight (1.9 ± 0.3 g/day) throughout the 21 day post transplantation period. Glucose tolerance tests were performed at 20 days post transplantation and demonstrated a near normal glucose clearance (Fig. 4.11). Graft removal by nephrectomy resulted in rapid reversal to the diabetic state in all of the animals. Histology of the resected grafts confirmed the presence of endocrine tissue beneath the renal capsule with no sign of rejection.

ii) Thyroid

Five non-diabetic nude rats were successfully transplanted with human thyroid tissue and all survived the required post transplantation period (2-12 weeks). Histological examination of non-transplanted thyroid fragments confirmed the presence of thyroid follicles in the tissue retrieved from the 4 patients with thyrotoxicosis. In the case

of the patient with multinodular goitre only colloid was seen with no evidence of cellular material.

Histology of the resected grafts removed at 2,4,6 and 12 weeks confirmed the presence of thyroid tissue beneath the renal capsule (Fig. 4.12). There was no evidence of rejection seen in any of the multiple sections examined. In one animal, transplanted with tissue from the patient with a multinodular goitre, histological examination at 8 weeks showed the presence of fibrous tissue but no evidence of thyroid follicles.

DISCUSSION

There are only a few reports of pancreas/islet transplantation in the diabetic nude rodent, which reflects the poor survival of such animals once made diabetic (Andersson, 1976; Lundgren, 1977). Streptozotocin has been used extensively since 1972 (Ballinger) to induce diabetes in rodents for islet transplantation studies. Although the action of streptozotocin, when given by intravenous bolus injection is rapid and diabetes is induced within 24-48 hours (Rerup, 1970), it is conventional to maintain animals for between 10-14 days post-induction of diabetes prior to islet transplantation. This makes it possible to detect any reversion of the diabetic state. Thus most previous studies of islet transplantation in the nude rodent have used the standard 10-14 day induction protocol and as the health of these animals is severely affected by the diabetic state, this long pre-transplantation period probably contributes to the overall poor survival of even successfully transplanted animals.

In this study, diabetes was induced rapidly in the nude rat, with hyperglycaemia becoming apparent within 24-48 hours after streptozotocin injection (Figs. 4.6, 4.7 and 4.10). Although the nude rat was utilised, which is more robust than the nude mouse (Festing, 1981), the

mean survival of the diabetic control animals was still only 23 days with many animals being in a relatively poor condition from dehydration and unfit for a transplant operation, from as early as 5-7 days.

The prompt reversal of diabetes and maintenance of normoglycaemia by transplantation of both freshly isolated and 48 hour cultured allogeneic islets in the initial part of the study confirmed that successful islet transplantation could be performed in the diabetic nude rat (Fig. 4.6 & 4.7). The shortened post-induction period of 48 hours was an important innovation because it allowed demonstration of the onset of diabetes but ensured that this state was present for only a short period. The effects of dehydration and infection as seen in the control animals were thus limited in the transplanted animals.

Successful transplantation was also confirmed when transplanted animals overcame an initial weight loss post-induction of diabetes and demonstrated a weight gain (identical to that seen in normal animals) over the transplant period (Fig. 4.5). Following graft removal the majority (15/16) of the animals (Figs. 4.6 & 4.7) became diabetic and lost weight (Fig. 4.5) confirming excision of viable islet tissue. The failure of one animal to become diabetic, following graft removal (Fig. 4.7), most probably occurred because of 'recovery' of the recipient's own islets from the streptozotocin treatment (spontaneous reversal).

Histological examination of the resected allogeneic islet grafts demonstrated a band of endocrine tissue with no sign of rejection (Fig. 4.9). The allogenicity of the islet grafts was confirmed by the transplantation of the WAG/Ola rat islets into the heterozygous Rowett rats. These rats had a mean graft rejection time of 9 days. Histological examination showed a dense lymphocytic cell infiltration (Fig. 4.4). The failure of two of the heterozygous rats to reject the

grafts within 21 days probably reflects the fact that these animals have a partially impaired immunocompetence (Festing, 1981) as a moderate lymphocytic cell infiltrate was present on resection with early signs of rejection.

In the second part of the study the 48 hour induction protocol was applied to the transplantation of 48 hour cultured xenogeneic (mouse) islet tissue. Diabetes was promptly reversed following transplantation and normoglycaemia was maintained for 21 days until graft removal with subsequent hyperglycaemia (Fig. 4.10). Transplantation of viable islet tissue was thus confirmed. Functional assessment of xenograft performance was also made by glucose tolerance testing and the clearance of a glucose load was demonstrated (Fig. 4.11). The sub-optimal glucose clearance of these transplanted animals in relation to that of normal controls may have been due to the transplantation of insufficient numbers of islets.

In the xenogeneic islet experiments, the dose of streptozotocin was deliberately increased from 70 to 80mg/kg because of spontaneous reversal of one animal in the allogeneic experiments (Fig. 4.7); all seven animals promptly became diabetic on graft removal with no evidence of reversion. Furthermore, histological examination of the excised kidney grafts confirmed that the islet xenografts were accepted by the recipient rats for the 21 day period; with no evidence of rejection in any of the grafts.

The short series of human thyroid transplants was performed to determine whether human endocrine tissue would be rejected by the nude rats once transplanted. This study demonstrated that human endocrine tissue could be transplanted into the renal subcapsular space of the nude rat and indeed could be left in vivo for periods of up to three months with no evidence of rejection (Fig. 4.12).

SUMMARY

A method was developed which allowed successful transplantation and assessment of function of allogeneic and xenogeneic islet tissue in the diabetic nude rat.

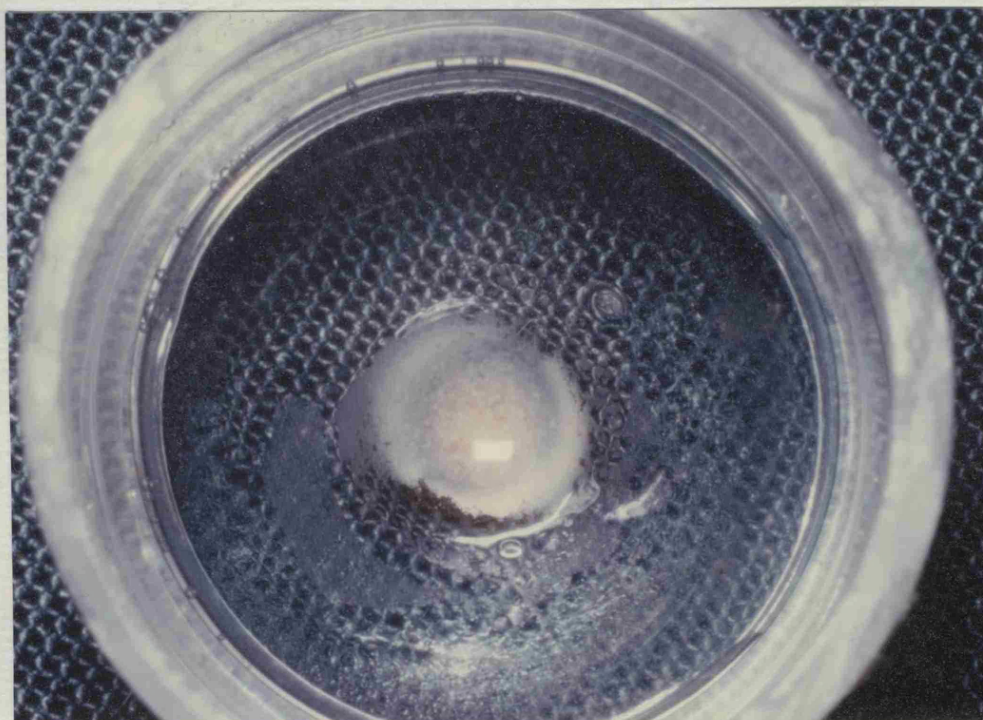


Figure 4.1 Isolated rat islets centrifuged into a pellet in the conical base of a cut-off universal vial prior to addition of recipient's blood.

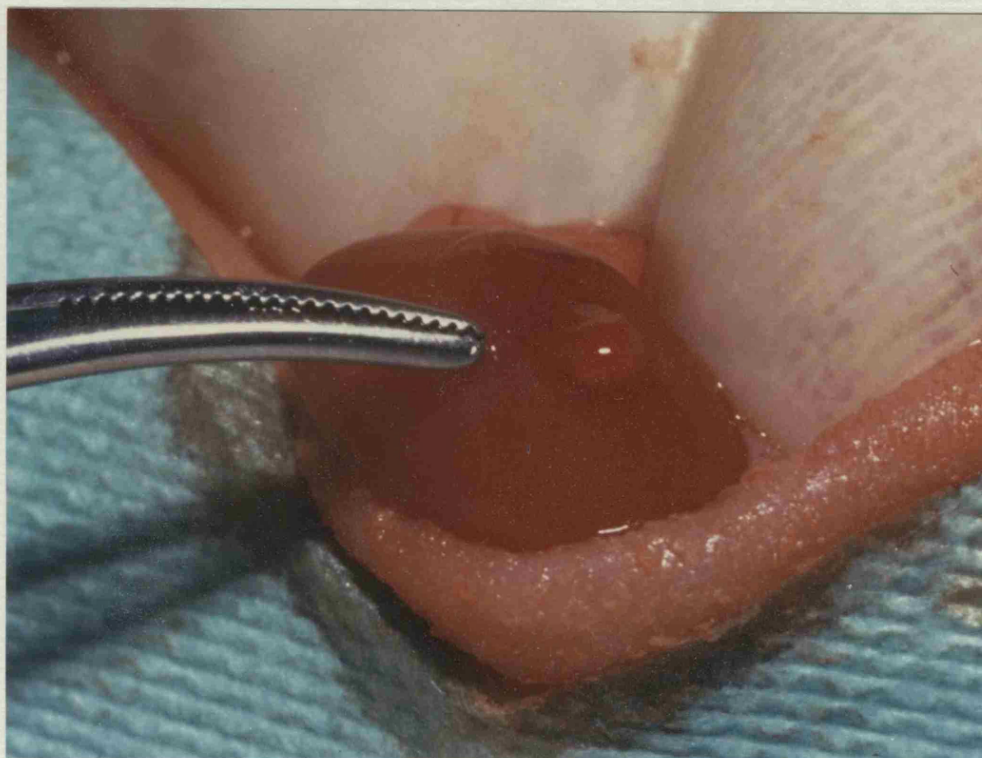


Figure 4.2 Transplantation of isolated islets, contained within blood clot, under the renal capsule.



Figure 4.3 Rowett nude rat.

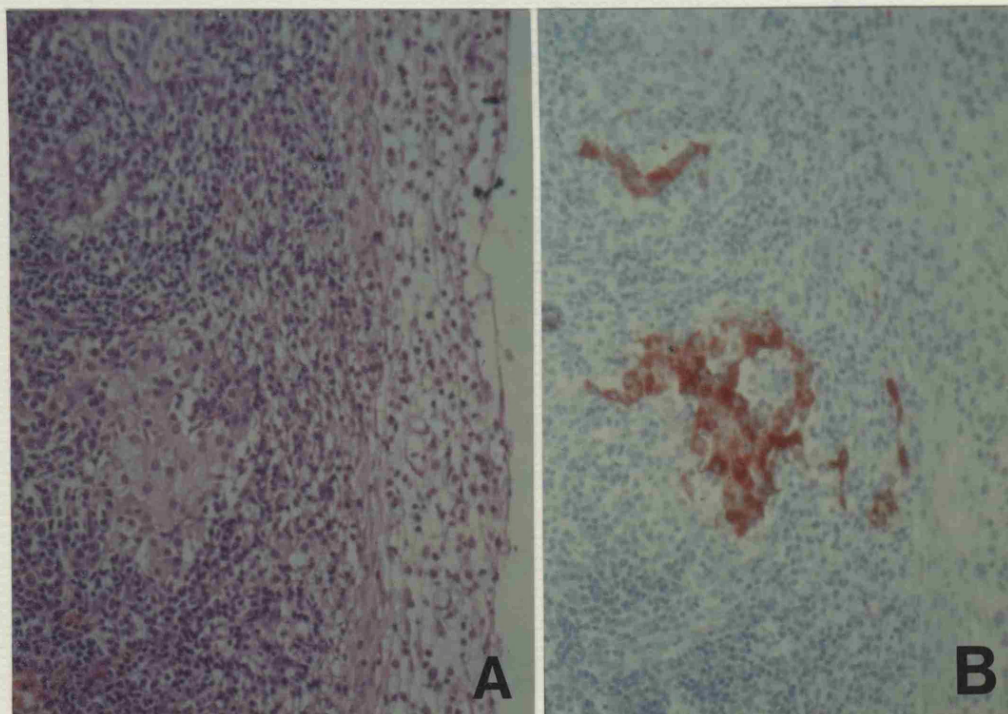


Figure 4.4 Photomicrographs of representative sections of renal subcapsular islet grafts. WAG/Ola rat islets transplanted into 1 of 6 heterozygous Rowett rats which rejected the grafts rapidly, showing dense lymphocytic cellular infiltration and destruction of islet tissue.

A- stained with haematoxylin & eosin; B- stained for insulin. (x 200)

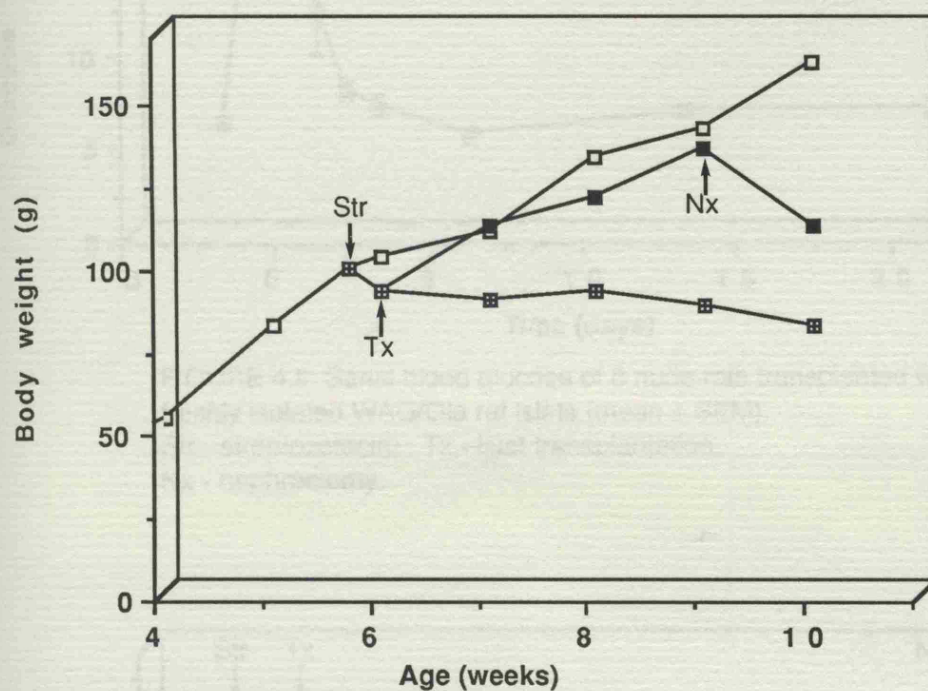


FIGURE 4.5 Mean serial body weights of normal (□, n=6), diabetic (■, n=7) and allogeneic islet transplanted (■, n=14) nude rats. Str - streptozotocin; Tx - islet transplantation; Nx - graft removal by nephrectomy.

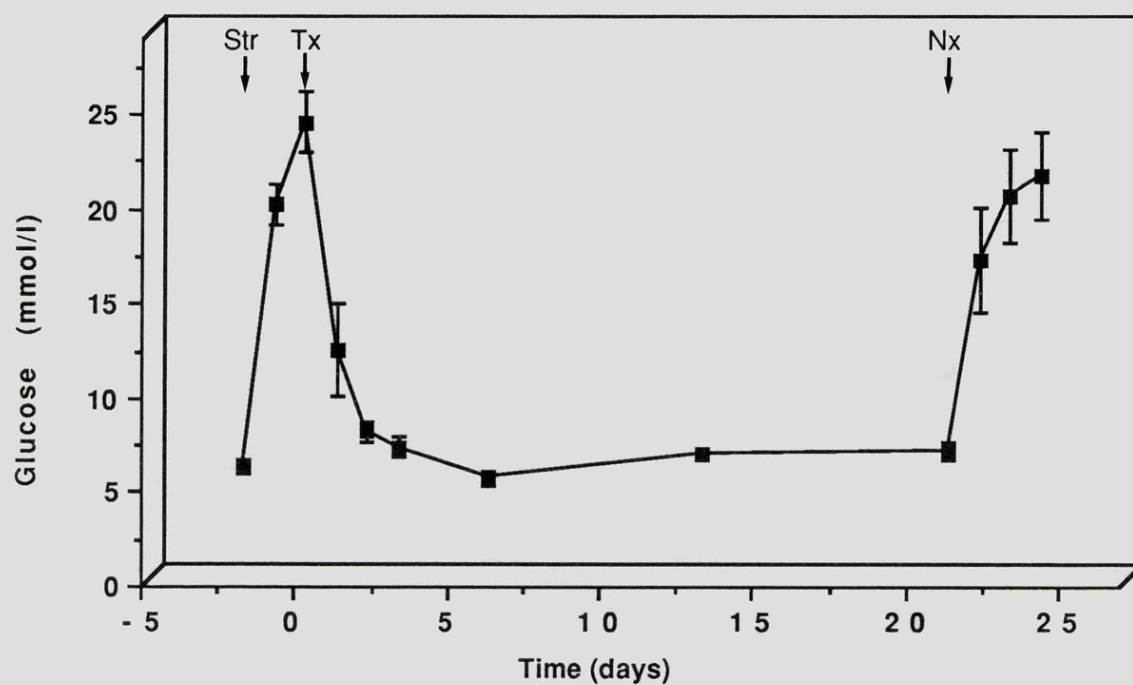


FIGURE 4.6 Serial blood glucose of 8 nude rats transplanted with freshly isolated WAG/Ola rat islets (mean $\pm$ SEM).
Str - streptozotocin; Tx - islet transplantation;
Nx - nephrectomy.

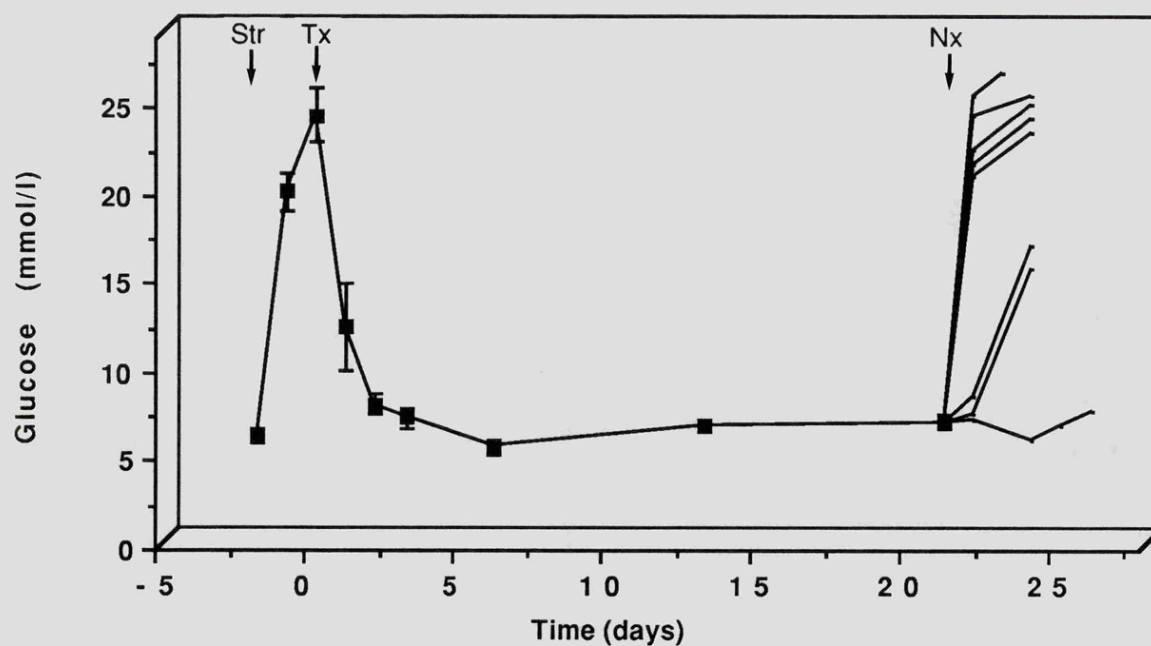


FIGURE 4.7 Serial blood glucose of 8 nude rats transplanted with 48 hour cultured WAG/Ola rat islets (mean $\pm$ SEM, with individual values after graft removal).
Str - streptozotocin; Tx - islet transplantation;
Nx - nephrectomy.



Figure 4.8 Photograph of islet engrafted kidney from 1 of the 16 nude rats transplanted with allogeneic islets showing the macroscopic appearance of the graft.

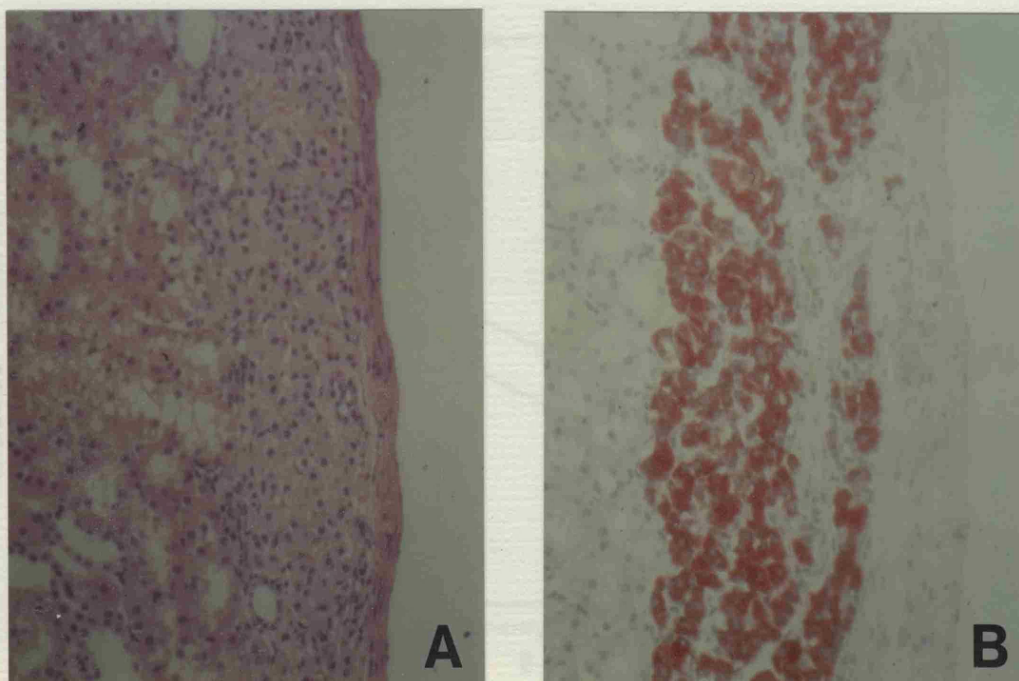


Figure 4.9 Photomicrograph of representative sections of the allogeneic renal subcapsular islet graft showing islet tissue with no evidence of rejection. A- stained with haematoxylin & eosin; B- stained for insulin. (x 200)

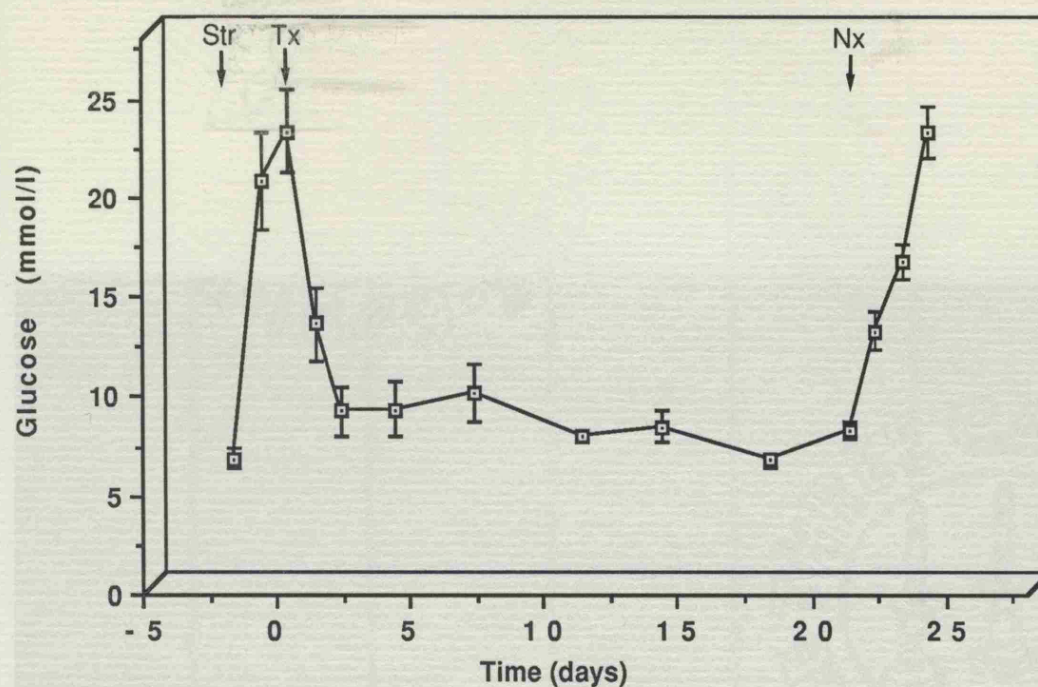


FIGURE 4.10 Serial blood glucose of 7 nude rats transplanted with 48 hour cultured xenogeneic (mouse) islets (mean $\pm$ SEM). Str - streptozotocin; Tx - islet transplantation; Nx - graft nephrectomy.

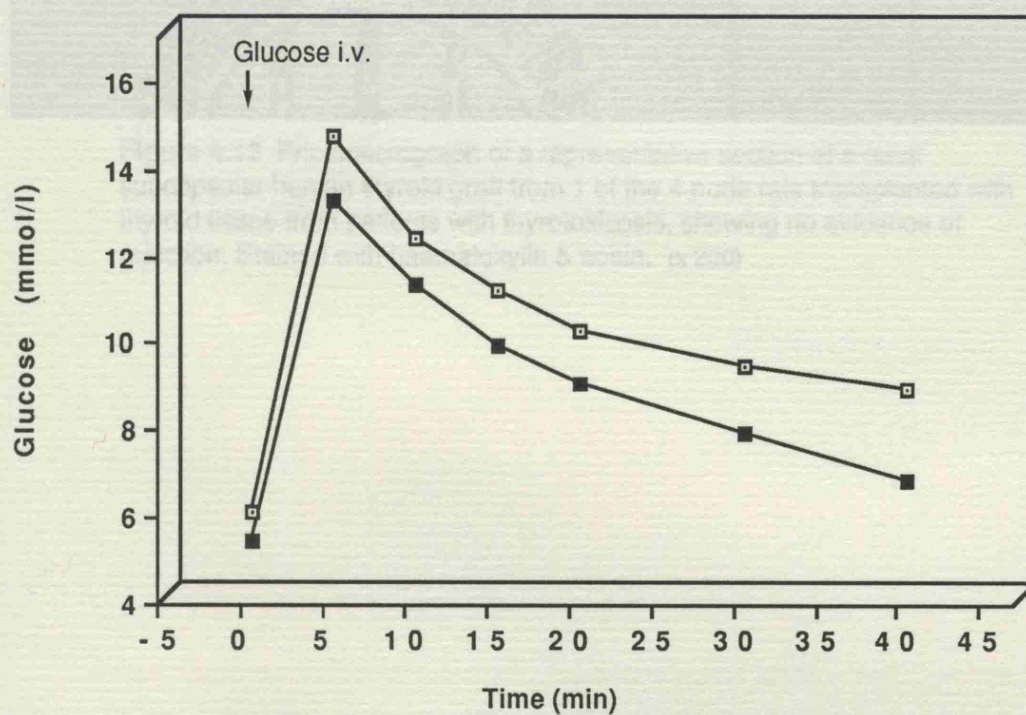


FIGURE 4.11 Glucose tolerance test of normal (■, n=6) and xenogeneic (mouse) (□, n=7) islet transplanted nude rats. (, $p < 0.05$)

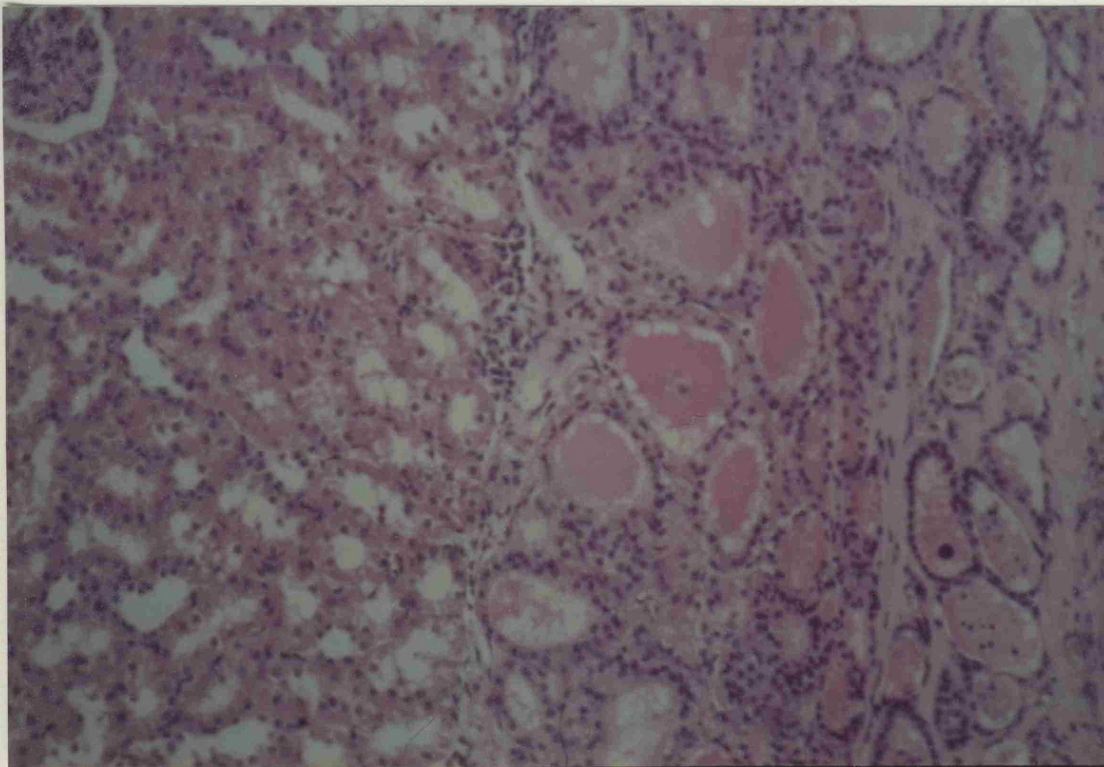


Figure 4.12 Photomicrograph of a representative section of a renal subcapsular human thyroid graft from 1 of the 4 nude rats transplanted with thyroid tissue from patients with thyrotoxicosis, showing no evidence of rejection. Stained with haematoxylin & eosin. (x 200)

CHAPTER 5

LARGE SCALE PURIFICATION OF HUMAN PANCREATIC ISLETS

INTRODUCTION

Collagenase digestion is the standard method of releasing islets from the pancreas and it has recently been demonstrated that intraductal delivery of this enzyme can produce large volumes of digest containing islets and dispersed exocrine cells (Gray, 1984). To prepare human islets for potential human islet transplantation a second stage is required to purify the islets and eliminate the contaminating exocrine tissue.

A variety of purification techniques have been employed for human islet isolation, including hand-picking, serial sieving and density gradient isolation using Ficoll (Sutherland, 1976; Gray, 1984; Scharp, 1987). Although pure islets can be obtained by hand-picking, this method is unsuitable for the large numbers required for human transplantation. Islet yield can be improved by the technique of serial sieving but this technique is open to infection and there may be significant loss of the tissue on the meshes (Gray, 1984). For efficient density gradient isolation multiple gradient tubes have been required (Scharp, 1987).

In Chapter 2 of this study a discontinuous gradient of Bovine Serum Albumin was shown to improve the yield and purity of rat islets compared with Ficoll. In order to adapt this method to the purification of human islets, the difficulties involved in processing the large volumes of digest produced from the human pancreas had to be overcome. The large volume density gradients, as described in Chapter 3 for canine islet isolation, were thus adapted for the purification of human islets to determine whether the use of this medium and technique could produce large numbers of purified islets. In order to determine whether the isolated islets were viable, in vitro and ultimately, in vivo, methods of assessment were used.

MATERIALS AND METHODS

Preparation of Pancreatic Digest

Adult human pancreata were obtained from individuals undergoing cadaveric organ retrieval for transplantation. Following in situ perfusion with cold Hyperosmolar Citrate solution (Baxter Healthcare Ltd., Thetford, UK), the tail segment of the pancreas was resected (Fig. 5.1). The surrounding fat was excised (Fig. 5.2) and a size 4 or 5 FG cannula (Portex) was inserted into the exposed duct and ligated in place (Fig. 5.3). The pancreas was then transported to the laboratory, on ice, within sterile plastic bags (Fig. 5.4).

Intraductal collagenase digestion of the pancreas was performed by modification of the method described by Gray (1984), using the apparatus described in Chapter 3 (Fig. 3.4). The pancreas was weighed and distended with 1 ml/g warm (37°C) MEM containing 2.5 mg/ml Type X1 collagenase (Sigma; Batch no. 46F-6838) and 0.02 mg/ml DNAase (Sigma) prior to incubation in warm (37°C) MEM. The pancreas was then perfused intraductally with warm enzyme solution at 4 ml/minute. Digestion was considered to be complete when gentle teasing with toothed forceps demonstrated that the pancreatic structure disrupted easily (usually after 19-25 min). The pancreas was rapidly chilled by immersion in ice-cold MEM and disrupted by teasing apart with forceps. The fragmented pancreas was suspended in 200 ml ice-cold MEM and mechanically dispersed by shaking in a 500 ml bottle for 1 minute. The digest was poured through a 500 µm mesh and washed three times with MEM containing 0.5% BSA.

BSA Test Gradient Isolation

In a series of preliminary experiments the optimum density of BSA for human islet isolation was determined as described in Chapter 3 for

canine islets except that a lower range of densities was required (1.055–1.070 g/ml).

Large Scale Islet Isolation

The IBM 2991 Cell Separator was used as described in Chapter 3. The sequence of events to produce a discontinuous BSA density gradient for human islet isolation was as follows:- the washed digest was thoroughly dispersed in 200 ml of BSA stock solution (density 1.10) in a sterile 500 ml glass bottle. The digest/BSA mixture was then run into the centrifuge bag under gravity. At slow speed centrifugation a second layer of BSA (75 ml, density 1.063) was loaded on to the gradient using the peristaltic pump. This layer was then overlaid with 75 ml MEM. The bag was centrifuged at 800g for 20 minutes. After centrifugation the supernatant (MEM) was pumped off to waste and the islet containing (inner) interface was collected in a sterile bag. The islets were washed with MEM/BSA, in 50 ml centrifuge tubes and aliquots were taken for the appropriate assays.

Assessment of Islet Yield and Purity

Islet counts were performed as described in Chapter 2. Purity was assessed by eye using serial dilution and an inverted microscope with side illumination and by measurement of amylase content.

Measurement of Insulin Release of Isolated Islets

Aliquots of 5 islets were taken randomly by micropipette from the initial digest (hand picked) and from the final islet preparation (BSA isolated). The aliquots were incubated at 37°C for 1 hour in MEM containing low glucose (5.5 mmol/l) or in MEM with added glucose (20 mmol/l) as described in Chapter 3.

Transplantation of Isolated Islets into Diabetic Nude Rats

Handpicked and BSA isolated islets were transplanted into nude rats made diabetic with streptozotocin (80 mg/kg) as described in Chapter 4. Islets (800-1000) were transplanted into the renal subcapsular space at 48 hours post streptozotocin injection. Regular blood glucose measurements were performed until graft removal (21 days). In addition, a glucose tolerance test was performed on day 20 during which blood glucose and serum insulin levels were measured. Glucose clearance (K value) was calculated by the method of Moorhouse (1964) with use of all glucose values between 5 and 40 minutes.

Islet Culture

Culture was performed at 37°C in an atmosphere of humidified air with 5% CO₂ as described in Chapter 4.

Sterility of Preparation

Aliquots (10ml) of transport fluid and of the final washings of the islet containing preparation were taken and were sent for routine microbiological assessment.

RESULTS

BSA Test Gradient

The optimum density for human islet isolation was determined by the test gradient system using an initial series of 6 human pancreata. Insulin and amylase determinations were performed and as with the canine pancreas (Fig. 3.15) incomplete separation of the 2 components was observed (Fig. 5.5). The density of BSA for optimum islet isolation (i.e. maximum insulin yield for minimal amylase contamination) was judged to be 1.063 and this density was then used for the consecutive series of human islet isolations.

Islet Yield

Fifteen human pancreata were digested with collagenase and the mean digest volume produced was 11.9 ± 1.5 ml (Table 5.1). Islets were isolated by BSA density gradient centrifugation and after purification a mean 'islet' volume of 430 ± 60 μ l (3.6% of original starting digest volume) was obtained (Table 5.1). The mean number of islets recovered was 2643 ± 284 islets per ml pancreatic digest. The final islet preparation contained a mean insulin content of 63.7% of the total available on the gradient (Fig. 5.6). The estimated purity of this preparation was $63 \pm 7\%$ with a minimal amylase content (Fig. 5.7). An example of the high degree of purity that was achieved is seen in Figure 5.8.

Insulin Release of Isolated Islets

To compare in vitro viability of the hand-picked and BSA density gradient isolated islets, static incubations were performed in both low and high glucose media (Fig. 5.9). There was no significant difference between insulin release of hand-picked and BSA isolated islets in response to either concentration of glucose (Fig. 5.9).

Transplantation of Isolated Islets in Diabetic Nude Rats

For the in vivo viability studies, the methods developed in Chapter 4 were used, once the technique had been established. Purified islets were isolated from 4 human pancreata and were cultured for 48 hours. For each of 3 pancreata processed, 2 nude rats were transplanted, 1 with hand-picked islets and 1 with BSA isolated islets. In the case of the fourth pancreas only BSA islets were obtained. Therefore 7 recipient nude rats were made diabetic with intravenous streptozotocin and all became diabetic within 48 hours (mean blood glucose pre-induction of diabetes 6.4 ± 0.3 mmol/l, 24 hours 17.1 ± 0.8

mmol/l and 48 hours 22.2 ± 1.1 mmol/l)(Fig.5.10). Diabetes was effectively reversed in 5/7 rats within 48 hours of transplantation and these 5 rats remained normoglycaemic until graft removal by nephrectomy, when all 5 rats became hyperglycaemic (Fig. 5.10). The other 2 rats received islets from the same donor pancreas and remained diabetic until culled for histology on day 15.

A mean body weight increase of 2.2 ± 0.3 g/day was seen post-islet transplantation in the 5 successfully transplanted rats. This was comparable to the body weight gain of normal nude rats of the same age (2.1 ± 0.2 g/day; $p = \text{NS}$, Mann-Whitney) (Fig. 4.5).

Intravenous glucose tolerance tests, performed 20 days post transplantation in the 5 successfully transplanted rats, demonstrated a mean glucose clearance ($K = 1.6 \pm 0.1$) similar to that seen in normal nude rats ($K = 1.7 \pm 0.2$, $p = \text{NS}$) (Fig. 5.11a). A rapid and biphasic rise in serum insulin levels was demonstrated during the glucose tolerance tests (Fig. 5.11b).

Histological examination of the grafts from the successfully transplanted animals demonstrated a band of endocrine tissue beneath the renal capsule which stained strongly positive for insulin (Fig. 5.12). There was no evidence of an inflammatory cell infiltrate or rejection. Sections of the 2 failed grafts showed scant insulin staining tissue with vacuolation and an inflammatory infiltrate which had the morphological and histological appearances characteristic of macrophages (Fig. 5.13).

Preparation Sterility

In this series the transport fluid was shown, by microbiological assay, to be sterile in all 15 cases. In addition, in all isolations, islets were successfully cultured without, in the short term, evidence of infection.

DISCUSSION

Attempts to treat patients with insulin dependent diabetes by transplantation of isolated adult islets of Langerhans has been largely unsuccessful to date with failure or only temporary control of hyperglycaemia in most cases. There are a number of possible reasons for the disappointing results to date. Although rejection of allografted islets has been suggested as the main cause of failure, poor viability and inadequate islet dosage probably play a significant role.

Although transplantation of the collagenase digested pancreas (dispersed graft) has been shown to reverse diabetes in the dog, this technique is inappropriate for use in humans. The reasons for this include the difficulty in transplanting large volumes of tissue and the likely inflammatory response due to the contaminating exocrine cells. For further progress to be made it will be necessary to establish efficient means of purifying large numbers of viable islets from human pancreatic digest.

In this study a technique which will isolate large numbers of islets in a rapid and efficient manner from the human pancreas has been developed. This method (utilising a BSA gradient generated on the IBM 2991 cell separator) gave an average yield of 2643 islets per ml of digest, with a maximum yield of greater than 90,000 islets, despite the fact that only a portion of the pancreas was used. Purified islets were recovered in a relatively small tissue volume (on average 430 μ l) which represents only 3% of the original digest yet contained greater than 60% of the available islet (insulin containing) tissue (Fig. 5.6). The high degree of islet purity obtained is important as this should facilitate both pre-transplantation culture and cryopreservation of islet tissue, as well as the actual transplant itself. Furthermore, the degree of

purity may be important for the potential immunomodulation of the islets prior to transplantation.

Confirmation that the purification procedure did not damage the islets came from assessment of viability. In vitro testing, by measurement of insulin release showed no difference in function between hand-picked and BSA isolated islets (Fig.5.9). In vivo viability was assessed using the protocol described in Chapter 4 and isolated adult human islets were transplanted into the renal subcapsular space of 7 nude rats. Diabetes was rapidly reversed in 5 animals and normoglycaemia was maintained throughout the 21 day study period (Fig. 5.10). Successful islet transplantation was confirmed by demonstration that the body weight of the transplanted rats steadily increased, with the rate of increase being almost identical to that seen in normal nude rats. In addition, the reversal to the diabetic state on graft removal (Fig. 5.10) verified that normoglycaemia was maintained by the human islet graft and was not due to reversion of the animal's own islets. Furthermore no difference in viability of the hand-picked and BSA isolated islets was seen in vivo.

For effective treatment of diabetes by islet transplantation blood glucose levels must not only be maintained within normal limits but also the grafted tissues should be able to respond appropriately to a glucose load. Thus the 5 successfully transplanted nude rats were challenged with glucose (0.5 g/kg; i.v.) and these demonstrated prompt return of blood glucose to normal, with glucose clearance ($K = 1.6 \pm 0.1$) being not significantly different from that seen in normal nude rats ($K = 1.7 \pm 0.2$) (Fig. 5.11a). To confirm that the transplanted islets were functioning normally, serum insulin levels were also measured following glucose stimulation, and were found to correspond to the classic biphasic profile observed in man (Fig. 5.11b). Histological examination of the

grafts from the successfully transplanted animals showed a band of endocrine tissue beneath the renal capsule which stained strongly positive for insulin with no evidence of rejection (Fig. 5.12).

The remaining 2 of the 7 nude rats used in the study did not reverse diabetes after islet transplantation. These animals received islets from the same donor pancreas and both grafts failed to reverse diabetes. The histological appearance in the two failed grafts was markedly different from that seen in the successful grafts. Scant insulin staining tissue was present with marked vacuolation and an inflammatory cell infiltrate (Fig. 5.12). The cell infiltrate was characterised histologically as macrophages (which are present in normal numbers in nude rodents) with no evidence of T lymphocytes being present. This infiltrate was probably due to a foreign body reaction rather than immunologically mediated rejection process. The reason for the failure of these particular grafts is unclear, however, it could be due to poor viability of the islet tissue secondary to the explantation procedures and the inflammatory cell infiltrate was in response to cellular death. These findings indicate the value of an *in vivo* method of assessing islet viability.

The 48 hour streptozotocin induction period for diabetes has many advantages in the nude rat. The animals tolerate this short period relatively well but soon begin to deteriorate when left diabetic for longer. The supply of human pancreata is unpredictable. Using this protocol animals were made diabetic only following successful human islet isolation had been demonstrated. The islets were then kept in tissue culture for the 48 hour period during the diabetic induction phase. There was thus no requirement for nude rats to be kept continually available in the diabetic state.

Finally, a further advantage of this large scale method is that the purification took place in an enclosed sterile system. This factor no doubt contributed to the sterility of the final islet preparation.

SUMMARY

A method for the large scale isolation of intact human pancreatic islets using albumin density gradients and an IBM 2991 cell separator is described. The islets so isolated were shown to be viable both in vitro, and in vivo, following transplantation into diabetic nude rats.

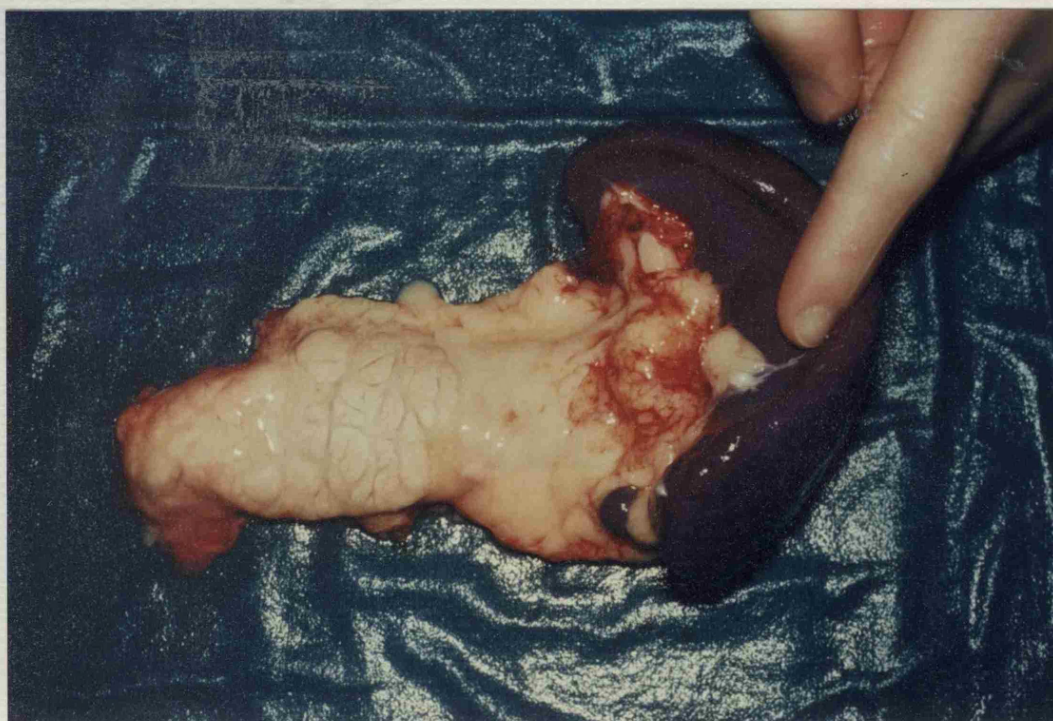


Figure 5.1 Tail segment of human pancreas after resection from cadaveric organ donor.



Figure 5.2 Segment of pancreas after removal of surrounding fat and spleen.

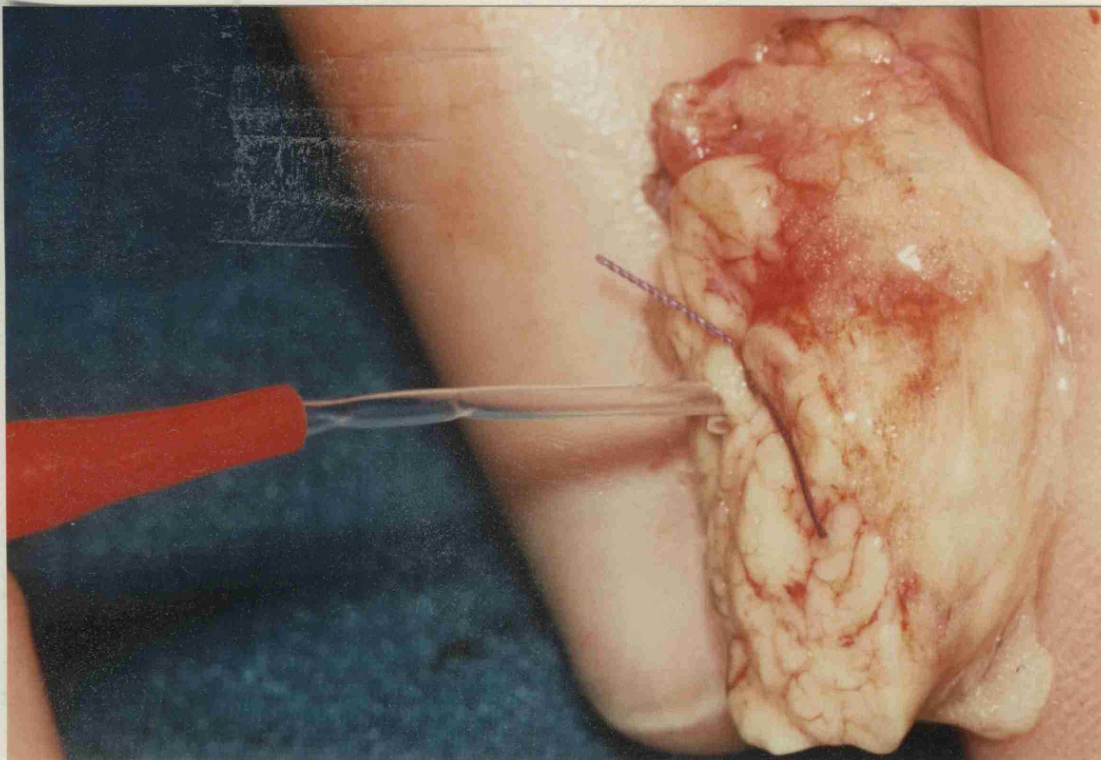


Figure 5.3 Cut proximal portion of pancreas after cannulation of duct for later collagenase digestion.

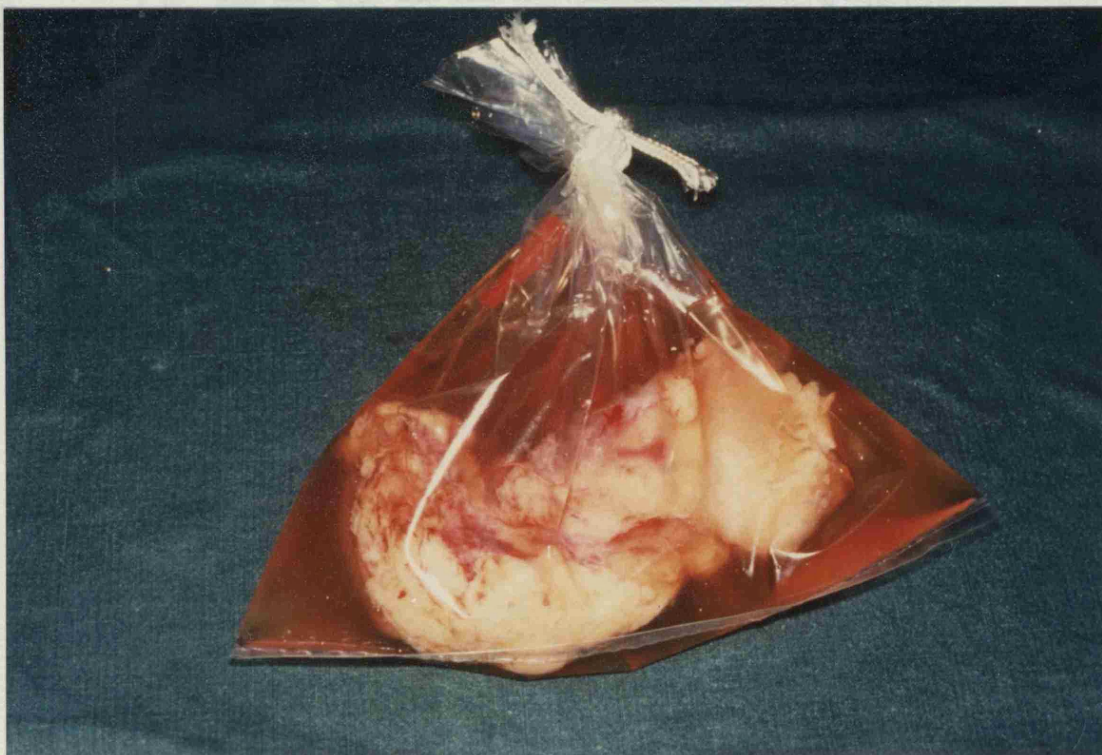


Figure 5.4 Pancreas enclosed in sterile plastic bag for transportation to the laboratory for processing.

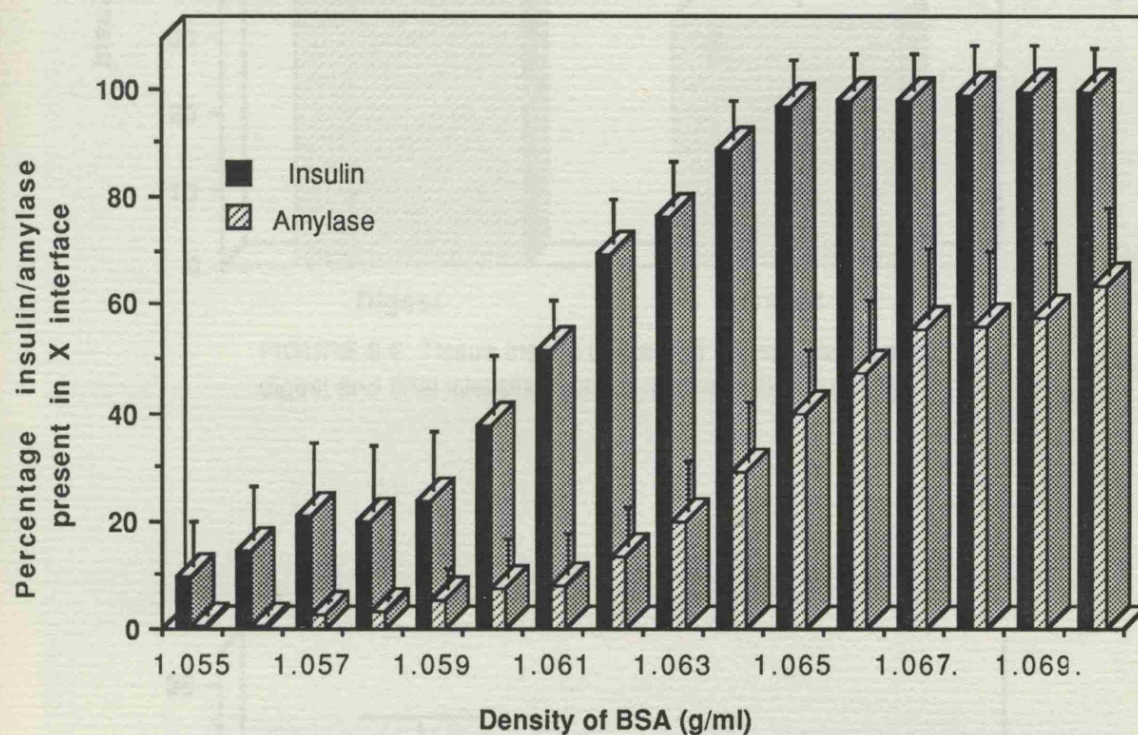


FIGURE 5.5 Analysis of insulin and amylase containing tissue present in the X interface of the human BSA test gradient system. Results expressed as the mean $\pm$ SEM ($n=5$) percent of insulin/amylase present compared with the total within the gradient.

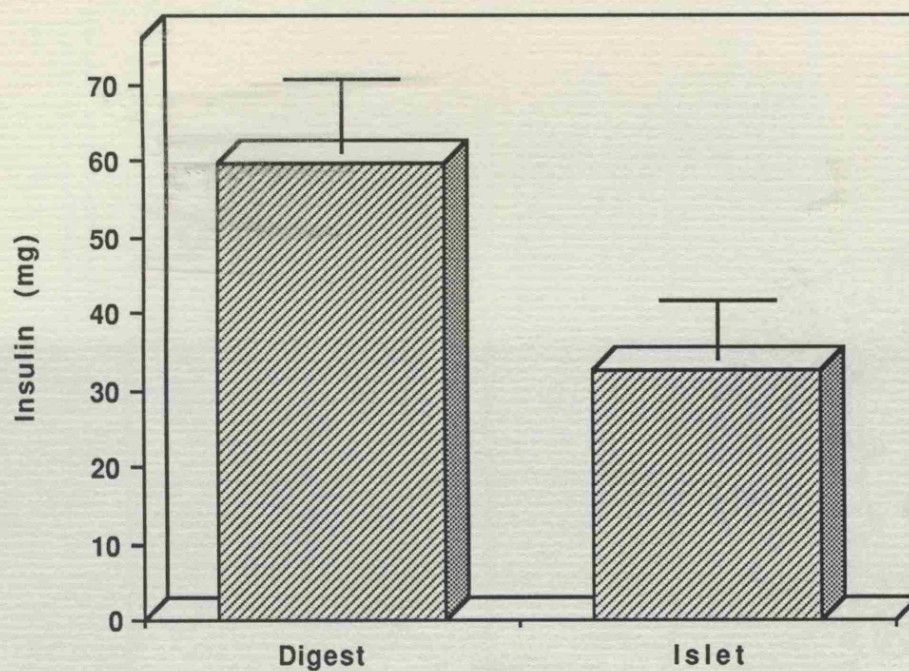


FIGURE 5.6 Tissue insulin content of human pancreatic digest and final islet preparation (mean $\pm$ SEM, $n=15$).

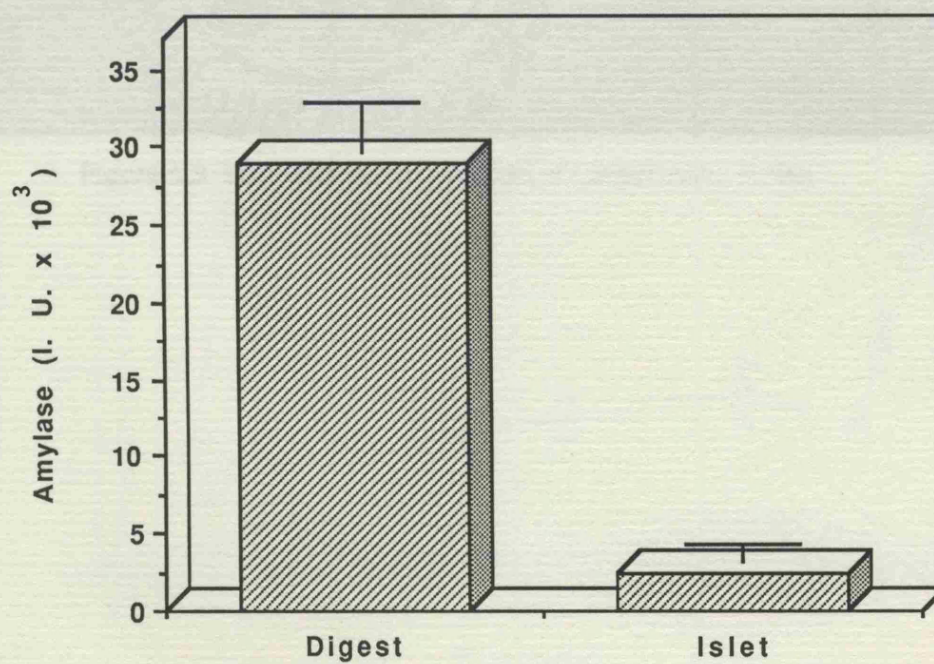


FIGURE 5.7 Amylase content of human pancreatic digest and final islet preparation (mean $\pm$ SEM, $n=15$).

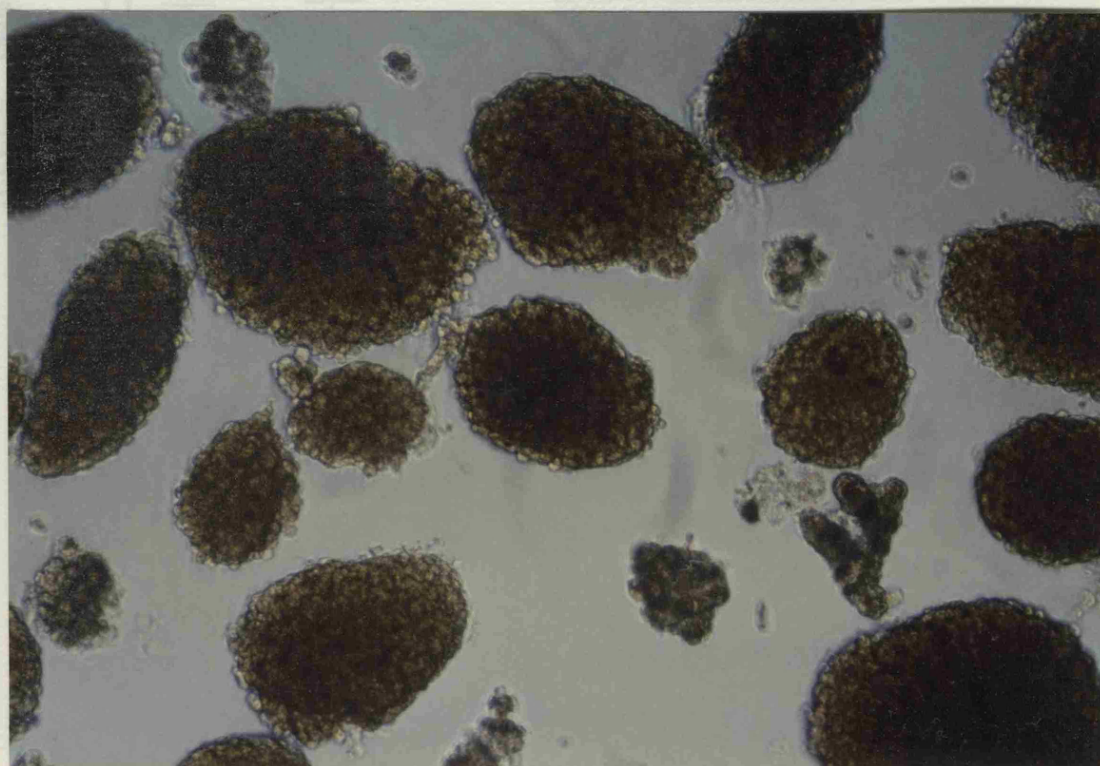


Figure 5.8 BSA isolated human islets of Langerhans. (x 100)

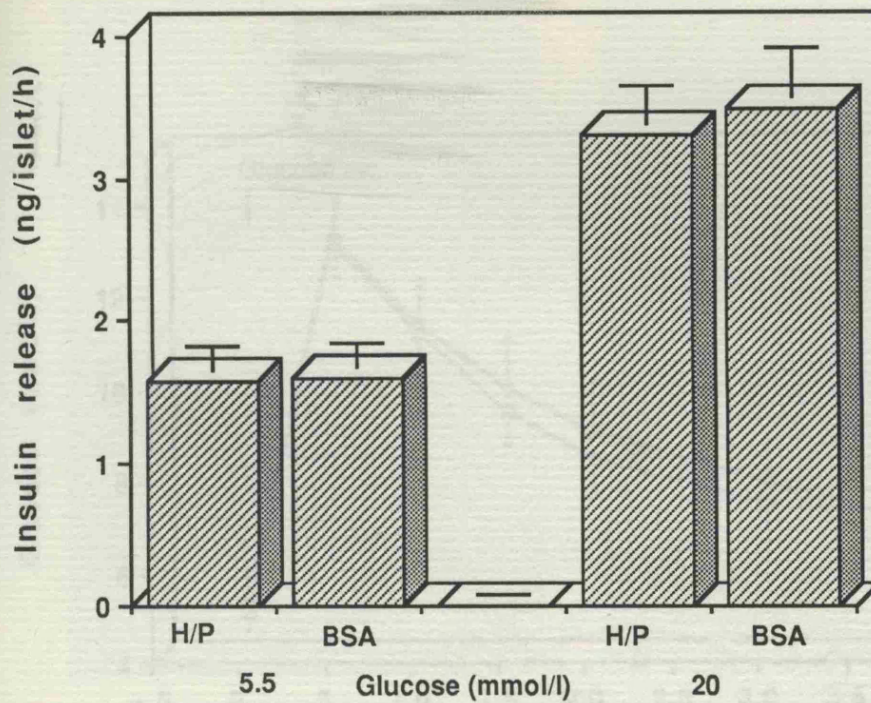


FIGURE 5.9 Insulin release of hand-picked and BSA density gradient isolated human islets in response to stimulation with glucose (mean $\pm$ SEM, $n=6$ pancreata).

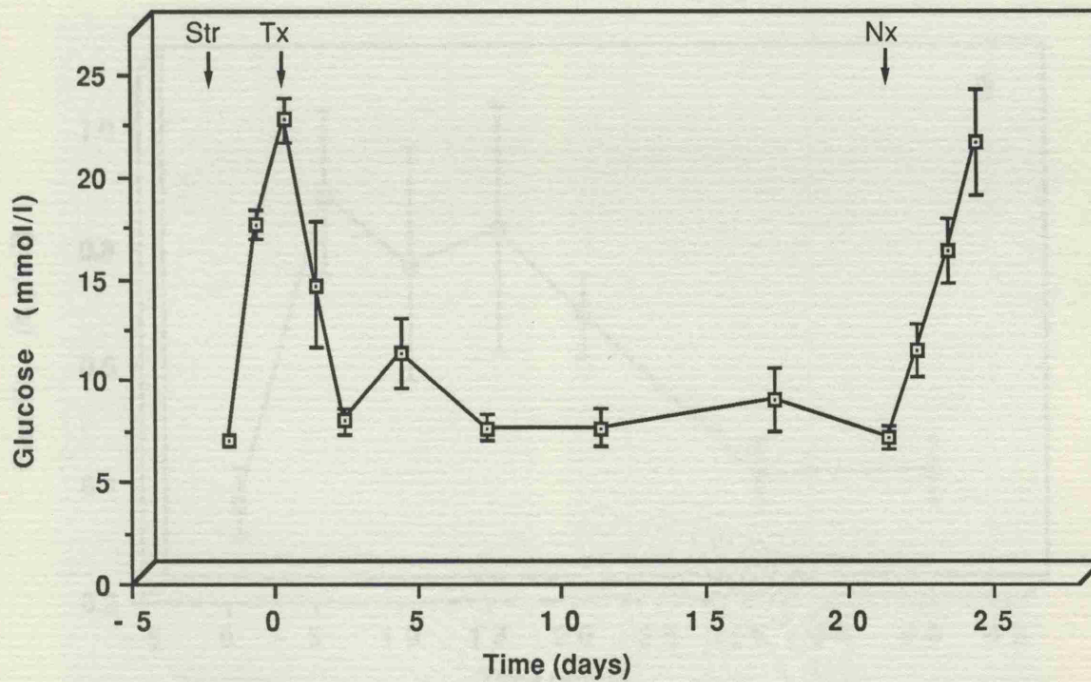


FIGURE 5.10 Serial blood glucose of nude rats ($n=5$) successfully transplanted with 2-day cultured isolated adult human islets (mean $\pm$ SEM).

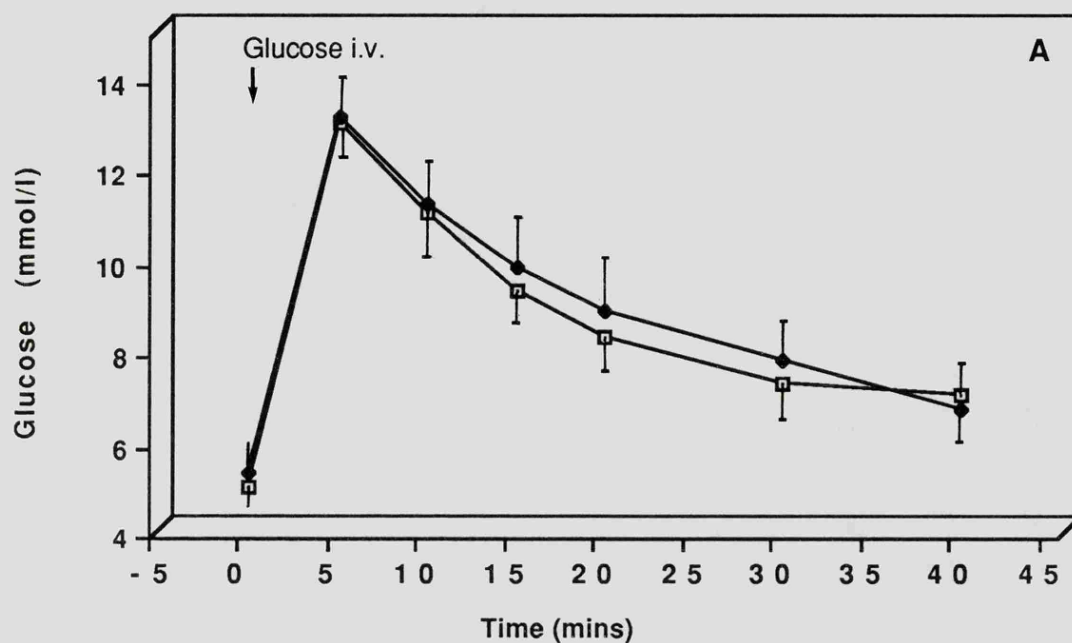
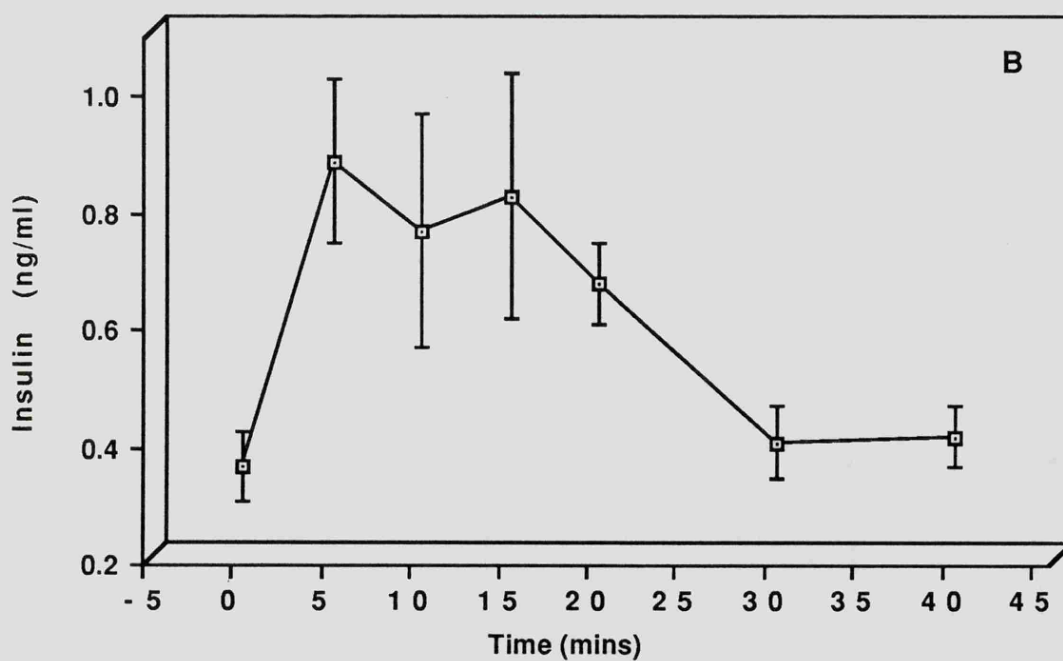


FIGURE 5.11 Glucose tolerance tests performed on successfully transplanted nude rats.

A. Serial blood glucose of normal (◆, n=6) and transplanted (□, n=5) nude rats following glucose stimulation.
 B. Serum insulin measurements of transplanted rats.



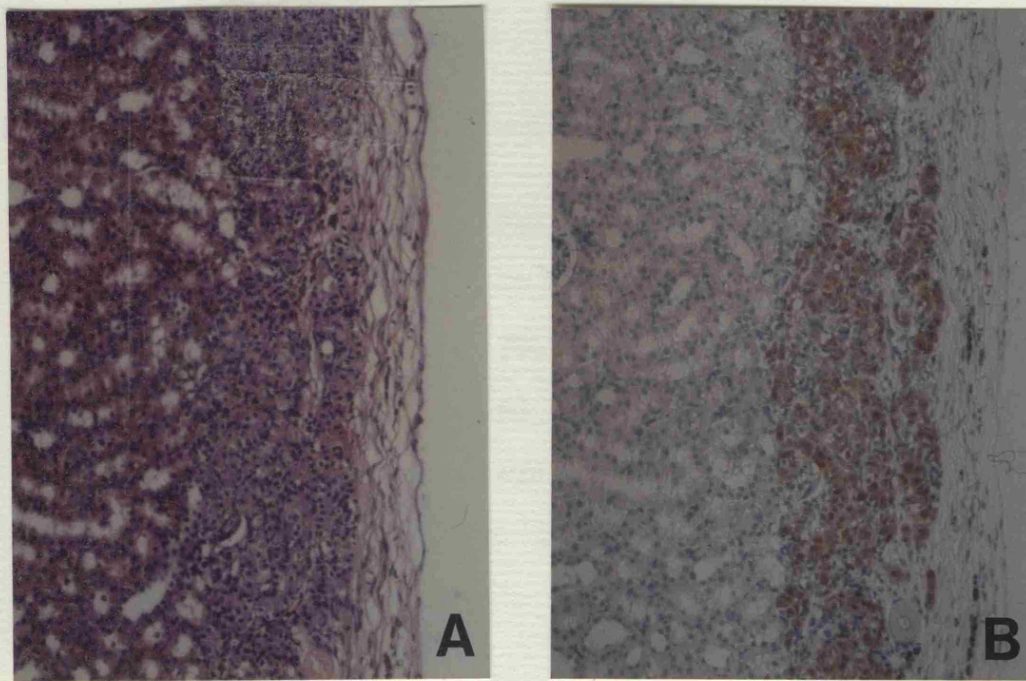


Figure 5.12 Representative sections of renal islet grafts taken from 1 of 5 successfully transplanted nude rats showing a band of endocrine tissue beneath the renal capsule with no sign of rejection. (x 200)

A) stained with haematoxylin/eosin;

B) stained for insulin.

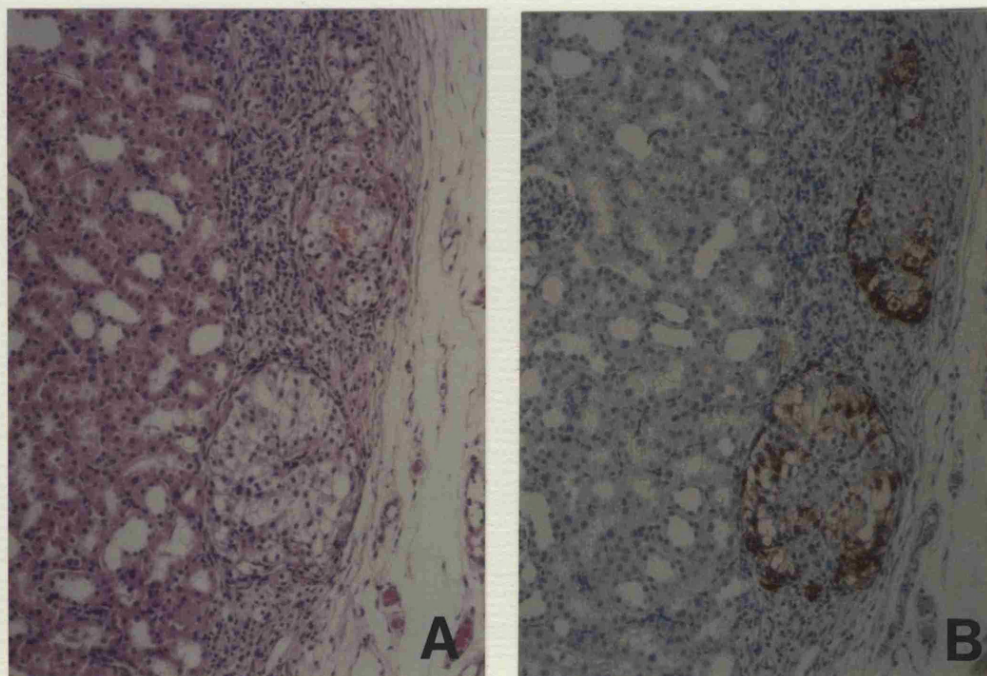


Figure 5.13 Representative sections of renal subcapsular islet grafts taken from 1 of 2 nude rats in which the human islet transplant failed to reverse diabetes showing an inflammatory cell infiltrate. (x 200)

A) stained with haematoxylin/eosin;

B) stained for insulin.

Table 5.1 Results of IBM 2991/BSA isolation of human pancreatic islets

	Mean	SEM	Range
Digest volume (ml)	11.9	1.5	5-27
No. islets/ml digest	2643	284	1100-4500
Estimated purity (%)	63	7	10-90
Islet volume (μ l)	430	110	150-900
Sterile preparation (%)	100		

Further details given in Appendix 2B

CHAPTER 6

OVERVIEW, CONCLUSIONS AND PROSPECTS FOR FUTURE RESEARCH

The transplantation of isolated islets of Langerhans for the treatment of diabetes has been the objective of many transplant surgeons for a number of years. In this study I have set out to improve one aspect of the islet transplantation field, that of the purification phase of islets, post collagenase digestion of the pancreas. Using the rat as a model system I first demonstrated that the standard method for islet purification (Ficoll density gradients) could be significantly improved by using BSA as the density gradient medium (Lake S.P. et al., 1986 & 1987). As my knowledge of the mechanisms by which density gradients separate different cell populations extended, I improved rodent islet isolation further by devising a test gradient system to determine the optimal density of BSA (or other medium) to separate islets (Lake S.P. et al., 1989c). This can be used for the purification of islets from other animals, as I have shown that islet densities differ considerably between different species.

Although it was possible to optimise the BSA gradient system for human islet isolation there was a further problem to overcome. The amount of tissue (collagenase digested pancreas) to be processed from a human pancreas is in the region of 500 fold more than the amount obtained from a rat pancreas. In addition, because of the nature of the pancreas which contains exocrine cells full of potentially cytotoxic proteolytic enzymes, it is important that the process of separating the endocrine cells from the acinar cells is performed rapidly. I therefore devised a method which utilises a centrifuge, which was originally developed for blood processing, and adapted it for the large scale isolation of human islets (Lake S.P. et al., 1989a). This technique was initially used with the canine pancreas and although the large scale purification of islets was demonstrated, it was disappointing that the islet autotransplants failed to prevent diabetes developing in the

pancreatectomised dog. The overall method was then adapted to the purification of human islets and this technique is now being used by a number of groups around the world (e.g. Alejandro, Miami; Hering, Giessen).

It is not sufficient to isolate islets and simply transplant them into patients. A number of investigations are important, particularly to confirm the viability of the islets. Most tests of viability, in the past have been performed in vitro but this may not reflect the situation in vivo. For this reason I set out to determine whether the nude (athymic) rat (which will accept human tissue grafts) could be used. Previous workers have had limited success with such animals for this purpose because they are very susceptible to dehydration once made diabetic. To overcome this problem I utilised a much shortened diabetic induction period and was the first to publish the use of this procedure (Lake S.P. et al., 1988). Using it I was able to show that human islets isolated by the BSA method were just as viable as hand-picked (non-density gradient isolated) islets (Lake S.P. et al., 1989b).

In conclusion I have demonstrated that:- BSA is a superior medium for density gradient separation of rodent and human islets; that the 2991 blood cell processor can be adapted for the large scale isolation of human islets; that the streptozotocin-diabetic nude rat can be used to assess viability of isolated human islets in vivo.

Several aspects of this study deserve comment with regard to future research. The islet preparation purity did vary from pancreas to pancreas despite the optimised system. The precise reason for this is unclear, however, possible reasons include:- a biological variation between pancreata in the density of the exocrine/endocrine components per se; that the intrinsic density is altered by the explantation procedures e.g. length of warm ischaemic time or storage time prior to

islet isolation. In addition, of interest is that aliquots of intact islets from 1 pancreas failed to reverse diabetes in the nude rat. This clearly not only demonstrates the value of the diabetic nude rat model, but also indicates that there may be factors, such as ischaemic time and storage time which will affect islet viability. With specific regard to the 2991 cell processor I believe that it may be possible to extend its role to incorporate the washing phase which is performed to remove the collagenase prior to islet purification. This would have advantages in that a further stage in the islet isolation process which requires multiple test tubes would be eliminated. These problems give rise to good prospects for future research and will clearly require substantial numbers of human pancreata to achieve definitive answers.

Finally, the studies in this thesis have not allowed successful transplantation of human islets of Langerhans into a diabetic patient, but will no doubt assist in achieving this important final goal.

The following publications resulted from the work described in this thesis:-

1. SP Lake, RFL James, J Anderson, J Chamberlain, S Gardner, PRF Bell.
An improved isolation method for rat pancreatic islets.
Trans Proc (1986); 18(6): 1817-1818.
2. SP Lake, J Anderson, J Chamberlain, S Gardner, PRF Bell, RFL James.
Bovine serum albumin density gradient isolation of rat pancreatic islets.
Transplantation (1987); 43(6): 805-808.
3. SP Lake, J Chamberlain, P. Husken, PRF Bell, RFL James
In vivo assessment of isolated pancreatic islet viability using the streptozotocin-induced diabetic nude rat.
Diabetologia (1988); 31(6): 390-394.
4. SP Lake, PD Bassett, A Larkins, J Revell, K Walczac, J Chamberlain, GM Rumford, NJM London, PS Veitch, PRF Bell, RFL James.
Large scale isolation of human islets utilising a discontinuous albumin gradient on an IBM 2991 cell separator.
Diabetes (1989a); 38(Suppl.1): 143-145.
5. SP Lake, J Chamberlain, PD Bassett, NJM London, K Walczac, PRF Bell, RFL James.
Successful reversal of diabetes in nude rats by transplantation of isolated adult human islets of Langerhans.
Diabetes (1989b); 38: 244-248.
6. SP Lake, J Chamberlain, K Walczac, PRF Bell, RFL James.
A test gradient system for optimising density gradient isolation of pancreatic islets.
Transplantation (In press, 1989c).

APPENDIX 1AMeasurement of Insulin by Radio-Immunoassay**Reagents:-**

Insulin standards - prepared from Wellcome Human Insulin,
8, 4, 2, 1, 0.5 and 0.25 ng/ml.

Insulin Binding Reagent - Wellcome Diagnostics, Dartford, UK.

¹²⁵I - labelled insulin (Chloramine T method)

Diluent buffer - Phosphate buffered saline plus 1% BSA and
0.05% sodium azide.

Equipment:-

LP3 tubes and stoppers.

Centrifuge - CR 422, Jouan, Tring, UK.

Gamma counter - LKB 1280, Pharmacia LKB, Milton Keynes, UK.

Procedure:-**1. Assay Tubes**

Sufficient assay tubes were set up and numbered for:-

- (a) Standard insulin solutions.
- (b) Test samples.
- (c) Maximum binding - to which labelled insulin and Binding Reagent were added.
- (d) Blanks - in which Binding Reagent was replaced by buffer.
- (e) Totals - to which only labelled insulin was added.

Each test was carried out in duplicate.

2. Addition of Insulin Standards and Samples

100 µl of insulin standard or sample was added to each tube as appropriate. 100 µl of diluent buffer was added to the zero and blank tubes.

3. Addition of Binding Reagent

100 µl of Insulin Binding Reagent was added to all tubes except totals and blanks. The solutions were then mixed using a vortex mixer and the tubes were stored at 4°C for 6 hours.

4. *Preparation of the Labelled Insulin*

The stock solution of iodinated insulin was diluted with diluent buffer in order to achieve 50,000 cpm per 100 μ l.

5. *Addition of the Labelled Insulin*

100 μ l of the working solution of ^{125}I -insulin was added to every tube and mixed again prior to storage at 4°C overnight.

6. *Separation of the Precipitate containing Antibody-bound Tracer*

1 ml of diluent buffer was placed in each tube (except totals) and the tubes were centrifuged at 2,000 g for 20 minutes.

7. *Determination of Radioactivity*

The supernatant was removed by aspiration and the radioactivity of the precipitate was measured by counting each assay tube, in the gamma counter, for one minute.

8. *Calculation of Results*

The mean count rate for each duplicate assay was calculated and the the mean 'blank' count rate was subtracted from all other counts. The percent bound for each sample was then calculated in the following way:-

$$\frac{\text{Counts bound in sample} - \text{'blank'}}{\text{Maximum binding} - \text{'blank'}} \times 100$$

The percent inhibition of binding of each sample was then calculated by the following:-

$$100 - \% \text{ Bound} = \% \text{ Inhibition}$$

A semi-log graph of % Inhibition (log) plotted against concentration of insulin standards (linear) was used to obtain a calibration curve. The concentration of insulin in the test samples could then be determined from the graph.

APPENDIX 1B

Measurement of Amylase

This procedure used the hydrolysis of a water-insoluble cross-linked starch polymer carrying a blue dye by α -amylase to form water-soluble blue fragments.

Reagents:-

Human α -amylase control

Starch polymer in tablet form - Pharmacia, Milton Keynes, UK.

Equipment:-

10 ml conical test tubes

Spectrophotometer - SP 1800, Pye Unicam, Cambridge, UK.

Procedure:-

1. 4 ml of distilled water was pipetted into 10 ml conical tubes.
2. 200 μ l samples, at appropriate dilutions, were added to the tubes, including distilled water & MEM as blanks and human α -amylase at 1/5 dilution as control.
3. The tubes were pre-incubated at 37°C for at least 5 minutes in a waterbath.
4. Starch polymer was added in tablet form using forceps. Each tube was then immediately vortexed for 10 seconds and replaced in the waterbath.
5. Tubes were incubated for exactly 15 minutes at 37°C.
6. 1 ml of 0.5N NaOH was added to each tube and vortexed to stop the reaction.
7. Tubes were centrifuged at 1500g for 5 minutes.
8. Absorbance of the supernatant was measured at 620 nm against distilled water, using plastic cuvettes, with 1 cm light path.
9. The absorbance of the blank was subtracted from that of the samples and the values were then read from a standard curve.

APPENDIX 1C

Measurement of Glucose

The principle of this assay is the measurement of oxygen change when the enzyme glucose oxidase reacts with glucose under controlled semi-aerobic conditions. The resultant oxygen tension change is monitored by a high-sensitivity oxygen sensor. The maximum rate of change is proportional to the glucose concentration initially.

The machine used for these assays was in regular use within the Department of Clinical Pathology, Leicester Royal Infirmary.

Reagents:-

Glucose standard solution - 8 mmol/l

Glucose oxidase solution - 40 Baker units/ml

Equipment:-

GM6 Oxidase Analyser - Analox Instruments Ltd., London, UK.

Procedure:-

1. The machine was set up on a daily basis and calibrated prior to and during each run of samples with the glucose standard.
2. 10 μ l of glucose solution or sample were added, by pipette, the reaction chamber containing the glucose oxidase solution. Following withdrawal of the injection pipette the solutions were mixed automatically to commence the reaction. The resulting peak rate of oxygen production was measured by the oxygen sensor and the glucose concentration determined.

APPENDIX 2A

Details of canine autotransplants

	Weight Body (kg)	Pancreas (g)	Digest Volume (ml)	Islet Count	Islet Volume (μ l)	
DA1	13.2	33	8	44,000	150	
DA2	12.7	37	15	180,000 ^F	200	
DA3	13.5	33	7	-	-	CONTROL DOG
DA4	10.2	25	10	30,000	150	
DA5	10	20	7.5	65,000	180	
DA6	15	30	15	54,500	250	
DA7	15	46	17	63,000	200	
DA8	12	36	10	46,000	300	
DA9	13.2	33	19.5	198,000 ^F	750	
Mean		32.5	12.1	85,100	272	
SEM		2.5	1.5	23,100	70	

F = Islet fragments present in preparation.

APPENDIX 2B

Results of BSA gradient/IBM 2991 isolation of human islets

Pancreas No.	Donor Age	Digest Volume (ml)	Islet Count No./ml digest	Estimated Purity (%)	Islet Volume (μ L)	Sterility
1	40	15	1200	90	100	N.G.
2	56	3	1800	60	600	N.G.
3	51	5	1400	60	400	N.G.
4	52	13	4500	70	500	N.G.
5	61	12	1140	70	150	N.G.
6	56	4	3270	60	450	N.G.
7	39	10	2800	20	650	N.G.
8	43	16	2940	90	100	N.G.
9	41	11.5	1680	30	550	N.G.
10	67	13	1500	10	900	N.G.
11	16	27	3500	80	400	N.G.
12	55	12	3300	50	250	N.G.
13	38	15	3750	90	350	N.G.
14	42	10	2915	90	350	N.G.
15	50	13	3950	85	700	N.G.
Mean		11.9	2643	63	430	
SEM		1.5	284	7	59	

N.G. = No bacterial/fungal growth in the transport fluid nor culture.

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STUDIES ON THE ISOLATION AND TRANSPLANTATION
OF MAMMALIAN PANCREATIC ISLETS

by

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A review is made of the relationship between the disease diabetes mellitus and the insulin-secreting B cells of the islets of Langerhans. In addition, an account is given of the clinical and experimental methods of treatment including insulin therapy and transplantation of the pancreas both as an intact organ and as isolated endocrine tissue (islet transplantation). In particular the methods which have been used to release and isolate islets from the rodent, large animal and human pancreas are discussed. Furthermore, the metabolic effects of transplantation, results of allogeneic transplantation and techniques of islet preservation are described.

In this thesis I have set out to determine whether the standard islet isolation method (using density gradients of Ficoll medium) could be improved by utilising an alternative density gradient medium (Bovine Serum Albumin). Using a rodent model, islet yield, purity and viability (both in vitro & in vivo) were shown to be improved with the BSA method over the standard Ficoll technique. A second stage was subsequently performed, using a novel test gradient system, to optimise the BSA gradient method further by definition of the precise density of rat islets and exocrine tissue.

The BSA gradient method was then adapted to the isolation of canine islets. To overcome the difficulties in processing large volumes of digest produced from the collagenase digested pancreas, an IBM 2991 cell separator was adapted to produce large volume density gradients. Although high yields of highly purified and viable islets were obtained, these were insufficient to prevent diabetes ensuing in autotransplanted pancreatectomised dogs.

An animal model was developed which would potentially be applicable for the in vivo assessment of human islet viability. The nude (athymic) rat, allogeneic and xenogeneic (mouse) islet transplants and a short diabetic induction period with streptozotocin were all used.

Finally, the BSA gradient methodology and IBM 2991 cell processor were used for the large scale purification of intact human islets which were shown to be viable both in vitro, and in vivo following transplantation into diabetic nude rats.